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Role of the Low Molecular Weight Protein Phosphatases PtpA and PtpB on Infectivity of *Staphylococcus aureus*

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Declaration

The work presented in this thesis was carried out in the period from April 2017 till July 2021 in the Institute of Medical Microbiology and Hygiene (IMMH) at Saarland University.

This thesis is written in a cumulative way and the corresponding publications are presented in the results section of this thesis after having a permission from the participating authors. A complete list of publications of the author of this thesis is presented in the publications and conferences section. The author of this thesis is the main author and/or a co-author in the following peer-reviewed publications:

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Dedicated to…

My Family

My Mother’s Soul

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Abbreviations

Abbreviation	Full name
agr	Accessory gene regulator
AMPs	Antimicrobial peptides
Arg	Arginine
Asp	Aspartate
ATP	Adenosine triphosphate
Aur	Aureolysin
B. subtilis	*Bacillus subtilis*
C3	Complement component 3
CC	Clonal complex
CA-MRSA	Community acquired-methicillin resistant *Staphylococcus aureus*
CDC	Centers of Disease Control and Prevention
CHIPS	Chemotaxis inhibitory protein of *S. aureus*
ClfA	Clumping factor A
ClfB	Clumping factor B
Cna	Collagen adhesion
C. burnetii	*Coxiella burnetii*
CtsR	Class three stress gene repressor
Cys	Cysteine
CWA	Cell wall-anchored
Da	Dalton
dAdo	2′-deoxyadenosine-5′-monophosphate into 2′-deoxyadenosine
DEA/NO	Diethylamine NONOate
DNA	Deoxyribonucleic acid
DSps	Dual specific phosphatases
Eap	Extracellular adherence protein
ECDC	European Centre of Disease Control and Prevention

eDNA	Extracellular DNA
Efp	Extracellular fibrinogen binding protein
ECM	Extracellular matrix
Efb	extracellular fibrinogen binding protein
Emp	Extracellular matrix protein
ETA	Exfoliative toxin A
ETB	Exfoliative toxin B
ETs	Exfoliative toxins
FAK	Focal adhesion kinase
Fc region	Fragment crystallizable region
FLIPr	Formyl peptide receptor-like 1 inhibitor
FnBPs	Fibronectin binding proteins
FnBPA	Fibronectin binding protein A
FnBPB	Fibronectin binding protein B
fMLP	Formyl-Methionine-Leucine-Phenylalanine
GC	Guanine cytosine
HA-MRSA	Health-care associated methicillin resistant *S. aureus*
His	Histidine
Hla	Hemolysin α
Hlb	Hemolysin β
IE	Infective endocarditis
IAIs	Implant-associated infections
ICAM-1	Intercellular adhesion molecule-1
IgG	Immunoglobulin G
IL	Interleukin
iNOS	inducible nitric oxide synthase
kDa	kiloDalton
LipA	Listeria phosphatase A
L. monocytogens	*Listeria monocytogens*
LMW-PPs	Low molecular weight protein phosphatases
LMW-PTPs	Low molecular weight-protein tyrosine phosphatases
Mbp	Mega base pairs
MDMs	Monocyte-derived macrophages
MOF	Multiple organ failure
MPtpA	Mycobacterium Protein tyrosine phosphatase A
MPtpB	Mycobacterium Protein tyrosine phosphatase B

MRSA	Methicillin resistant *Staphylococcus aureus*
Mtb	*Mycobacterium tuberculosis*
NADPH	Nicotinamide adenosine dinucleotide phosphate
NETs	Neutrophil extracellular traps
nm	Nanometer
NO˙	Nitric oxide
NPPCs	Non-professional phagocytic cells
nuc	Nuclease
P-loop	Phosphate binding loop
PAMPs	Pathogen-associated molecular patterns
PAPs	Protein arginine phosphatases
pArg	Phosphorylated arginine
PGN	Peptideolycan
PHPs	Polymerase and histidinol family of phosphoesterases
PMNs	Polymorphonuclear leukocytes
PPs	Protein phosphatases
PRRs	Pattern recognition receptors
PTMs	Post-transnational modifications
PtpA	Protein tyrosine phosphatase A
PtpB	Protein tyrosine phosphatase B
PTPs	Protein tyrosine phosphatases
PTSAgs	Pyrogenic toxin superantigens
psm-α	Phenol soluble modulin-α
PVL	Panton-Valentine leukocidin
qRT-PCR	Quantitative real-time polymerase chain reaction
RKI	Robert Koch Institute
ROS	Reactive oxygen species
SAK	Staphylokinase
S. aureus	*Staphylococcus aureus*
S. typhi	*Salmonella typhi*
S. typhimurium	*Salmonella typhimurium*
SaPIs	*Staphylococcus aureus* pathogenicity islands
SCIN	Staphylococcal complement inhibitor
ScpA	Staphopain A
SEM	Scanning electron microscope
Ser	Serine

SEs	Staphylococcal enterotoxins
SERAMS	Secretable expanded repertoire adhesive molecules
S. flexneri	*Shigella flexneri*
SpA	Staphylococcal protein A
spp.	Species
S. pneumoniae	*Streptococcus pneumoniae*
SptP	*Salmonella* protein tyrosine phosphatase
SSIs	Surgical site infections
SSSS	Staphylococcal scalded skin syndrome
SSTIs	Skin and soft tissue infections
StK1	Serine/Threonine protein kinase
STKs	Serine/Threonine kinases
Thr	Theronine
TLRs	Toll like receptors
TNF-α	Tumor necrosis factor-alpha
TSB	Trypticase soy broth
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin-1
Tyr	Tyrosine
WHO	World Health Organization
H_2O_2	Hydrogen peroxide
µM	Micromolar
µm	Micrometer
α	Alpha
β	Beta
γ	Gamma
δ	Delta
Δ	Deletion

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1. Summary

The bacterium, *Staphylococcus aureus* (*S. aureus*) is an opportunistic pathogen which can infect a variety of tissues resulting in a wide spectrum of infections ranging from mild cutaneous lesions to serious clinical manifestations such as endocarditis and osteomyelitis. This pathogen is also a common cause of implant-associated infections (IAIs), which are usually difficult to treat. A major characteristic of infections caused by *S. aureus* is being recurrent and long-standing. This latter characteristic is probably due to the ability of this pathogen to penetrate and survive within different types of cells in the human body including both professional and non-professional phagocytic cells (NPPCs). Many bacterial pathogens that are capable of surviving intracellularly in host immune cells secrete signaling molecules to modulate host cell signaling in order to survive in these cell types. The molecular mechanisms promoting the intracellular survival of *S. aureus* in professional phagocytes are not fully understood. However, the survival of *S. aureus* in these immune cells contributes to the dissemination of this pathogen to different body organs during infections using the so-called “trojan-horse delivery system” mechanism. This mechanism is clearly dangerous when macrophages particularly are occupied with viable *S. aureus*, owing to the mobility and long-living nature of macrophages compared to other immune cells.

Across evolution, bacterial pathogens adapt their genomes in order to be able to counteract adverse environmental conditions during infections. Post-translational modifications (PTMs) of proteins are common mechanisms used by bacterial pathogens to modulate their immune evasion strategies. One common PTM mechanism utilized by many bacterial pathogens is phosphorylation/dephosphorylation of bacterial and host proteins. *S. aureus* is known to use this reversible phosphorylation of proteins to modulate metabolic processes and the activity of diverse global regulators, however its relation to staphylococcal pathogenesis is not fully characterized and asks for further investigation.

This thesis focuses on the characterization of potential roles of two low molecular weight protein phosphatases on the infectivity of *S. aureus*. These two proteins are called PtpA (Protein tyrosine phosphatase A) and PtpB (Protein tyrosine phosphatase B). Both these phosphatases were not fully characterized in *S. aureus* till the beginning of this study, despite

the fact that homologues for both proteins have been already reported to promote infectivity of pathogens such as *Mycobacterium tuberculosis (Mtb)*, *Salmonella typhimurium (S. typhimurium)*, or *Yersinia* spp. By studying the impact of these proteins on the interactions of *S. aureus* with host cells, especially macrophages, it became clear that both, PtpA and PtpB, play important roles in pathogenesis of *S. aureus* by enhancing the bacterium's ability to survive inside macrophages. Both proteins also promoted the *in vivo* infectivity of *S. aureus* in a mouse model of infection. Moreover, a number of intracellular host proteins were identified as putative binding candidates for PtpA after being secreted inside macrophages during infections. Importantly, the protein Coronin-1A was phosphorylated on tyrosine residues when macrophages were infected with *S. aureus*. This protein is a crucial component of the cytoskeleton of highly motile host cells and is implicated in various immune-mediated responses. Thus, PtpA could be identified as a tyrosine phosphatase secreted by *S. aureus* to promote the intramacrophage survival capacity of this pathogen during infections, presumably by interacting with intracellular host proteins including Coronin-1A.

In the second half of this study, i investigated the impact of a *ptpB* deletion on the stress response and infectivity of *S. aureus*. Here, i observed that this protein arginine phosphatase (PAP) is also required for the intracellular survival of *S. aureus* inside human macrophages. Subsequent analyses revealed that the phosphatase activity of PtpB in *S. aureus* is modulated by the oxidative status of the bacterial cell. When mimicking different kind of stresses encountered by *S. aureus* upon engulfment by macrophages, i noticed that the deletion of *ptpB* reduced the capacity of *S. aureus* to cope with oxidative-, nitrosative- and acidic stress, suggesting that PtpB enhances the intracellular survival capacity of *S. aureus* inside macrophages by increasing the bacterial fitness against the major stresses generated inside these immune cells to kill the internalized bacterial cells. Additionally, PtpB also exerted a protective effect in *S. aureus* against phagocytosis by polymorphonuclear leukocytes (PMNs). In this regard, cells of the *ptpB* mutant displayed additionally a decreased ability to release nucleases, which are important to degrade the Neutrophil Extracellular Traps (NETs) produced by PMNs upon activation. PtpB is also required for the overall proteolytic activity of *S. aureus*. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis uncovered a modulatory effect of PtpB on the expression of various virulence factor encoding genes including *psmα* (endocing phenol-soluble module α), *aur* (encoding aureolysin), *nuc* (encoding nuclease) and also RNAIII (a regulator of the *agr* locus). Finally, i found that PtpB is also involved in maintaining the cell wall integrity of *S. aureus*, presumably by modulating the activity of selected autolysins/regulators involved in cell wall homeostasis.

2. Zusammenfassung

Der opportunistische Krankheitserreger *S. aureus* ist dazu imstande, verschiedene Organe/Gewebetypen zu infizieren, und dadurch Infektionen der Haut bis hin zu ernsten klinischen Komplikationen wie Endokarditis, Pneumonie oder Osteomyelitis auszulösen. Dieser Erreger ist zudem ein häufiger Verursacher von Implantat-assoziierten Infektionen, die in der Regel nur schwierig zu behandeln sind. *S. aureus*-Infektionen sind zudem oft wiederkehrend und chronisch, wobei die letztere Eigenschaft vermutlich auf die Fähigkeit des Pathogens zurückzuführen ist, in verschiedene Wirtszelltypen, wie professionelle und nicht-professionelle Phagozyten inserieren zu können und in ihnen für mehrere Tage zu überleben. Die intrazelluläre Überlebensfähigkeit von *S. aureus* in diesem Immunzelltyp ist eine wichtige Pathogenese-Eigenschaft dieses Bakteriums, die zur Verbreitung des Bakteriums im Körper des Menschen bis in entfernte anatomische Bereiche beiträgt, ein Mechanismus, der im Englischen als “trojan-horse delivery system” bezeichnet wird. Die molekularen Mechanismen, die die intrazelluläre Überlebensfähigkeit von *S. aureus* in diesen professionellen Phagozyten erhöhen, sind bisher nicht vollständig aufgeklärt. Für verschiedene andere pathogene Bakterien ist jedoch bekannt, dass sie sich intrazellulär in Immunzellen behaupten können, indem sie dort Signalmoleküle sekretieren, die die Wirtszell-Signalwege so modulieren, dass die internalisierten Bakterienzellen in diesem Wirtszelltyp zu überleben vermögen. Viele dieser Pathogene nutzen dabei post-translationale Modifikationen (PTM) von Proteinen, um ihre Immunevasionsstrategien zu modulieren. Die Phosphorylierung/Dephosphorylierung von Wirtszellproteinen stellt dabei für viele Pathogene eine wichtige Form der PTM dar, die Abwehrmechanismen des Wirtes zu umgehen. Die reversible Phosphorylierung wird auch von *S. aureus* dazu genutzt, metabolische Prozesse und die Aktivität verschiedener globaler Regulatoren zu steuern. Die Bedeutung dieses PTM Mechanismus für die Infektiosität von *S. aureus* wurde bisher jedoch nur unzureichend charakterisiert, weshalb weitere Untersuchungen in diesem Bereich wünschenswert sind.

Diese Arbeit fokussierte sich daher auf die Charakterisierung zweier Niedermolekulargewichts-Proteinphosphatasen, PtpA und PtpB, hinsichtlich ihrer Rolle während der Pathogenese von *S. aureus*. Beide Phosphatasen sind in *S. aureus* schon seit

längerem bekannt, wurden bis dato aber noch nicht in Hinblick auf ihre Bedeutung für die Infektiosität von *S. aureus* untersucht, obwohl für andere pathogene Bakterien wie *Mycobacterium tuberculosis (Mtb)*, *Salmonella typhimurium (S. typhimurium)* und *Yersinia* spp. gezeigt werden konnte, dass Homologe der beiden Proteine wichtige Virulenzfaktoren darstellen. Durch meine Untersuchungen konnte ich zeigen, dass sowohl PtpA als auch PtpB wichtige Virulenzfaktoren für *S. aureus* darstellen, die beide die Überlebenskapazität von *S. aureus* in Makrophagen steigern und die Virulenz des Pathogens während der Infektion erhöhen.

PtpA wird dabei vom Bakterium in das umgebende Milieu sekretiert, um mutmaßlich mit Wirtsfaktoren, wie Coronin-1A zu interagieren. Dieses Protein ist eine wichtige Komponente des Zytoskeletts migrierender Wirtszellen und ebenso für die Immunantwort der Wirtszelle von Bedeutung. Da PtpA auch nach Phagozytose durch Makrophagen innerhalb der Wirtszelle sekretiert wird, liegt die Vermutung nahe, dass auch *S. aureus* durch die Sekretion dieser Protein-Tyrosin-Phosphatase das intrazelluläre Überleben der Bakterienzelle innerhalb der Immunzelle fördert. In Einklang mit dieser Hypothese konnte in Untersuchungen mit einem *S. aureus*-basierten Leberabszessmodell der Maus gezeigt werden, dass PtpA für die *in vivo* Infektiosität von *S. aureus* von großer Bedeutung ist.

Im zweiten Teil meiner Promotion beschäftigte ich mich mit der Protein-Arginin-Phosphatase PtpB. Ebenso wie PtpA fördert PtpB das intrazelluläre Überleben von *S. aureus* in Makrophagen, anders als PtpA wird diese Phosphatase von *S. aureus* jedoch nicht in das umgebende Milieu sekretiert, sondern übt ihren regulatorischen Einfluss innerhalb der Bakterienzelle aus. PtpB scheint dabei die Fähigkeit von *S. aureus* zu fördern, mit Stressbedingungen, wie sie im Phagolysosom von Makrophagen nach Aufnahme von Cargo anzutreffen sind, umzugehen. So verminderte die Deletion von *ptpB* in *S. aureus* die Fähigkeit des Bakteriums, mit oxidativem-, nitrosativem- oder Säurestress umzugehen. Weiterführende Untersuchungen zeigten zudem, dass die Protein-Arginin-Phosphatase auch eine protektive Rolle für *S. aureus*, der Phagozytose durch polymorphkernige Leukozyten (PMNs) zu entgehen, einnimmt. Ebenso förderte PtpB die Sekretion von extrazellulären Nukleasen, einem weiteren wichtigen Immunevasionsmechanismus von *S. aureus*, den von Neutrophilen gebildeten extrazellulären Netzen zu entgehen. Zusätzlich unterstützte PtpB die proteolytische Aktivität von *S. aureus* und dessen Widerstandsfähigkeit gegenüber lytischen Agenzien wie Triton X-100 oder Lysostaphin. Darüber hinaus konnte ich zeigen, dass PtpB die Transkription von verschiedenen, für Virulenzfaktoren kodierende Gene beeinflusst, darunter *psm*α (codiert für die phenollöslichen Moduline α1-4), *aur* (codiert für die Proteinase Aureolysin), *nuc* (codiert für die Nuklease 1) und *RNAIII* (eine regulatorische RNA und Masterregulator des *agr* Lokus). Last not least zeigte eine *ptpB* Deletionsmutante in dem *S.*

aureus-basierten Leberabszessmodell der Maus eine deutlich verminderte Fähigkeit, vier Tage nach Infektion eine erhöhte Bakterienlast in der Leber und in den Nieren hervorzurufen, und unterstreicht damit die hohe Bedeutung auch dieser Phosphatase für die Virulenz von *S. aureus*.

3. Introduction

3.1. *Staphylococcus aureus*

In 1880, the Scottish surgeon Sir Alexander Ogston reported grape-like clusters of bacteria from slides prepared of pus extracted from an abscess in a septic knee joint and he named them *Staphylococcus* (Greek: *staphyle*, “a bunch of grapes”; *kokkos*, “berry or grain”) (Ogston, 1881; Ogston, 1882). Shortly thereafter, the German physician Friedrich Julius Rosenbach was able to successfully isolate and identify the species *S. aureus*, based on the yellowish-orange (golden) pigmentation of their colonies (Latin: *aurum*, “Gold”) (Rosenbach, 1884). This golden pigmentation is due to the production of a carotenoid pigment called staphyloxanthin, which exerts an antioxidant activity in this pathogen (Clauditz *et al.,* 2006; Xue *et al.,* 2019). Nowadays, *S. aureus* is described as Gram-positive bacterium that appears in pairs or grape-like clusters when observed using Gram-staining and light microscope (Foster, 1996; Harris *et al.,* 2002). Moreover, the scanning electron microscope (SEM) reveals roughly spherical cells with a relatively smooth surface and a diameter of 0.5 to 1.0 µm (Greenwood and O’Grady, 1972) **(Figure 3.1)**.

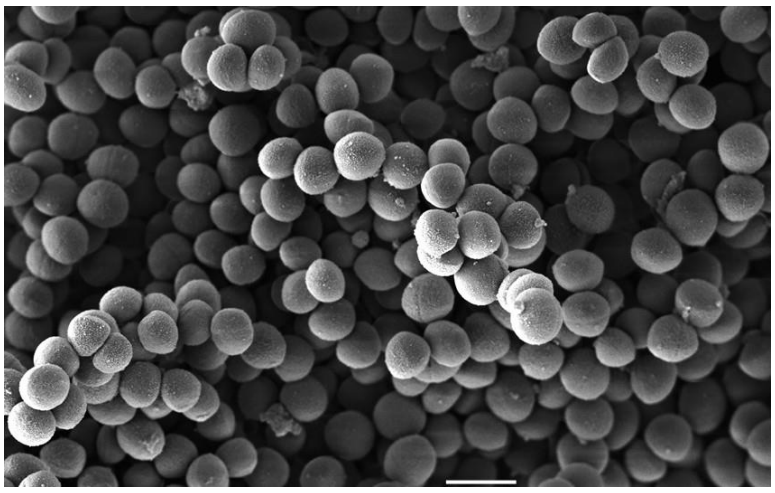

Figure 3.1: SEM of *S. aureus.* Cells of *S. aureus* are clustered in colonies and appeared spherical with a relatively smooth surface (scale bar is 2 µm) (N. Bannert, K. Madela, Robert Koch Institute (RKI), 2011).

This organism is non-motile, non-spore forming and grows aerobically or facultative-anaerobically at a wide temperature range of 15-45°C (Foster, 1996; Missiakas and Schneewind, 2013). Furthermore, it is catalase-positive and is often hemolytic when grown on blood agar plates owing to the production of four types of hemolysins known as α-, β-, γ- and δ-hemolysins (Dinges *et al.,* 2000). In addition, nearly all isolates of *S. aureus* are coagulase-positive (Brown *et al.,* 2005; Preda *et al.,* 2021). The genome of *S. aureus* is nearly 2.8 mega base pairs (Mbp) in size with a relatively low genomic GC-content (Kuroda *et al.,* 2001). In addition to this "core genome", *S. aureus* harbors an additional assortment of extrachromosomal accessory genetic elements such as prophages, plasmids, pathogenicity islands (SaPIs), transposons, and insertion sequences (Baba *et al.,* 2008; Malachowa and DeLeo, 2010). This accessory genome constitutes genes mostly encoding virulence factors and antibiotic resistance genes (Jamrozy *et al.,* 2017), making *S. aureus* a very heterogeneous bacterial species (Lindsay *et al.,* 2006).

3.2. *S. aureus* infections and diseases

Being a part of the human normal flora, *S. aureus* asymptomatically colonizes the human body (Sollid *et al.,* 2014). Although the nasopharynx is the most important site for *S. aureus* colonization (Williams, 1963; Peacock *et al.,* 2001), this bacterium can be also found in the intestine, perineum, axillae, and on the skin (Armstrong-Esther, 1976; Wertheim *et al.,* 2005; Bhalla *et al.,* 2007; Acton *et al.,* 2009; Sollid *et al.,* 2014). It is now well-established that, *S. aureus* permanently colonizes nearly 20% of the human population all the time and an estimated 30% of humans carry *S. aureus* transiently (Eriksen *et al.,* 1995; Kluytmans and Wertheim, 2005; van Belkum *et al.,* 2009). This commensal relationship is usually unproblematic and harmless to the immunocompetent host, however certain immune-related conditions including a weakened immune system and altered microbiota can stimulate the bacteria to attack its hosting body, which may result in a wide range of diseases ranging from mild infections of the skin to severe and even life-threatening forms of pneumonia or septicemia (Lowy, 1998; Tong *et al.,* 2015). Nasal colonization is now well-known to be closely linked to the incidence of *S. aureus*-induced bacteremia, where the risk in colonized patients is 3-fold higher than in patients who are not colonized (Marzec and Bessesen, 2016). Besides humans, *S. aureus* further colonizes mammals including many farming- and household animals such as dogs, horses, sheep, pigs and cattle (Sutra and Poutrel, 1994; Quinn *et al.,* 2011; Smiet *et al.,* 2012), a characteristic representing a potential risk factor for dissemination of *S. aureus* infections back to humans (Lowder *et al.,* 2009; Haag *et al.,* 2019).

The pathogenesis of *S. aureus* infections involves up to four phases: local infection, systemic dissemination, metastatic infections, and toxinosis (Thomer *et al.,* 2016; Pietrocola *et al.,* 2017; Horino and Hori, 2020). Localized cutaneous infections develop when *S. aureus* is inoculated into the skin through a cutaneous incision or a breach. When reaching the underlying tissue, this pathogen creates its characteristic local abscesses or even induces more complicated clinical manifestations such as furuncles, carbuncles, cellulitis and pyomyositis (Roberts and Chambers, 2005). In this context, *S. aureus* is one of the major nosocomial pathogens, and the most common cause for surgical site infections (SSIs) (Tong *et al.,* 2015, Bhattacharya *et al.,* 2016; Pal *et al.,* 2019), with nearly 2% of all surgical incisions being reported to be infected with *S. aureus* in the United Kingdom (Lindsay, 2008).

If *S. aureus* reaches the lymphatic channels or blood, it may induce bacteremia and spreads systemically to different body organs causing a wide range of infections such as pneumonia, endocarditis, septic arthritis and epidural abscesses (Bamberger, 2007; Clerc *et al.,* 2011). *S. aureus* is reported as a leading cause of blood stream- and infective endocarditis (IE) infections in industrialized countries (Malachowa and DeLeo, 2011; Tong *et al.,* 2015). Additionally, *S. aureus* is one of the main causes of osteomyelitis (Lew and Waldvogel, 2004; Kavanagh *et al.,* 2018), a severe inflammatory disease of bones characterized by necrosis and loss of the bone matrix (Marriott, 2013). Furthermore, implant-related infections induced by colonization of medical devices such as artificial heart valves, intravascular catheters or synthetical joints with *S. aureus* are highly problematic for patients and difficult to be eradicated (Darouiche, 2004; El-Ahdab *et al.,* 2005; Stuart *et al.,* 2013). These infections are induced and even maintained by *S. aureus* owing to a variety of features supporting this pathogen including adherence molecules, invasive determinants and biofilm induction factors (Archer *et al.,* 2011; Heilmann, 2011).

Even without a systemic infection, several specific syndromes can arise due to extracellular toxins secreted by *S. aureus*. The main syndromes induced by such toxins include toxic shock syndrome (TSS), scaled skin syndrome, and food-borne gastroenteritis (Archer, 1998; Tong *et al.,* 2015). Importantly, the toxic-shock syndrome toxin-1 (TSST-1) is a superantigen expressed by several strains of *S. aureus* and induces the TSS (Kulhankova *et al.,* 2014), a severe manifestation characterized by several symptoms including high fever, erythematous rash and multiple organ failure (MOF) (Raumanns *et al.,* 1995). Staphylococcal scalded skin syndrome (SSSS) is another life-threatening condition resulting from disruption of keratinocytes adhesion by the secreted staphylococcal exfoliative toxins (ETs) (Mishra *et al.,* 2016).

3.3. Multi-drug resistance of *S. aureus*

Infections caused by *S. aureus* were initially tried to be controlled by penicillin since its implementation on the market in the 1940s (Rammelkamp and Maxon, 1942). However, the extensive use of penicillin resulted in the rapid emergence of penicillin-resistant strains of *S. aureus* (Rayner and Munckhof, 2005). Nearly all clinical isolates of this pathogen are nowadays resistant to penicillin, and most of these strains secrete a β-lactamase enzyme called penicillinase to hydrolyze the peptide bond in the β-lactam ring of penicillin, rendering it inactive (Heesemann, 1993). Attempting to control the newly emerging resistant strains, the penicillinase-resistant semisynthetic penicillin, methicillin was introduced into the clinical field (Chambers and Deleo, 2009; Jokinen *et al.,* 2017). However, the resistance against methicillin was also reported by the evolution of methicillin-resistant *S. aureus* (MRSA) strains (Jevons, 1961). Notably, these MRSA strains did not replace other *S. aureus* strains, instead they represent additional infectious strains (Lindsay, 2008). A major characteristic of MRSA strains is their tendency to accumulate additional resistance determinants that are functional against other classes of antibiotics such as aminoglycosides and tetracycline, rendering these isolates often to be described as multidrug-resistant strains (Trzcinski *et al.,* 2000; Benito *et al.,* 2014; Khosravi *et al.,* 2017).

The glycopeptide vancomycin and other semisynthetic lipoglycopeptides were thus developed and proved to be efficient, despite side effects, against severe infections caused by MRSA strains (Hiramatsu, 2001; Zeng *et al.,* 2016). However, *S. aureus* managed again to develop a quite fast resistance against vancomycin, and the number of vancomycin-resistant *S. aureus* (VRSA) isolates is still increasing in the hospital setting, making treatment options against these infections highly limited (Tenover *et al.,* 2001; McGuinness *et al.,* 2017; Cong *et al.,* 2020; Shariati *et al.,* 2020). The Centers of Disease Control and Prevention (CDC) reported at least 11.000 death cases and over 80.000 severe MRSA infections in the United States in 2011 (Dantes *et al.,* 2013). Furthermore, MRSA was reported to be the most prevalent antimicrobial resistant bacterial pathogen detected in hospitalized patients in Europe, North Africa and the Middle East (ECDC, 2012). The percentage of MRSA isolates among *S. aureus* infections in the hospital setting is still highly alarming, with over 50% of invasive infections in Portugal and Romania being reported to be caused by MRSA strains (ECDC, 2015). In the USA, the CDC reported a slow rate in controlling MRSA bloodstream infections in hospitals, despite of an overall decrease in MRSA infections in this country (CDC, 2019). Initially, infections caused by MRSA strains were thought to be only limited to hospitals (health care-associated methicillin resistant *Staphylococcus aureus* or HA-MRSA), however, beginning in the 90th of the last century, they were also increasingly detected outside the hospital setting and known as “community acquired methicillin resistant *Staphylococcus aureus”* (CA-MRSA)

infections (Raygada and Levine, 2009; David and Daum, 2010). In this regard, several geographically and genetically distinct CA-MRSA isolates were already observed worldwide (Stegger *et al.,* 2014). In a study performed by Self *et al*. (Self *et al.,* 2016), CA-MRSA strains were reported to be a major cause of community-acquired pneumonia in adults and healthy individuals even without having contact to health care settings. Similarly, several other studies concluded that CA-MRSA isolates are a common cause of severe necrotizing pneumonia (Gillet *et al.,* 2002; van der Flier *et al.,* 2003; Gómez *et al.,* 2009). In addition, Moran and colleagues (Moran *et al.,* 2006). reported that MRSA is considered the predominant causative agent of community associated skin and soft tissue infections (SSTIs) in patients admitted to the emergency departments in 11 hospitals in the USA. Because of this, both MRSA and VRSA strains have been classified by the World Health Organization (WHO) as high priority pathogens emphasizing the urgent need to develop new therapies to treat infections induced by these isolates (Govindaraj and Vanitha, 2018).

3.4. *S. aureus* is a well-armed pathogen

The ability of *S. aureus* to invade different cells of the body and induce such a wide range of severe diseases is attributed among others to its large arsenal of virulence factors **(Figure 3.2)** (Lowy, 1998; Foster, 2004; Gordon and Lowy, 2008; Otto, 2014; Jin *et al.,* 2021). *S. aureus* harbors genes encoding a variety of virulence factors including adhesion molecules, immune-modulatory molecules and toxins (Li *et al.,* 2019). The expression of these virulence factors in *S. aureus* is tightly regulated by a sophisticated network of regulatory molecules ensuring that they are expressed only as needed (Bischoff *et al.,* 2001; Bischoff and Romby, 2016). These factors and regulatory molecules work together in a coordinated manner to support this pathogen to attach to host cells, break down the host immune protective shield, enhance tissue invasion, induce persistence, and establish infections (Kim *et al.,* 2016).

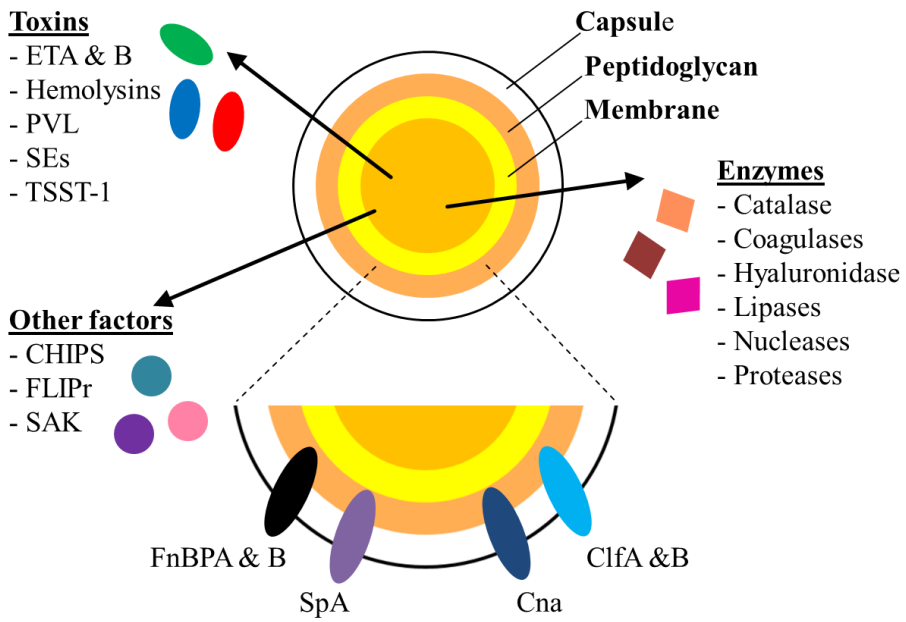

Figure 3.2: Virulence factors of *S. aureus*. Several virulence factors are expressed by *S. aureus* in order to establish infections and subvert the host immune responses. These virulence factors include adherence factors, invasive molecules, toxins and immunomodulatory molecules (adapted from Jin *et al.,* 2021).

A number of cell wall components of *S. aureus* are considered crucial virulence factors in this pathogen. Of importance, the capsule plays a central role in the resistance of this pathogen to the phagocytic clearance by host immune cells (Kuipers *et al.,* 2016). Being a Gram-positive pathogen, *S. aureus* contains a 20-30 nm thick peptidoglycan (PGN) layer which has a protective function in addition to providing a scaffold for attachment of other virulence factors, especially those required for adhesion (Sharif *et al.,* 2009). In addition, PGN fragments released during cell wall turnover induce the release of tumor necrosis factor alpha (TNF-α) (Wang *et al.,* 2000), a pleiotropic pro-inflammatory cytokine contributing to the inflammatory cascade (Wajant *et al.,* 2003).

Early in the initial stages of growth of *S. aureus*, a variety of cell wall associated proteins are synthesized in order to promote the attachment process in this pathogen. One major class of *S. aureus* adherence molecules comprises proteins which are covalently attached to the cell wall PGN and are commonly known as cell wall-anchored (CWA) proteins (Geoghegan and Foster, 2017). Members of the CWA proteins are staphylococcal protein A (SpA), collagen adhesin (Cna), Fibronectin binding proteins A and B (FnBPA and FnBPB respectively), and clumping factors A and B (ClfA and ClfB respectively) (Foster and Höök, 1998; Lowy, 1998). In addition to adhesion, some of these molecules also mediate immune-evasive functions

during staphylococcal infections. As an example, the staphylococcal protein A (SpA) is 42 kilodalton (kDa) protein that can bind the Fc region of immunoglobulin-G (IgG), thus hindering opsonophagocytosis, because IgG-coated bacterial cells avoid being recognized by Fc-receptors on PMNs (Jansson *et al.,* 1990; Foster, 2005). SpA also contributes to adherence of *S. aureus* to platelets and damaged endothelial cells by binding to a specific host factor (von-Willebrand factor) under low shear stress (Hartlieb *et al.,* 2000; Viela *et al.,* 2019). Fibronectin binding proteins A and B (FnBPA and FnBPB respectively) attach to fibronectin in the extracellular matrix (ECM) and on the cell surface mediating adherence of *S. aureus* to human endothelial cells (Peacock *et al.,* 1999). Moreover, the intracellular invasion of *S. aureus* inside NPPCs depends on the interaction between staphylococcal fibronectin binding proteins (FnBPs) and the cellular integrin α5β1 (Sinha *et al.,* 1999). Clumping factors A and B (ClfA and ClfB) are two *S. aureus* adhesion factors which are crucial for staphylococcal infections (Moreillon *et al.,* 1995). ClfA is required to the adhesion of *S. aureus* to the soluble plasma fibrinogen by binding to the C-terminal region of the fibrinogen γ-chain (McDevitt *et al.,* 1997; Herman-Bausier *et al.,* 2018). Similarly, ClfB binds to fibrinogen (Walsh *et al.,* 2008) and also to the squamous epithelial cell envelope protein, loricrin promoting nasal colonization of *S. aureus* (Schaffer *et al.,* 2006; Mulcahy *et al.,* 2012).

Another important category of adhesion factors in *S. aureus* is the so-called “secretable expanded repertoire adhesive molecules” (SERAMS). Members of this group of adherence molecules are secreted but partially rebound to the cell wall and includes exoproteins such as coagulases, extracellular adhesive protein (Eap), extracellular fibrinogen binding protein (Efb), and extracellular matrix protein (Emp) (Chavakis *et al.,* 2005). The main function of these adhesion molecules is to mediate the attachment of the pathogen to host cells and/or to modulate the host immune response. Remarkably, Eap interferes with migration of leucocytes towards sites of *S. aureus* infections by interacting with intercellular adhesion molecule-1 (ICAM-1) on the surface of endothelial cells (Haggar *et al.,* 2004; Chavakis *et al.,* 2005). Similarly, Efb contributes to complement inactivation through changing the conformation of the complement component 3 (C3) (Hammel *et al.,* 2007).

In later phases of the infection process, the synthesis of extracellular proteins is activated in *S. aureus*, converting the bacterial cells from being adherent to invasive (Kubica *et al.,* 2008). To accomplish this invasive role, *S. aureus* secrets several exotoxins and enzymes including nucleases, proteases, lipases, hyaluronidases, and collagenases (Dinges *et al.,* 2000). Importantly, *S. aureus* secretes large numbers of cytolytic toxins such as hemolysins, leukocidin, and Panton-Valentine leukocidin (PVL), if equipped with the corresponding genes (Aarestrup *et al.,* 1999; Kaneko and Kamio, 2004). α-hemolysin (Hla) is one of the best characterized and most potent membrane-damaging toxins of *S. aureus*. This toxin acts by

creating pores into the cell membrane of diverse host cells and is particularly active against erythrocytes, lymphocytes, platelets and monocytes (Liang *et al.,* 2011; Vandenesch *et al.,* 2012). Several strains of *S. aureus* also secrete β-hemolysin (Hlb), which is a type C-sphingomyelinase damaging the sphingomyelin of a variety of host cells (Dinges *et al.,* 2000). A two-component toxin is the γ-hemolysin (formed as HlgAB or HlgCB), which targets mainly the human red blood cells and leukocytes (Seilie and Bubeck Wardenburg, 2017). The δ-hemolysin is a low molecular weight exotoxin of *S. aureus* and acts by forming multimeric structures and lysing many cell types (Novick *et al.,* 2003). This toxin is also regarded as a member of the phenol-soluble modulins (Cheung *et al.,* 2014). Panton-Valentine leucocidin (PVL) is another bicomponent toxin produced only by specific *S. aureus* isolates and consists of two components, LukS-PV and LukF-PV. Both components assemble in high concentrations into a pore-forming heptamer within neutrophil membranes, thereby inducing neutrophil lysis, or internalize in low doses into the cell to form pores into the membranes of mitochondria, thereby inducing apoptosis of the cell (Kaneko and Kamio, 2004; Boyle-Vavra and Daum, 2007). Furthermore, *S. aureus* may produce additional types of exotoxins, if containing certain pathogenicity islands, including TSST-1, the staphylococcal enterotoxins (SEs) and the exfoliative toxins A and B (ETA and ETB respectively) (Bohach *et al.,* 1990). TSST-1 and SEs are known to act as pyrogenic toxin superantigens (PTSAgs), owing to their ability to stimulate the proliferation and activation of T-lymphocytes, thereby inducing a cytokine storm during infections (Marrack and Kappler, 1990). ETA and ETB are epidermolytic toxins largely responsible for the induction of the SSSS (Ladhani *et al.,* 1999), a severe desquamating skin rash characterized by hydrolysis of keratinocytes and formation of pus-filled bullae (Leung *et al.,* 2018).

Specifically, human-adapted variants of *S. aureus* also express a variety of secreted and cell surface-associated immunomodulatory molecules to evade the host immune response (Okumura and Nizet, 2014). Remarkably, the chemotaxis inhibitory protein of *S. aureus* (CHIPS) and the formyl peptide receptor-like 1inhibitor (FLIPr) interfere with PMNs extravasation and chemotaxis during infections (Thammavongsa *et al.,* 2015). In addition, *S. aureus* secretes the exoprotein staphylokinase (SAK) in order to inactivate the α-defensins and disturb the human complement system, thus modulating the immune evasion mechanisms used by this pathogen (Foster, 2005; Buchan *et al.,* 2019).

3.5. *S. aureus*-host immune cells battles

Upon infection, the host immune system immediately starts a battle against invading pathogens such as *S. aureus* by using several protective mechanisms (Koch *et al.,* 2012). As a result, numerous host immune cells, including among others neutrophils and macrophages, are activated and recruited to sites of infection (Chaplin, 2010, Selders *et al.,* 2017).

Neutrophils constitute 50-70% of all leukocytes in the human body, and thus represent a very important part of the host innate immune system (Mortaz *et al.,* 2018). While circulating in blood, neutrophils are attracted to site of infection by chemical mediators which are formed and secreted through diverse host cells at the infection site (Rosales, 2018). After approaching the infection focus, neutrophils recognize the pathogen via interactions mediated by pattern recognition receptors (PRRs) such as Toll like receptors (TLRs). These receptors interact with conserved microbial molecules called pathogen-associated molecular pattern (PAMPs), allowing neutrophils to target and internalize bacterial cells. Uptake of bacteria by the immune cell induces the formation of a phagosome around the internalized bacteria, which will subsequently maturate into a highly cytotoxic compartment called phagolysosome to kill the phagocytosed bacteria (Abbas and Lichtman, 2009). Alternatively, neutrophils have the ability to kill extracellular bacteria without engulfing them by releasing the so-called NETs upon activation (Kaplan and Radic, 2012; Apel *et al.,* 2021). These traps consist of decondensed DNA as backbone known as extracellular DNA (eDNA) together with proteases and antimicrobial peptides (AMPs) such as defensins and myeloperoxidases (Brinkmann *et al.,* 2004). This combined activity of trapping and destroying pathogens have been reported to be very effective in controlling *S. aureus* infections (Wang *et al.,* 2017). However, *S. aureus* is not helpless against activated neutrophils and possesses many strategies to resist neutrophil-mediated killing. Interestingly, *S. aureus* may counteract the trapping by NETs via the secretion of nucleases which degrade these immune-decorated DNA nets (Berends *et al.,* 2010; Thammavongsa *et al.,* 2013). Furthermore, *S. aureus* may modulate NETosis by secreting the SERAM Eap, which is a potent inhibitor of neutrophil elastase (Stapels *et al.,* 2014), a major host factor triggering the decondensation of chromatin and thus NET formation (Papayannopoulos *et al.,* 2010). Additionally, Eap prevent the formation of NETs by aggregating the decondensed DNA on the neutrophil cell surface (Eisenbeis *et al.,* 2018). The *S. aureus* nuclease-driven degradation of NETs is also deleterious to macrophages because *S. aureus* converts the DNA degradation products 2′-deoxyadenosine-3′-monophosphate and 2′-deoxyadenosine-5′-monophosphate into 2′-deoxyadenosine (dAdo), which in turn stimulate apoptosis of macrophages (Thammavongsa *et al.,* 2013). Notably, Hoppenbrouwers *et al.* (Hoppenbrouwers *et al.,* 2018) demonstrated that SpA mediates NETosis in *S. aureus* in a process linked to the ability of this pathogen to kill neutrophils. Another way of protection

against neutrophils is the secretion of proteases by *S. aureus* in order to inactivate neutrophils. Staphopain A (ScpA) is such a protease produced and secreted by *S. aureus* in order to render neutrophils to be unresponsive to CXCR2 chemokines, thus interfering with neutrophil migration during infection (Laarman *et al.,* 2012). The second Staphopain, ScpB, is involved in staphylococcal spread of infections by interacting with CD31 on the surface of neutrophils (Smagur *et al.,* 2009). Aureolysin (Aur) is a zinc-metalloprotease produced by *S. aureus* which is particularly important for the bacterium after being phagocytosed by the immune cell to protect them inside the phagolysosome by enhancing its resistance against AMPs (Kubica *et al.,* 2008).

Macrophages are another important partner in the immediate battle against infections with *S. aureus*. In contrast to neutrophils, macrophages are less immunoreactive cells, a requisite for these cells to patrol tissues searching for pathogens (Davies and Taylor, 2015). When facing *S. aureus* at the site of invasion/infection, macrophages phagocytose *S. aureus* and subsequently destroy the bacterial cells by using a multitude of killing mechanisms (Pidwill *et al.,* 2021). The importance of macrophages in controlling *S. aureus* infections was clearly demonstrated when animal models with depleted macrophages were infected. Surewaard and colleagues (Surewaard *et al.,* 2016) reported that depletion of macrophages in mice infected with *S. aureus* yielded increased bacterial loads and mortality. Likewise, depletion of alveolar macrophages interfered with the clearance of *S. aureus* infections in murine lung infection models (Cohen *et al.,* 2016). However, macrophages do not necessarily succeed to control *S. aureus* infections, and they may even act as intracellular niches for *S. aureus*, thus allowing bacterial persistence and dissemination. Michailova and colleagues (Michailova *et al.,* 2000) reported that *S. aureus* can survive phagocytosis by rat and mice macrophages. Human monocyte-derived macrophages (MDMs) also failed to kill all internalized *S. aureus* cells and allowed for an intracellular survival of *S. aureus* for at least seven days after internalization without killing the hosting macrophages (Kubica *et al.* 2008). These long-term intracellularly persisting *S. aureus* cells may even start to kill the hosting cell later to release themselves into the extracellular milieu, which, together with the migrating ability of macrophages within the body, is suspected to significantly contribute to the dissemination of *S. aureus* within the host by seeding infections at distant anatomic sites (Lacoma *et al.,* 2017). Such a scenario is exemplified by phagocytosis of *S. aureus* by Kupffer cells in the liver of mice, which is followed by a bacterial escape into the peritoneal cavity, where the bacterial cells were again phagocytosed by peritoneal macrophages. Surprisingly, these two waves of phagocytosis by macrophages were not successful to eliminate *S. aureus*, instead they provided new intracellular niches for the bacterium, and dissemination of the pathogen to other internal organs was observed (Jorch *et al.,* 2019).

The complement system is a central part of the human immune response against bacterial pathogens (Dunkelberger and Song, 2010). It is composed mainly of several proteins which are activated by cleavage in three different ways: the classical, the alternative and the lectin pathway (Seelen *et al.,* 2005). During bacterial infections, proteins of this system are activated in order to recognize the invading bacterial pathogens, thereby forming enzyme complexes known as C3 convertases (Rooijakkers *et al.,* 2009). These complexes cleave the complement component C3 into two proteins, C3a and C3b (Ricklin *et al.,* 2016). The protein C3b is required for opsonization of *S. aureus* cells, making them palatable for phagocytic cells during phagocytosis (Cunnion *et al.,* 2004). Deficiency of C3 in mice has been shown to increase their suitability to *S. aureus* infections (Na *et al.,* 2016). Inactivation of the complement system is one of the major mechanisms used by *S. aureus* to override host innate immunity and to establish infections. To accomplish this, *S. aureus* secretes among others Aur to cleave C3 to C3b, which is then degraded by the combined actions of factor H and Factor I in the serum (Laarman *et al.,* 2011). This depredation of C3b results in poor opsonization and phagocytosis of *S. aureus* by phagocytic cells. Similarly, *S. aureus* secretes the SERAM Efb to inhibit the classical and alternative pathways of activation by binding to the α-chain of C3, thereby blocking its deposition (Lee *et al.,* 2004). Reduction of the enzymatic activity of C3 convertase complexes is also accomplished by the staphylococcal complement inhibitors (SCIN) (Jongerius *et al.,* 2007). Moreover, SpA and SAK inhibit recognition of *S. aureus* by C1q component resulting in inhibition of the classical complement activation pathway (Laarman *et al.,* 2010; Cruz *et al.,* 2021).

3.6. Protein phosphorylation in bacteria

Reversible protein phosphorylation is a PTM controlling several biological processes in all living organisms (Ubersax and Ferrell, 2007; Sacco *et al.,* 2012; Salomon and Orth, 2013). In these organisms, a wide range of amino acid residues such as arginine (Arg) aspartate (Asp), histidine (His), serine (Ser), threonine (Thr) and tyrosine (Tyr) can be modified (Roskoski, 2012). Bacteria utilize these reactions mainly to adapt to adverse environmental conditions which usually change rapidly (Kobir *et al.,* 2011). This way, adding of negatively charged phosphate groups, usually originating from adenosine triphosphate (ATP) to side chains of amino acids induces a variety of fast transcriptional responses (Johnson and Barford, 1993; Hunter, 1995). Thus, phosphorylation can result in a direct modification of the activity of targeted proteins or establish docking sites for other phospho-binding domains (Waksman *et al.,* 1992; Seet *et al.,* 2006). These domains are required for other downstream protein-protein

interactions and are thus central for phosphorylation-based signal transduction pathways (Jin and Pawson, 2012).

Major characteristics of theses reactions are being dynamic and reversible (Soulat *et al.,* 2002). Thus, phosphorylation/dephosphorylation reactions are controlled by two kinds of opposing enzymes: phosphotransferases (also known as protein kinases) and protein phosphatases. The opposing functions of these enzymes thus control the extent of phosphorylation status of proteins, where kinases add phosphate groups on amino acid residues and phosphatases remove the phosphate moieties from the amino acid side chains (Mijakovic and Macek, 2012). In addition of being important for the regulation of numerous bacterial intrinsic pathways, several pathogens translocate these enzymes into host cells to interfere with host signal transduction pathways (Canova *et al.,* 2014). Excellent overviews on pathogen-encoded phosphatases reported to modify host-pathogen interactions by directly interacting with protein targets within host cells are given by (Whitmore and Lamnot, 2012; Heneberg, 2012).

3.6.1. Protein tyrosine phosphatases (PTPs) in bacteria

Phosphorylation of proteins on Tyr residues has been long considered as a mechanism specific only to eukaryotes, where it comes at the head of many signal transduction pathways, and thus controlling a variety of cellular processes including cell division, cell growth and metabolism (Fantl *et al.,* 1993; Hunter, 1995). Later on, protein phosphorylation on Tyr has been reported to occur in both, Gram-negative and Gram-positive bacteria as well (Cozzone, 1993). In addition to its considered role as a regulating device of bacterial physiology, previous work linked this PTM also to the virulence and survival capacities of some pathogens (Ge and Shan, 2011; Whitmore and Lamont, 2012). As such, numerous bacterial tyrosine phosphatases were also reported to be secreted into host cells during infections (Whitmore and Lamont, 2012).

Structure:

Bacterial PTPs can be categorized into three distinct groups: eukaryotic-like and dual-specificity phosphatases (DSPs), the polymerase and histidinol family of phosphoesterases (PHPs), and the low-molecular-weight protein tyrosine phosphatases (LMW-PTPs) (Aravind and Koonin, 1998; Standish and Morona, 2014). DSPs catalyze the dephosphorylation of substrates on phosphorylated Ser and Thr residues in addition to Tyr. The DSPs and LMW-PTPs enzymes utilize a common catalytic mechanism that includes the conserved cysteine-X5-arginine (C-X5-R) motif in the phosphate binding loop, where cysteine attacks the

phosphorus atom of the phosphotyrosine residue of the substrate (Whitmore and Lamont, 2012). The Arg residue also interacts with the phosphate moiety of the phosphotyrosine (Tiganis, 2002). Members of the LMW-PTPs and PHPs families are specific for phosphorylated Tyr residues and are found across a spectrum of genera including bacteria and eukaryotes (Aravind and Koonin, 1998). In addition, phosphatases of the PHPs family have four motifs in common, termed domains I to IV, which are required for the metal binding (Davis *et al.,* 1994).

Functions:

Since the discovery of the first bacterial tyrosine phosphatase in 1992 (Zhang *et al.,* 1992), a large set of phosphatases has been identified in different bacterial pathogens. The biological significance of these PTPs is still not fully understood, however, in some bacteria, they have been reported to play an important role as secreted effector proteins and are integrated in the potential manipulation of signal transduction pathways of host cells during infections (Kennelly and Potts, 1996; Caselli *et al.,* 2016). In **Figure 3.3**, PTPs and their proposed functions in different pathogens during infections are summarized.

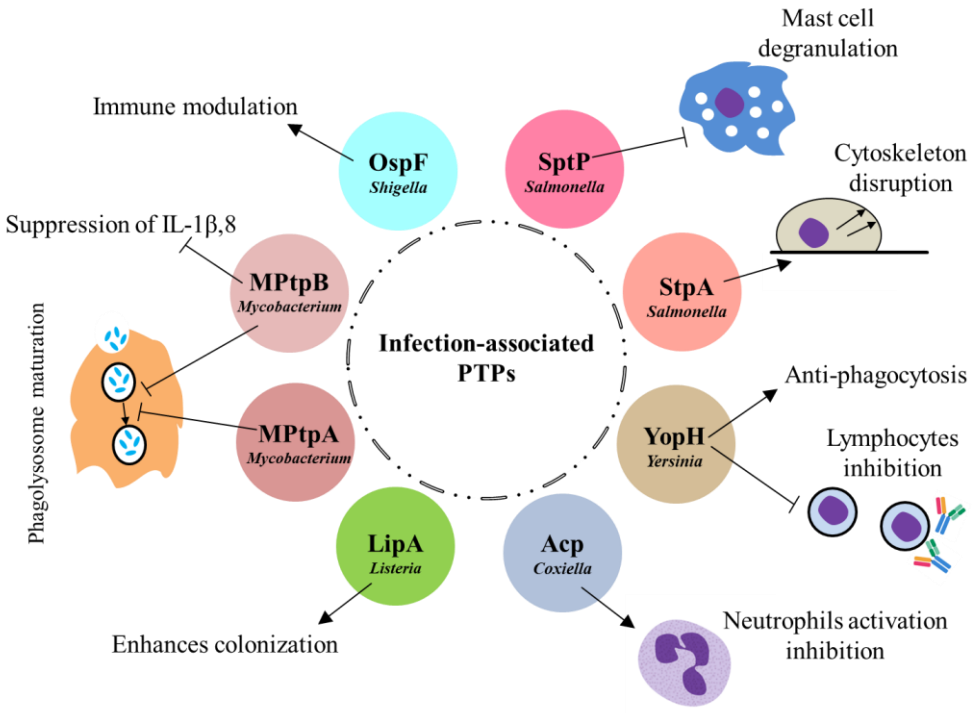

Figure 3.3: PTPs in bacterial pathogens and their impact on host cells. Several bacterial PTPs are reported in bacteria to induce cellular changes in host cells. In this schematic representation, proteins names and genera of bacteria and their impact on host cells are indicated.

The Gram-negative bacterium, *S. typhimurium* secretes the LMW-PTP SptP (*Salmonella* protein tyrosine phosphatase), which is a required for full virulence of the intracellular pathogen in the mice infection model (Kaniga *et al.,* 1996). SptP suppresses mast cell degranulation, which in turn reduces the recruitment of PMNs and facilitates bacterial dissemination (Kawakami and Ando, 2013). *Salmonella typhi (S. typhi)* is another bacterial pathogen secreting a SptP-like phosphatase called StpA in order to disturb the host cell cytoskeleton during infection (Arricau *et al.,* 1997). Similarly, the PTP YopH is secreted by pathogenic *Yersinia* spp. into mammalian host cells to block phagocytosis by subverting phosphotyrosine signaling pathways in the host, thus inhibiting bacterial uptake by host cells (Hamid *et al.,* 1999; Montagna *et al.,* 2001). This process is achieved by dephosphorylation of proteins of the focal adhesion complex such as p130Cas and focal adhesion kinase (FAK) which in turn inhibits phagocytes by detaching of actin structures (Persson *et al.,* 1997). In addition, YopH suppresses the degranulation of neutrophils by blocking Ca^{2+} signaling and prevents T-cell activation (Persson *et al.,* 1999; Yao *et al.,* 1999). *Coxiella burnetti (C. burnetti)* is Gram-negative obligate intracellular pathogen encoding a phosphatase called Acp, which is reported to inhibit the activation of neutrophils via repressing the metabolic burst of formyl-Met-Leu-Phe (fMLP)-stimulated cells (Li *et al.,* 1996). *Shigella flexneri* (*S. flexneri*) is another example of a Gram-negative bacterium producing a DSP called OspF, which dephosphorylates mitogen-activated protein kinase and prevent histone H3 phosphorylation (Arbibe *et al.,* 2007). A reduction in histone H3 phosphorylation interrupts the access of the transcription factor NF-kB to the chromosome, allowing *S. flexneri* to modulate host cell immune response strategies (Whitmore and Lamont, 2012).

In Gram-positive bacteria, PTPs were documented in both pathogenic and non-pathogenic bacterial species. The pathogen *Listeria monocytogens* (*L. monocytogens*) secretes the protein *Listeria* phosphatase A (LipA), which exerts a dual function as a tyrosine and phosphoinositide phosphatase (Beresford *et al.,* 2009; Kastner *et al.,* 2011), and is reported to enhance the bacterial load and inflammatory cytokine production in *L. monocytogens*-infected mice. Of importance, the genome of *L. monocytogens* does not encode proven Tyr kinases (Glaser *et al*., 2001). Thus, LipA produces these effects mostly by dephosphorylating Tyr-phosphorylated host cell proteins or lipids (Kastner *et al.,* 2011). Likewise, the human pathogen *Mtb* secretes two LMW-PTPs known as MPtpA and MPtpB (abbreviation of Mycobacterium Protein tyrosine phosphatase A and B respectively) (Koul *et al.,* 2000; Grundner *et al.,* 2005). Expression of MPtpA is upregulated when the bacterial cells are internalized in host cells, and it interacts with the host vacuolar-H^+-ATPase complex and blocks its trafficking to the mycobacterial phagosome, thus hindering the process of phagosome maturation (Bach *et al.,* 2008; Wong *et al.,* 2011). The phosphatase MPtpB

suppresses the innate immune response by inhibiting the production of inducible nitric oxide synthase (iNOS) as well as the expression of interleukin (IL)-1β and IL-6 (Zhou *et al.,* 2010, Fan *et al.,* 2018). In addition, it contributes to the intracellular survival of *Mtb* inside host cells by acting on cytoskeleton proteins such as Coronin-1 and Profilin-1and blocking phagosome-lysosome fusion (Dhamija *et al.,* 2019).

3.6.2. Protein arginine phosphatases (PAPs) in bacteria

In fact, phosphorylation of proteins on Arg residues has become a hot topic in bacterial physiology only recently (Suskiewicz and Clausen, 2016). In a few Gram-positive bacteria such as *Bacillus subtilis* (*B. subtilis*) and *S. aureus*, hundreds of phosphoarginine (pArg) sites were described in the bacterial proteomes (Elsholz *et al.,* 2011; Junker *et al.,* 2018). Initially, YwlE was identified as the first PAP to be discovered in bacteria, which counteracts the phosphorylating functions of McsB, a protein arginine kinase in Gram-positive bacteria (Elsholz *et al.,* 2011; Fuhrman *et al.,* 2009; Fuhrmann *et al.,* 2013). Trentini *et al.* (Trentini *et al.,* 2016) stated that both proteins are required for the regulation of pArg-based cellular pathways. Further studies categorized the pArg-phosphatase YwlE within the LMW-PTPs family and it was initially thought that YwlE acts only on phosphorylated Tyr residues (Xu *et al.,* 2006), as it adopts the conserved set of active site residues specific for this family of phosphatases (Zhang, 2003). Surprisingly, Fuhrman and colleagues (Fuhrmann *et al.,* 2013) observed that YwlE is highly specific to target pArg residues, and its activity towards pArg residues is attributed to a Thr residue within the catalytic loop CXXXTCR (S/R).

Arg phosphorylation is also known to affect the regulation of various cellular functions in bacteria (Schmidt *et al.,* 2014). The McsB/YwlE pair was shown to regulate the activity of the heat shock response regulator CtsR (Class three stress gene Repressor) of *B. subtilis*, a central transcription factor involved in stress adaptation in Gram-positive bacteria. Under stress conditions, *B. subtilis* releases McsB to phosphorylate and inhibit CtsR by displacing it from DNA preventing the protein-DNA complex formation, which intern increases the expression of the two heat shock genes: *clpC* and *clpP* (Derre *et al.,* 1999; Fuhrmann *et al.,* 2009). Subsequently, the products of these two genes constitutes the ClpCP degradation system required for degradation of misfolded proteins (Michel *et al.,* 2006). Importantly, Trentini and colleagues (Trentini *et al.,* 2016) reported also that McsB-mediated Arg phosphorylation functions as signal for this degradation system, allowing it to recognize stress-damaged proteins. In addition, the highly nucleophilic cysteine (Cys) residue of YwlE acts also as a sensor for oxidative stress forming a reversible disulfide bridge inactivating the protein Arg dephosphorylating activity (Fuhrmann *et al.,* 2016).

3.7. PtpA and PtpB in *S. aureus*

S. aureus contains a tyrosine phosphatase, called Phosphatase C (du Plessis *et al.,* 2002). This phosphatase is a homologue of protein CpsB, which shows a PHP-activity in *Streptococcus pneumoniae* (*S. pneumoniae)* (Morona *et al.,* 2002). Interestingly, the genome of *S. aureus* encodes two additional phosphatases of the LMW-PTPs family termed PtpA and PtpB (Soulat *et al.,* 2002). Both proteins contain the two conserved active-site sequence motifs (D-P-Y) (amino acids 120 to 122: aspartic acid, proline, tyrosine) and (C-X_4-C-R) (amino acids 8 to 15: cysteine, 4 unknown amino acids, cysteine, arginine), which are designated to characterize this family of phosphatases (Soulat *et al.,* 2002). The genome analysis of different strains of *S. aureus* showed that *ptpA* and *ptpB* are widely conserved across this species. Furthermore, both genes are separated by ~221,000 nucleotides, suggesting that they do not belong to the same operon. By calculating the nucleotide sequence, the molecular mass for both proteins were estimated to be 17,493 Da for PtpA and 15,790 Da for PtpB (Soulat *et al.,* 2002). Moreover, Comparative sequence analysis revealed that both proteins share a sequence similarity of 50% and sequence identity of 27% (Vega *et al.,* 2011).

Structurally, both PtpA and PtpB follow the general architecture specific for LMW-PTPs. On one side, PtpA is a single domain protein with a central connected four-stranded parallel β-sheet forming the active site. Sheet 1 and helix 1 are connected by the phosphate binding loop (P-loop) which harbors the catalytic Cys residue (Cys8). The P-loop adopts the CX4CR motif of LMW-PTPs comprising residues 8 to 15. This motif is also surrounded by five helices. Helix 5 is present on one side of PtpA and two 12 and 15 amino acid long loop regions which are further stabilized by three β-turns are located on the other side **(Figure 3.4A)** (Vega *et al.,* 2011). In addition to Cys8, a second Cys residue (Cys13) is also found in the P-loop and functions to provide protection against oxidative inactivation of the catalytic site (Raugei *et al.,* 2002). On the other hand, PtpB is also a single α/β protein folding into five α-helices and four β-strands which are then connected by a number of unstructured loops. Long helices and loops flank the centrally located and twisted four-stranded β-sheet. The P-loop is located between the C-terminus of the β1 and the N-terminus of α1. In addition, a variable loop of residues connects the β2 and α2. A long flexible loop harboring the DPY motif connects the α4 and α5 helices. **(Figure 3.4B)** (Mukherjee *et al.,* 2009).

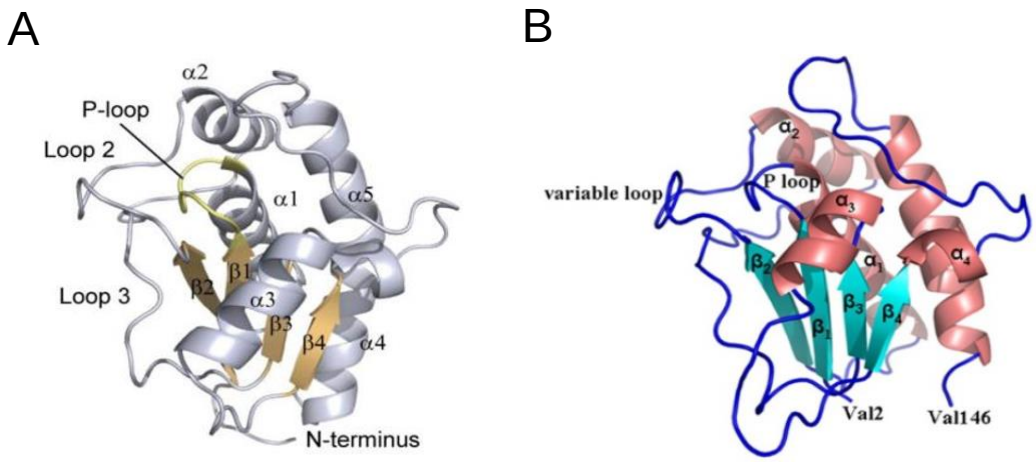

Figure 3.4: 3D-Structures of PtpA and PtpB in *S. aureus.* (A) PtpA consists of 4-chain β-sheets (beige) connected to the P-loop (yellow). This loop contains the catalytically active cysteine (Cys8) and is framed by the 5 α-helices (gray) (Vega *et al.,* 2011). (B) PtpB consists of 4-chain β-sheets (turquoise) and 5 α-helices (pink). The P-loop forms the active site and connects β-sheets and α-helices (Mukherjee *et al.,* 2009).

Both PtpA and PtpB showed reactivity toward phosphorylated Tyr substrates in *S. aureus* (Soulat *et al.,* 2002; Mukherjee *et al.,* 2009). Notably, deletion of *ptpA* or *ptpB* did neither influence the *in vitro* growth kinetics nor the cell wall integrity of *S. aureus* if cultured in rich medium (Vega *et al.,* 2011), leading to the assumption that both phosphatases might be involved in infectivity of this bacterium (Vega *et al.,* 2011). Interestingly, Brelle and colleagues (Brelle *et al.,* 2016) reported that *S. aureus* secretes substantial amounts of PtpA into the growth medium during the *in vitro* culturing, even though this protein shows no obvious export pathway signal at its N-terminus. PtpB was originally categorized as LMW-PTP in *S. aureus* with a specificity towards phosphorylated Tyr residues as mentioned previously (Soulat *et al.,* 2002). However, a striking feature of PtpB is its ability to dephosphorylate pArg residues in *S. aureus*. This feature was only recently revealed by Junker and colleagues (Junker *et al.,* 2018), who observed that deletion of *ptpB* in the *S. aureus* strain COL, a MRSA strain belonging to clonal complex 8 (CC-8), produced 207 proteins with pArg sites in the *ptpB* deletion mutant compared to only 8 sites in the wild-type strain. This finding indicated that the *ptpB* mutant strain lost its ability to dephosphorylate Arg-phosphorylated proteins. This overlap of Tyr and Arg phosphorylation by PtpB is of major concern, because it indicates that the conserved active site of LMW-PTP might target not only phosphorylated Tyr but also phosphorylated Arg residues, if the catalytic loop consists of a CXXXTCR motif. This dual function might be explained by the fact that phosphorylation of Arg involves the formation of high energetic phosphate ester bonds (Hunter *et al.,* 2012). The chemical properties of these

bonds suggest the possibility of the occurrence of phosphate transfer to other proteinogenic amino acids such as Tyr, if they are accessible for the catalytic domain.

Among the pArg proteome, a number of regulatory proteins were found to be phosphorylated on Arg residues in *S. aureus* when *ptpB* was deleted, including CtsR and MgrA (Junker *et al.,* 2018; Junker *et al.,* 2019). In *S. aureus*, CtsR act mainly as a repressor of heat shock genes, which is inactivated under oxidative and other stress conditions, thus ensuring a fast and efficient adaption of bacterial cells to such stresses (Elsholz *et al.,* 2011; Wozniak *et al.,* 2012). This inactivation results presumably from the McsB-induced phosphorylation of Arg residues within the DNA-binding domains of CtsR, as has been observed for the CtsR homologue of *B. subtilis* (Krüger *et al.,* 2001). Earlier work on McsB demonstrated that its staphylococcal homologue is involved in pathogenicity (Wozniak *et al.,* 2012), suggesting a similar role for its cognate phosphatase PtpB in *S. aureus*.

The Sar-family transcription factor MgrA is major global multiple gene regulator controlling the transcription of around 350 genes (Luong *et al.,* 2006), which are involved among others in capsule biosynthesis, toxin production, nuclease production and autolytic activity of *S. aureus* (Ingavale *et al.,* 2003). In addition, MgrA acts as an oxidant sensor via its unique redox-sensitive Cys12 residue which is located in the N-terminal helix α1 in the dimerization domain. Various reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) and organic hydroperoxide were reported to disturb the hydrogen bound network around Cys12, which in turn results in in dissociation of MgrA from its DNA target sequences. This oxidation-sensing mechanism is used to modulate several signaling pathways utilized by *S. aureus* during infections (Chen *et al.,* 2006).

3.8. Protein phosphatases as potential drug targets

Since protein phosphatases (PPs) represent a large family of bacterial signaling enzymes, they are considered as attractive key targets for new therapies (Martins *et al.,* 2015; Stanford and Bottini, 2017). Despite this, most of the clinical trials aiming to develop new drugs targeting protein phosphorylation are directed towards the inhibition of protein kinases (Cohen, 2002; Kurosu and Begari, 2010). Nevertheless, a number of protein phosphatase inhibitors were also developed as potential drugs, however, the majority of them are exploited against infections caused by *Mtb* and Yersinia spp. (Grundner *et al.,* 2008; Chiaradia *et al.,* 2008, Bahta and Burke, 2012). One such trial targeted the ClpC1 ATPase enzyme, an essential enzyme of the ClpC1P1P2 proteolytic complex required for viability of *Mtb* (Akopian *et al.,* 2012; Gavrish *et al.,* 2014). In addition, specific chemical inhibitors against MptpA and MptpB

were identified and considered as alterative strategy to using antibiotics. These inhibitors target different mechanisms including the disturbance of the protein catalytic loop (reviewed in Ruddraraju *et al.,* 2021). The PTP YopH produced by *Yersinia* spp. has been also investigated in depth in order to develop specific inhibitors, which act mostly by binding to the active site of this enzyme (Taylor *et al.,* 1996; Phan *et al.,* 2003). Similarly, Hu and colleagues (Hu *et al.,* 2004) performed docking study of the YopH and the *Salmonella* SptP in which they revealed distinctive characteristics in the binding modes of both proteins. In a trial to enhance the inhibitory potency against YopH, Lee *et al.* (Lee *et al.,* 2005) described a modification of the previously characterized monoanionic pTyr mimetic-based PTP inhibitors in which adding the 5-methylindolyl group at the N-terminus resulted in a potent inhibitory effect against the YopH.

Likewise, novel drugs are required to combat infections caused by *S. aureus* and encounter the multidrug resistance developed by this pathogen (Payne, 2008; Nelson *et al.,* 2021). Inhibitors of protein phosphatases have been already considered as promising putative agents to combat infections caused by this pathogen (Jarick *et al.,* 2018). However, the majority of trials focused mainly on developing specific inhibitors against Ser/Thr kinases (STKs), which are important virulence modulators in *S. aureus* (Zheng *et al.,* 2016). A challenge to be overcome is to develop selective therapeutic agents against PtpA and PtpB due to their highly conserved active site. However, several features of the active site and surface organization of the staphylococcal phosphatases, especially PtpA (Vega *et al.,* 2011), supporting the idea that developing new therapies specifically targeting these phosphatases in *S. aureus* might be feasible.

4. Aim of thesis

Infections caused by MRSA strains represent an ongoing health problem, causing high morbidity and mortality (Raygada and Levine, 2009; Turner *et al.,* 2019). This fact has been more complicated due to the emergence of several MRSA strains reported to be resistant against almost all currently available antibiotics (Vestergaard *et al.,* 2019). For these reasons, it is of highest priority to find new treatment methods against infections induced by this pathogen. In order to be able to develop new anti-infectious medicines, there is an urgent need to know more about the molecular mechanisms used by this pathogen to establish infections, especially those involved in interactions of *S. aureus* with the host immune cells.

The low molecular weight protein phosphatases (LMW-PPs) are already well investigated in other bacterial pathogens and linked to the ability of these pathogens to induce and establish infections. The fact that *S. aureus* contains two LMW-PPs, PtpA and PtpB, which were only partially characterized in this pathogen at the beginning of this thesis work, let to the motivation to investigate and characterize the roles of these two proteins on the infectivity of *S. aureus* in this thesis. Having the structure of *S. aureus* PtpA and PtpB available in hand from previous studies (Mukherjee *et al.,* 2009; Vega *et al.,* 2011), we are then potentially one step closer to target these proteins. Specifically, i intended to characterize the effects of deletion of *ptpA* or *ptpB* on the intracellular survival capacity of *S. aureus* inside macrophages, and the effect of both proteins on the *in vivo* infectivity of *S. aureus* in a mouse model of infection. Given the impact of PtpB on the Arg phosphorylation of CtsR and MgrA in *S. aureus* (Junker *et al.,* 2018; Junker *et al.,* 2019), the ability of the Δ*ptpB* deletion mutant cells to adapt to different stress conditions was also investigated. In addition, i intended to investigate the effects of a *ptpB* deletion on the exonuclease activity, and the proteolytic, hemolytic and autolytic activities of *S. aureus*. In order to gain deeper insights into the molecular mechanisms that are responsible for the PtpB-driven effects on stress adaptation and infectivity of *S. aureus*, qRT-PCR analyses were planned for stress resistance and virulence genes presumably influenced by PtpB.

5. Results

This cumulative thesis is based on three peer-reviewed publications which are presented in this part.

Article (I)

PtpA, a secreted tyrosine phosphatase from *Staphylococcus aureus*, contributes to virulence and interacts with coronin-1A during infection

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Edited by Ursula Jakob

Secretion of bacterial signaling proteins and adaptation to the host, especially during infection, are processes that are often linked in pathogenic bacteria. The human pathogen *Staphylococcus aureus* is equipped with a large arsenal of immune-modulating factors, allowing it to either subvert the host immune response or to create permissive niches for its survival. Recently, we showed that one of the low-molecular-weight protein tyrosine phosphatases produced by *S. aureus*, PtpA, is secreted during growth. Here, we report that deletion of *ptpA* in *S. aureus* affects intramacrophage survival and infectivity. We also observed that PtpA is secreted during macrophage infection. Immunoprecipitation assays identified several host proteins as putative intracellular binding partners for PtpA, including coronin-1A, a cytoskeleton-associated protein that is implicated in a variety of cellular processes. Of note, we demonstrated that coronin-1A is phosphorylated on tyrosine residues upon *S. aureus* infection and that its phosphorylation profile is linked to PtpA expression. Our results confirm that PtpA has a critical role during infection as a bacterial effector protein that counteracts host defenses.

The success of *Staphylococcus aureus* as a pathogen and its ability to cause a wide range of disease patterns are the result of its large arsenal of virulence factors that is controlled by a sophisticated network of regulatory molecules (reviewed in Refs. 1 and 2). A number of experiments assessing the invasion and the intracellular survival of *S. aureus* in endothelial and epithelial cells as well as osteoblasts suggest that such events may contribute to the persistence of *S. aureus* during infections such as endocarditis, bovine mastitis, and osteomyelitis (3). Other work demonstrated that *S. aureus* bacteria possess a high level resistance to neutrophil (4) and macrophage (5) mediated killing, and it has been proposed that professional phagocytes may serve as intracellular reservoirs of *S. aureus* (5, 6). It is nowadays well accepted that the facultative intracellular lifestyle of *S. aureus* contributes to recurrent infections that are frequently observed with this species (7). The pathogen is able to replicate in the phagosome or freely in the cytoplasm of its host cells, and may escape the phagolysosome of professional and nonprofessional phagocytes, subvert autophagy, induce cell death mechanisms, such as apoptosis and pyronecrosis, or may induce anti-apoptotic programs in phagocytes (reviewed in Ref. 8). Earlier work demonstrated that a subpopulation of ingested *S. aureus* bacteria can survive for up to 7 days within macrophages (5, 9), and a number of *S. aureus* global regulators and secreted virulence factors have been identified that contribute to this ability (5, 6, 10–13). However, one strategy utilized by a number of pathogenic bacteria, the secretion of bacterial signaling proteins into target host cells (14, 15), thereby directly modulating host signaling networks, has not yet been studied with *S. aureus*.

Recently, numerous host-pathogen interactions were found to be dependent on pathogen-secreted phosphatases (16–20). Bacterial tyrosine phosphatases catalyze the dephosphorylation of tyrosyl-phosphorylated proteins, which in turn can result in either the propagation or inhibition of phospho-dependent signaling. Whereas bacterial tyrosine phosphatases can be intimately involved in a number of cellular processes, one major theme has become apparent with the involvement of tyrosine phosphatases as secreted effectors with the potential for manipulation of host cell signal transduction pathways (18). Although a detailed picture is yet unavailable, a role of secreted bacterial protein-tyrosine phosphatases during host infection has been identified in different facultative and obligate intracellular pathogens, and the strategies employed by them are currently being elucidated. For instance, the protein-tyrosine phosphatase YopH is a major virulence factor of *Yersinia spp.* that is injected into epithelial cells by the type III secretion machinery of the pathogen. YopH can uncouple multiple signal transduction pathways (21), and in human epithelial cells, YopH dephosphorylates several focal adhesion pro-

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This article contains Figs. S1–S3.
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teins, including p130Cas (Cas), focal adhesion kinase, and paxillin (22–24). Similarly, *Salmonella typhimurium* translocates the low-molecular-weight (LMW)[5] protein-tyrosine phosphatase (PTP) SptP into epithelial cells to reverse mitogen-activated protein kinase activation (25). Moreover, SptP is required for full virulence in murine models of disease (26). *Mycobacterium tuberculosis* (Mtb) secretes two LMW-PTPs, termed PtpA and PtpB (27). Expression of PtpA in Mtb is up-regulated within monocytes, and PtpA is secreted from Mtb into the host macrophage cytosol and disrupts key components of the endocytic pathway, resulting in the arrest of phagosome maturation (20, 28). Human vacuolar protein sorting 33B (VPS33B), a regulator of membrane fusion, was identified as the cognate substrate of PtpA, and it is assumed that PtpA impairs phagolysosomal fusion in Mtb-infected macrophages by dephosphorylation of VPS33B (20). A Mtb *ptpB* mutant was shown to be impaired in its ability to grow in human macrophages (20), and to display a decreased survival rate in a guinea pig model (29).

A wealth of information has been gained from studies aimed at deciphering the pathophysiological events during *S. aureus*-macrophage infection (reviewed in Ref. 30), but the signaling pathways leading to these adaptations are still poorly understood. The Gram-positive pathogen is known to produce two LMW-PTPs, PtpA and PtpB (31). Earlier work demonstrated that the *S. aureus* PtpA dephosphorylates not only protein tyrosine phosphates, but also protein ribulosamine 5-phosphates as well as free ribuloselysine 5-phosphate and erythruloselysine 4-phosphate (32). However, deletion of *ptpA* and/or *ptpB* in *S. aureus* did neither affect the *in vitro* growth kinetics nor the cell wall integrity of the mutants, which led to the assumption that the *S. aureus* Ptp homologs might have some specialized functions during infection (33). Support for this hypothesis is given by our recent observations, indicating that PtpA is secreted during growth of *S. aureus*, albeit of the fact that the protein lacks a clear export pathway signal sequence (34).

Here we demonstrate that PtpA contributes to the intracellular survival capacity of *S. aureus* within macrophages, and participates in the infectivity of this pathogen. Additionally, we show that PtpA is secreted by *S. aureus* upon ingestion by macrophages, and identify potential PtpA interaction partners within this host cell type.

Results

S. aureus PtpA is required for intramacrophage survival

Given the impact of the Mtb PtpA homolog on the persistence capacity of this pathogen within macrophages (35, 36) and the findings that *S. aureus* persists readily within this host immune cell type we wondered whether the PtpA homolog of *S. aureus* might fulfill similar function(s). For this purpose, *ptpA* deletion mutants in *S. aureus* strains Newman, a frequently used laboratory strain, and SA564, a low passage clinical isolate, were generated. First, we determined the survival rates of *S. aureus* WT and *ptpA* mutants within RAW 264.7 cells at 45 min post-gentamicin treatment (pGt) (Fig. 1*A*). Already after this short period of time, a significantly smaller proportion of intracellular surviving cells were observed in RAW 264.7 cells infected with the *ptpA* mutants of Newman and SA564, respectively. *Cis*-complementation of the *ptpA* mutants with a functional *ptpA* locus reverted in both cases the intracellular survival rates to levels comparable with WT strains (Fig. 1*A*). Notably, ingested SA564 bacteria were killed much faster by RAW 264.7 cells than Newman cells. After 45 min pGT, about 80% of the ingested Newman bacteria were still viable and cultivable, whereas this was only the case for 16% of the ingested SA564 bacteria (Fig. 1*A*). Next, the effect of PtpA on intracellular survival of *S. aureus* Newman in macrophages was determined at a later infection stage (Fig. 1*B*). Similar to the situation seen at 45 min pGt, significantly reduced survival rates were observed in macrophages infected with the Newman Δ*ptpA* mutant at 22 h pGt when compared with the WT and the *cis*-complemented derivative, respectively, indicating that the survival defect of the *ptpA* mutant in macrophages is maintained over time. *In vitro* growth curves performed with WT and mutant Newman strains excluded that deletion of *ptpA* in *S. aureus* might affect the bacterial growth in suspension (Fig. 1*C*).

S. aureus PtpA contributes to infectivity of S. aureus in a murine abscess model

Because PtpA enhances the survival capacity of *S. aureus* within macrophages (Fig. 1, *A* and *B*), we hypothesized that PtpA may affect the infectivity of *S. aureus in vivo*. To address this hypothesis, we next assessed the ability of the strain triplet SA564/SA564 Δ*ptpA*/SA564 Δ*ptpA::ptpA* to cause disease in a murine abscess model (37). Consistent with our intramacrophage survival findings (Fig. 1), a significant decrease (about 2-*log*) in the bacterial loads in liver was detected in mice infected with the Δ*ptpA* mutant as compared with mice challenged with the WT strain (Fig. 2). Mice infected with the *cis*-complemented $ptpA^{+}$ derivative SA564 Δ*ptpA::ptpA* displayed significantly higher bacterial loads in liver than in Δ*ptpA* mutant-challenged mice, although the levels remained lower as seen in WT-challenged animals, however, this effect was not statistically significant ($p = 0.215$). These data suggest that PtpA positively contributes to the infectivity of *S. aureus* in mice.

PtpA is secreted intracellularly upon S. aureus macrophage infection

We recently demonstrated that PtpA was secreted into the extracellular milieu by *S. aureus* under *in vitro* growth conditions (34). To test whether PtpA might be also secreted by *S. aureus* into the host macrophage, we used the *cis*-complemented *S. aureus* derivative Newman Δ*ptpA::ptpA*_FLAG (34), which facilitates detection of the expressed PtpA by anti-FLAG immunoblotting. Macrophages were infected with the Newman Δ*ptpA* mutant or the *cis*-complemented Newman Δ*ptpA::ptpA*_Flag derivative expressing $PtpA_{\_FLAG}$, lysed at different time points pGt, separated from the intracellular bac-

[5] The abbreviations used are: LMW, low-molecular-weight; PTP, protein-tyrosine phosphatase; VPS, vacuolar protein sorting; pGt, post-gentamicin treatment; GFP, green fluorescent protein; GST, glutathione *S*-transferase; CorA, coronin-A; Ni-NTA, nickel-nitrilotriacetic acid; TSB, tryptic soy broth; cfu, colony forming unit; m.o.i., multiplicity of infection.

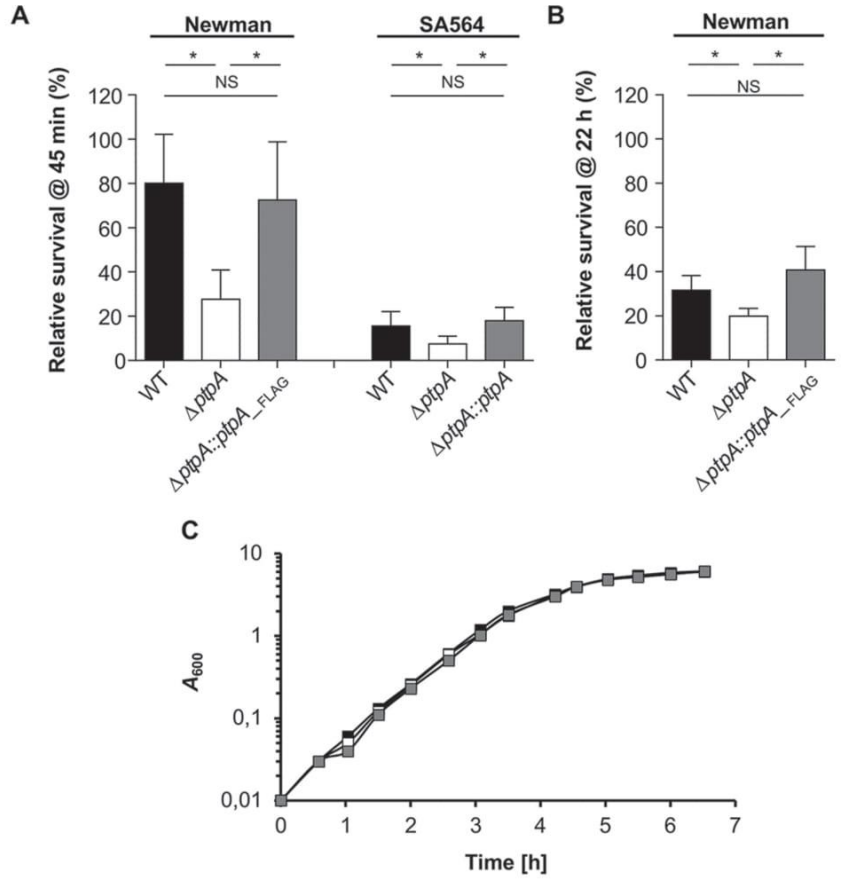

Figure 1. PtpA promotes *S. aureus* survival in macrophages. *A, S. aureus* short-term survival in infected macrophages. Cells of *S. aureus* strains SA564 and Newman and their isogenic Δ*ptpA* mutants and *cis*-complemented derivatives, respectively, were used to infect RAW 264.7 macrophages (5×10^5 cells/ml) at a m.o.i. of 20 for 1 h at 37 °C followed by 30 min incubation with gentamicin. Macrophages were then incubated for 45 min in complete media supplemented with lysostaphin to kill extracellular bacteria that might be released from lysed macrophages during the successive incubation time, and subsequently lysed in 0.1% Triton X-100. Survival rates are given in relationship to the intracellular bacterial cell numbers seen pGt treatment. Results represent the mean ± S.D. ($n = 5$). *, $p < 0.05$; *NS*, not significant (Mann-Whitney-*U* test). *B, S. aureus* long-term survival in infected macrophages. RAW 264.7 macrophages (5×10^5 cells/ml) were infected with a m.o.i. of 20 of *S. aureus* strains Newman (*black bar*), Newman Δ*ptpA* (*white bar*), and Newman Δ*ptpA::ptpA*_Flag (*gray bar*), respectively, and co-incubated for 2 h at 37 °C, followed by a gentamicin/lysostaphin treatment to eliminate external bacteria. Macrophages were lysed with Triton X-100 (0.1%) at 22 h pGt, and bacteria enumerated by plate counting. Survival rates are given in relationship to the intracellular bacterial cell numbers seen immediately after gentamicin treatment. Data represent the mean ± S.D. ($n = 5$). *, $p < 0.05$; *NS*, not significant (Mann-Whitney-*U* test). *C, in vitro* growth kinetics of the *S. aureus* Newman/Δ*ptpA*/Δ*ptpA::ptpA*_Flag strain triplet. Growth of *S. aureus* strains Newman (*black symbols*), Newman Δ*ptpA* (*white symbols*), and Newman Δ*ptpA::ptpA*_Flag (*gray symbols*) was measured in TSB at 37 °C and 150 rpm. Data represent the mean A_{600} readings at the time points indicated ($n = 3$).

teria and processed for anti-FLAG immunoprecipitation and Western blot analysis. $PtpA_{\_FLAG}$ could be detected in increasing amounts in macrophage lysates over time upon cell infection with the *ptpA* complemented Newman strain Δ*ptpA::ptpA*_FLAG, whereas no such signal was observed in Newman Δ*ptpA*-infected macrophages (Fig. 3*A*).

To exclude that the $PtpA_{\_FLAG}$ signal might originate from lysed bacteria, we transformed the Newman Δ*ptpA* mutant and the *cis*-complemented Newman Δ*ptpA::ptpA*_FLAG derivative with the green fluorescent protein (GFP) expressing vector pMK4_GFP. Following macrophage infection with these pMK4_GFP harboring derivatives, no GFP signal could be detected in the cleared macrophage lysates after concentration by GFP immunoprecipitation (GFP-trap, Chrommoteck), whereas strong GFP signals were detected in the bacterial pellets that were obtained from the macrophage lysates by centrifugation (Fig. 3*B*). In the same samples, $PtpA_{\_FLAG}$ was detected in the bacterial pellet and after FLAG-immunoprecipitation from the cleared lysates of the Newman Δ*ptpA::ptpA*_FLAG + pMK4_GFP-infected macrophages, whereas this signal was not seen in the cleared lysates of macrophages that were challenged with the pMK4_GFP-transformed Δ*ptpA* mutant (Fig. 3*C*). Taken together, these findings indicate that the $PtpA_{\_FLAG}$ signal detected in the lysate fractions of macrophages infected with the Newman Δ*ptpA::ptpA*_Flag derivative was not substantially caused by intracellular bacterial lysis, suggesting a secretion of PtpA into macrophages.

PtpA interacts with several host cell proteins in pulldown experiments

Because PtpA is most likely secreted into host cells, we next assessed whether this bacterial derived phosphatase might interfere with host cell signal transduction pathways. To identify host cell proteins that might interact with PtpA, we

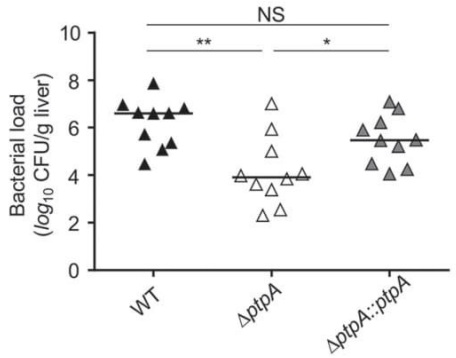

Figure 2. Effect of *ptpA* deletion on infectivity of *S. aureus* SA564 in a murine abscess model. C57BL/6N mice were infected via retroorbital injection with 1×10^7 cells of *S. aureus* strain SA564 (*black symbols*), SA564 Δ*ptpA* (*white symbols*), and the *cis*-complemented derivative SA564 Δ*ptpA*::*ptpA* (*gray symbols*), respectively ($n = 10$ per group). Mice were euthanized 4 days post-infection, the livers were removed and homogenized in PBS to determine the bacterial loads. Each *symbol* represents an individual mouse. *Horizontal bars* indicate the median of all observations. *, $p < 0.05$; **, $p < 0.01$; *NS*, not significant (Mann-Whitney-*U* test).

developed a strategy combining the use of the slime mold *Dictyostelium discoideum,* and our PtpA functional mutants. *D. discoideum* is a eukaryotic professional phagocyte amenable to genetic and biochemical studies, and in our case allowing the ectopic expression of PtpA in large culture volumes. In a second approach, we used a "substrate trapping" strategy, based on a methodology used to identify substrates for the *Yersinia* phosphatase YopH in HeLa cells (38), or Mtb PtpA host interactants (20). The latter mechanism-based approach utilized a catalytically defective mutant of PtpA to trap substrate complexes. PTPs contain a cysteine nucleophile (Cys-8 within the highly conserved sequence C-X_5-R) that forms a phosphocysteinyl intermediate during catalysis (39). We hypothesized that a cysteine to serine mutation at position 8 of the *S. aureus* Newman PtpA ORF (PtpA_C8S) would result in a catalytically defective PtpA variant that, like other similar PTP family mutants, might "trap" host substrate proteins by stabilizing the covalent enzyme-substrate complexes. To confirm that the C8S mutation in *S. aureus* PtpA affects its activity, we first tested the recombinant His-tagged versions of PtpA and PtpA-C8S with the substrate *p*-nitrophenylphosphate. Our results demonstrate that phosphatase activity of the *S. aureus* C8S mutant was indeed abrogated (Fig. S1).

Lysates from *D. discoideum* overexpressing PtpA_C8S$_{_FLAG}$, or expressing the FLAG alone as a control, were next incubated with beads coupled to an anti-FLAG antibody. Afterward, beads were extensively washed, and bound proteins were subsequently stripped off and separated by SDS-PAGE before MS analysis. The proteins identified under each condition were compared and purged of those interacting with FLAG alone (Table 1).

The putative interactants of PtpA identified by this approach are involved in different cellular pathways involving cell adherence or endosomal trafficking. Two of them, lysosomal-α-mannosidase ManA and Arf-GTPase–activating protein DDB0167328, likely play a role in cell adherence (40, 41). Lysosomal-α-mannosidases are members of the glycoside hydrolase family 38. They are involved in the catabolism of Asn-linked glycans of glycoproteins and play a vital role in

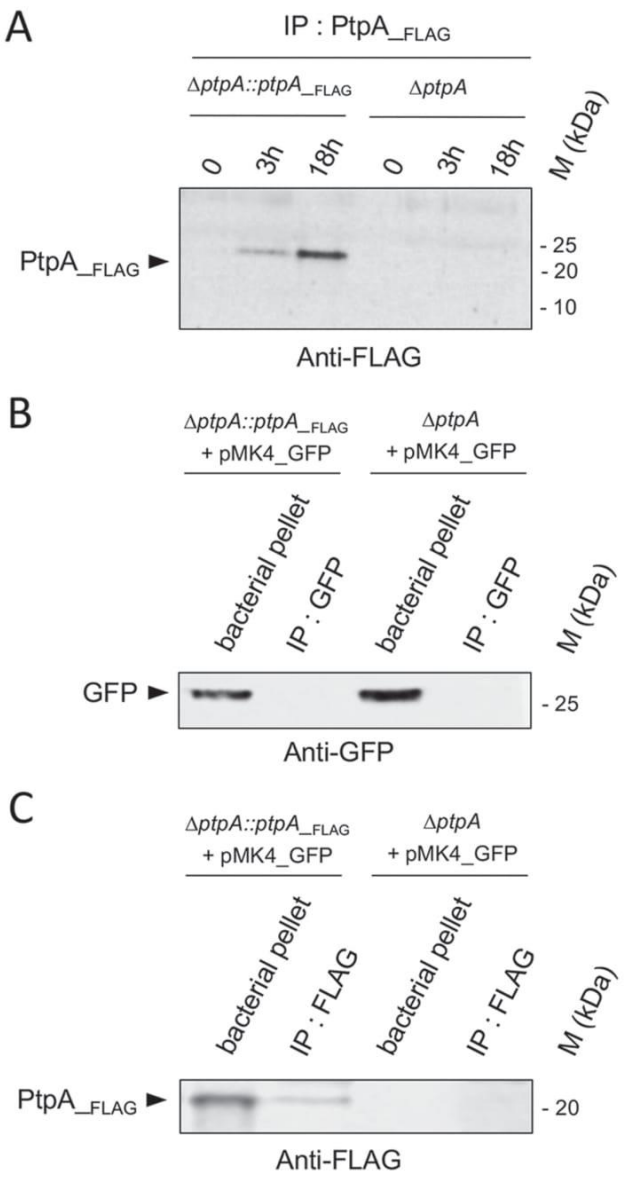

Figure 3. PtpA is secreted in macrophages during infection. *A,* PtpA secretion in macrophage lysates. RAW 264.7 macrophages (5×10^5 cells/ml) were incubated for 2 h with either *S. aureus* Newman Δ*ptpA* cells or *S. aureus* Δ*ptpA* cells complemented with a FLAG-tagged *ptpA* (Newman Δ*ptpA*::*ptpA*_Flag) at a m.o.i. of 20, and nonphagocytosed bacteria were subsequently removed by gentamicin/lysostaphin treatment. Macrophages were lysed at the time points indicated, centrifuged to eliminate intracellular bacteria, and macrophage lysates were subjected to immunoprecipitation and Western blotting analyses using anti-FLAG antibodies. *B* and *C,* control immunoprecipitations to rule out bacterial proteins leaking in macrophage lysates. RAW 264.7 macrophages (5×10^5 cells/ml) were infected with Newman Δ*ptpA*::*ptpA*_FLAG harboring plasmid pMK4_GFP and Newman Δ*ptpA* harboring plasmid pMK4_GFP at a m.o.i. of 20, respectively. At 3 h pGt, infected macrophages were lysed in 0.1% Triton X-100, and centrifuged at 14,000 × *g*. The obtained supernatants corresponding to macrophage lysates were immunoprecipitated with GFP-trap beads (Chromoteck) (*B*) or with anti-FLAG antibodies coated on agarose-beads (*C*), whereas the pellets containing intracellular bacteria were resuspended in an equal amount of PBS with protease inhibitor mixture and lysed in a bead-beater (Retsch, MM400). Immunoprecipitated proteins and bacterial pellets were resolved on SDS-PAGE and detected with an anti-GFP (*B*) or anti-FLAG (*C*) antibody. *M kDa*, molecular markers.

Table 1
Major PtpA interactants identified by mass spectrometry

Protein name	Accession number	Sum PEP score[a]	Coverage[b]	Number of peptides[c,d]	Mass
			%		kDa
V-type proton ATPase subunit A	P54647	3216	65	30	68.16
V-type proton ATPase subunit B	Q76NU1	454	35	12	54.84
Coronin-A	P27133	435	33	10	49.18
Cathepsin D	O76856	421	19	6	41.09
Vacuolin-A	O15706	196	13	6	66.25
Lysosomal α-mannosidase	P34098	131	7	4	113.36
Arf GTPase-activating protein	Q9Y2X7	110	10	3	64.89
Phox domain-containing protein vps5	Q86IF6	26	9	2	61.92

[a] Sum PEP score: sum of –log(PEP) (PEP: posterior error probability), which is a probability that a Peptide Spectra Matches (PSM) is incorrect. The lower the PEP, the higher the sum PEP score.
[b] % of protein sequence coverage.
[c] A peptide is identified by one or more PSM corresponding to relevant MS-MS mass spectra leading to the identification of a peptide. A protein is identified by several peptides (*# Peptides*).
[d] The cut-off is validated by the SEQUEST HT algorithm and corresponds to at least two peptides to identify the protein.

maintaining cellular homeostasis, cell adhesion during development, viral infection, or immune response (40). Among several functions, Arf-GTPase activating proteins are notably regulators of specialized membrane surfaces implicated in cell migration involving adhesive structures in which the cell membrane is integrated with the actin cytoskeleton (41). Additionally, proteins related to endosome function and trafficking have been co-immunoprecipitated with PtpA: Vacuolin A, Cathepsine D, and Phox domain-containing protein Vps5. Vacuolin A is a flotillin/reggie-related protein from *Dictyostelium* that oligomerizes for endosome association (42), and Cathepsin D is an aspartic endoprotease that is ubiquitously distributed in lysosomes to degrade proteins and activate precursors of bioactive proteins in pre-lysosomal compartments (43). Vps5 belongs to the family of sorting nexins containing a Phox homology domain and might be a component of the retromer complex. These proteins are involved in regulating membrane traffic and protein sorting in the endosomal system (44). Interestingly, V-ATPase, previously identified as an interactant of *M. tuberculosis* PtpA (28), was captured also in our substrate-trapping assay, suggesting that in *S. aureus* a similar interaction might occur. Moreover, the host protein coronin-A (CorA) was identified as putative PtpA interactant (Table 1). Interestingly, the mammalian homologue coronin-1A (Coro-1A) was reported as being retained on phagosomes containing living Mtb, while being rapidly released from phagosomes containing inactive mycobacteria (45). Furthermore, genetic depletion or RNAi-mediated gene silencing of Coro-1A were later reported to inhibit the survival of mycobacteria within macrophages (46–48).

PtpA interacts with coronin in vitro

As Coro-1A was shown to be important for the survival of mycobacteria in infected macrophages (45–49), we decided to investigate the putative interaction of *S. aureus* PtpA and Coro-1A in more detail. First, we performed GST pulldown assays to verify the interaction between PtpA and *D. discoideum* CorA (Dd_Coro-A) (Fig. 4*A*). Fusion proteins combining GST and PtpA ($PtpA_{\_GST}$) were immobilized onto GSH-agarose beads and incubated with *D. discoideum* cell lysates expressing a myc-tagged-Dd_Coro-A (myc-Dd_Coro-A). Bound proteins were stripped off the beads and subjected to Western blotting analyses with an anti-myc antibody. As displayed in Fig. 4*A*, myc-Dd_Coro-A was pulled down with the $PtpA_{\_GST}$ fusion, but not with GST alone. This observation strongly suggests that PtpA interacts with CorA from *D. discoideum*, thus supporting our MS findings.

Next, we assayed the interaction of the *S. aureus* PtpA with human Coro-1A (Hs_Coro-1A) by using a pulldown assay (Fig. 4*B*). His-tagged versions of PtpA ($PtpA_{\_His}$) and the catalytically inactive PtpA_C8S ($PtpA\_C8S_{\_His}$) were overexpressed and purified as previously described (34), bound on Ni-NTA-agarose beads and incubated with BL21 lysates expressing the recombinant Hs_Coro-1A protein harboring a GST tag ($Hs\_Coro\text{-}1A_{\_GST}$). As control, $PtpA_{\_His}$ beads were incubated with BL21 lysates expressing GST alone. Ni-NTA-agarose beads without PtpA were additionally incubated with GST or $Hs\_Coro\text{-}1A_{\_GST}$ to confirm that beads alone could not interact with GST fusion proteins (Fig. 4*B*, *upper panel*). Protein complexes were pulled down, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane before detection by anti-GST and anti-coronin-1A antibodies, respectively. In this assay, $Hs\_Coro\text{-}1A_{\_GST}$ was retained on beads when $PtpA\_C8S_{\_His}$ was present, whereas no signal was seen with the $PtpA_{\_His}$ version (Fig. 4*B*, *lower panel*). Additionally, GST alone did neither in absence nor presence of the His-tagged PtpA fusion proteins bind to the beads (Fig. 4*B*). Taken together, these data suggest that specific complexes were formed between *S. aureus* PtpA and Coro-1A either with the *D. discoideum* CorA or the human homologue Hs_Coro-1A.

Coronin-1A is phosphorylated on tyrosine residues upon infection

To test whether Coro-1A might serve as a tyrosine-phosphorylated substrate for PtpA *in vivo*, we infected RAW 264.7 cells with *S. aureus* Newman and its isogenic Δ*ptpA* mutant, respectively, and lysed the macrophages for 30 min and 3 h pGt. The murine macrophage Coro-1A homolog (Mm_Coro-1A) was subsequently immunoprecipitated with an anti-Coro-1A antibody, and the tyrosine phosphorylation status of Mm_Coro-1A was determined by Western blotting analyses using anti-phosphotyrosine antibodies. We observed that phosphorylation of Mm_Coro-1A on tyrosine residues was increased upon infection, and this effect seemed to be influenced by PtpA (Fig. 5*A*). Infection of RAW 264.7 cells with *S. aureus* clearly enhanced the tyrosine phosphorylation signal of Mm_Coro-1A at both time points analyzed. Interestingly, in lysates of Newman

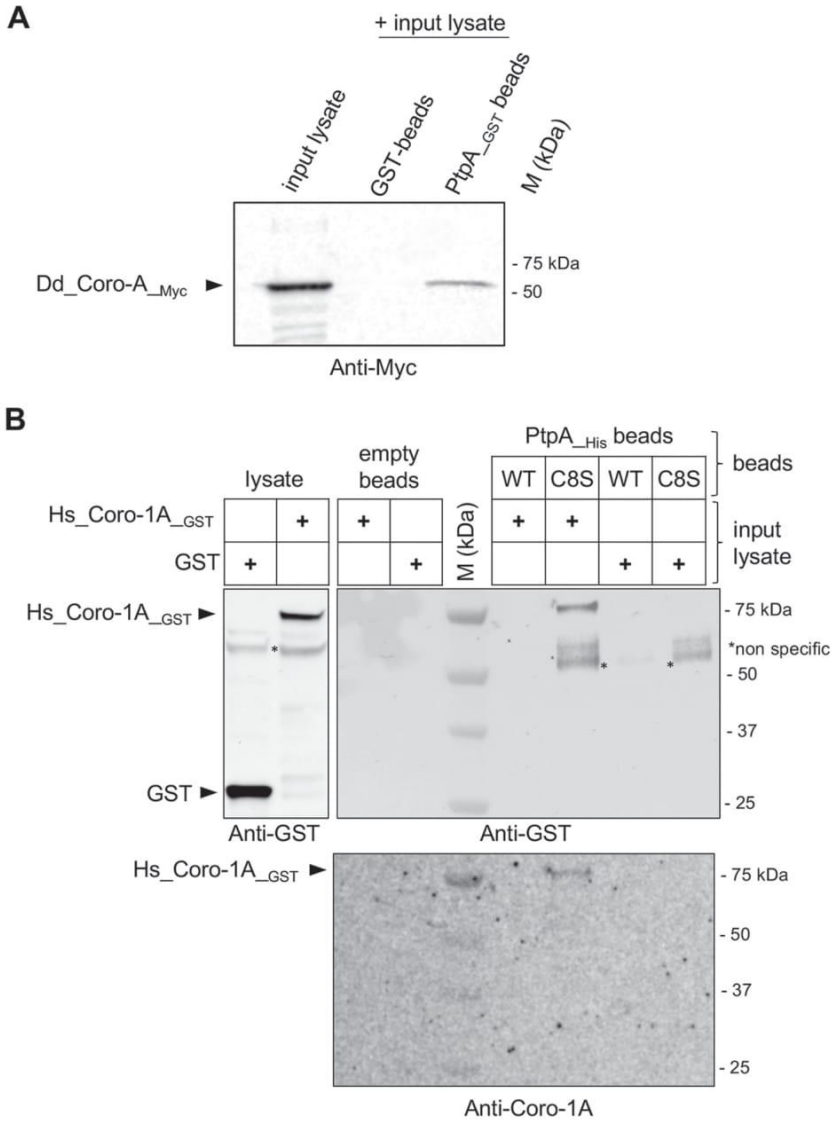

Figure 4. PtpA interacts with Coro-1A *in vitro*. *A*, the interaction between PtpA and *D. discoideum* CorA was confirmed by GST pulldown assay. The indicated GST fusion proteins were expressed in *E. coli*, bound to GSH beads, and then incubated with *D. discoideum* cell lysates expressing myc-tagged-Dd_Coro-1 (Dd_Coro-A$_{_myc}$). Beads were washed, eluted by boiling, and bound proteins were revealed by Western blot analysis with an anti-myc antibody. *B,* the interaction between PtpA and human Coro-1A was tested by pulldown analysis. His-tagged versions of PtpA (*WT*) and the catalytically inactive PtpA_C8S (*C8S*) were constructed, produced. and purified as described under "Experimental procedures." BL21 lysates expressing Hs_Coro-1A$_{_GST}$ or GST alone were prepared and incubated with the PtpA$_{_His}$ derivatives immobilized on Ni-NTA-agarose beads. The bound proteins were eluted and resolved by SDS-PAGE followed by immunoblotting using anti-GST and anti-Coro-1A antibodies. Empty Ni-NTA-agarose beads were used as a control. *, nonspecific signals co-precipitated with PtpA_C8S$_{_His}$. *M kDa*, molecular markers.

Δ*ptpA*-infected macrophages, about 2-fold higher phosphotyrosine signals (2.2 ± 0.7; $n = 6$) were observed at 3 h pGt than in lysates of RAW 264.7 cells that were challenged with the parental strain Newman. Therefore, our results reveal for the first time a tyrosine phosphorylation of Coro-1A, as previous records of phosphorylation for this protein were related only to Ser-Thr residues (50–52), as well as its PtpA-mediated dephosphorylation.

Coronin-1A is not dephosphorylated in vitro by recombinant PtpA

The observation that Coro-1A can be Tyr-phosphorylated, and that its Tyr-phosphorylation status seemed to be influenced by PtpA, prompted us to assess whether Coro-1A might be directly dephosphorylated by PtpA, as previously reported for some Mtb PtpA substrates (20). Thus we tested the ability of PtpA and of the catalytically inactive PtpA derivative PtpA_D120A (34) to dephosphorylate the murine variant of Coro-1A. PtpA_D120A was chosen for this dephosphorylation assay as it corresponds to the commonly used phosphatase-defective PtpA mutant PtpA_D126A in Mtb (20, 28, 53, 54). First, Mm_Coro-1A was purified by immunoprecipitation from *S. aureus* Newman-infected macrophages. Equal amounts of the immunoprecipitated Mm_Coro-1A were then incubated with recombinant PtpA derivatives for 30 min and 1 h, respec-

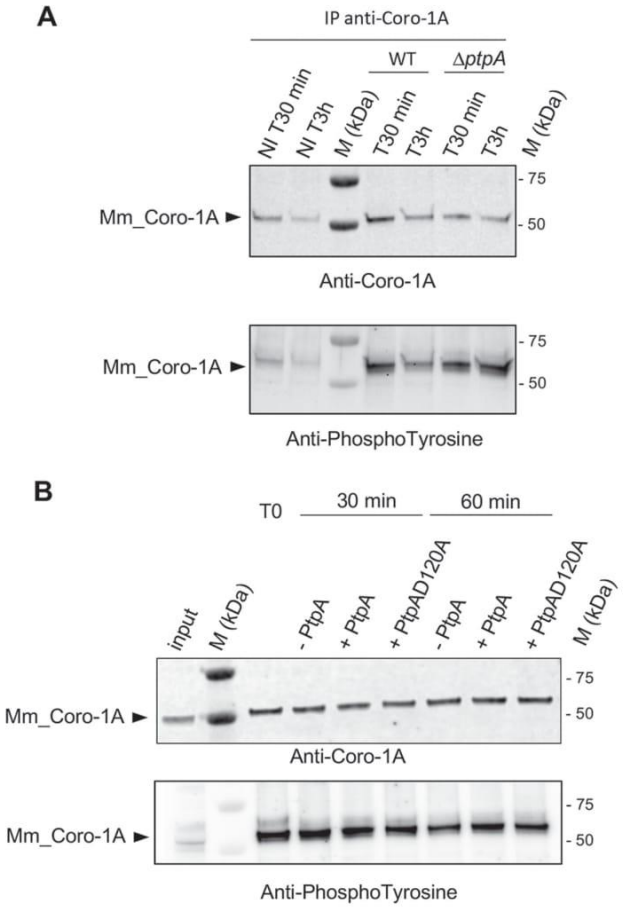

Figure 5. Coro-1A is phosphorylated on tyrosines *in vivo* but is not dephosphorylated by PtpA *in vitro*. *A,* immunoblot analysis with anti-Coro-1A (*upper panel*) or anti-phosphotyrosine antibodies (*bottom panel*) of immunoprecipitated (*IP*) endogenous Coro-1A (Mm_Coro-1A) from lysates of RAW 264.7 macrophages infected with cells of *S. aureus* Newman (*WT*) or the isogenic Δ*ptpA* mutant. Noninfected macrophages and infected macrophages were treated with gentamicin for 30 min and subsequently incubated with lysostaphin to kill extracellular bacteria that might be released from lysed macrophages during the successive incubation time. Noninfected and infected macrophages were lysed 30 min (*NI T30 min* and *T30 min*) and 3 h (*NI T3 h* and *T3 h*) pGt treatment. *B,* immunoprecipitated Mm_Coro-1A from lysates of macrophages infected with strain Newman for 30 min pGt were incubated in the absence (−) or presence (+) of 2 μg of PtpA$_{_His}$ or PtpA-D120A$_{_His}$ at 37 °C for the time points indicated (*T0*, 30 and 60 min). Proteins were resolved by SDS-PAGE and probed with anti-Coro-1A (*upper panel*) or anti-phosphotyrosine (*bottom panel*) antibodies on the same blot. Contents of Mm_Coro-1A and Tyr-phosphorylated Mm_Coro-1A in lysates of *S. aureus*-infected macrophages prior to concentration by IP are indicated in the input lane (*input*). *M kDa*, molecular markers.

tively. Proteins were subsequently separated by SDS-PAGE and electrotransferred to a membrane. The Coro-1A and Tyr-phosphorylation signals were revealed using anti-Coro-1A and anti-phosphotyrosine antibodies, respectively (Fig. 5). However, no clear reductions in the phosphorylation levels of Mm_Coro-1A were observed after incubation with either PtpA or the catalytically inactive PtpA_D120A. All together, these data indicate that Mm_Coro-1A is a bona fide substrate for tyrosine phosphorylation, and that PtpA seems to affect Coro-1A Tyr-phosphorylation in an indirect manner. Notably, a similar observation was made with the Mtb PtpA, which is able to interact with the V-ATPase subunit H without using this protein as a catalytic substrate (28).

To identify putative Tyr-phosphorylation sites of Coro-1A, we next performed an *in silico* analysis with the ORFs of the Coro-1A variants used in this study. The alignment of the amino acid sequences of the three Coro-1A homologues identified 94% of homology between the Mm_Coro-1A and Hs_Coro-1A ORFs, whereas the Dd_Coro-A homologue shared only 38% of homology with the human Coro-1A (Fig. S2). Despite the comparably low degree of conservation between Dd_Coro-A and the two mammalian Coro-1A variants, four Tyr-residues were identified as conserved among the three species. A subsequent alignment of 25 Coro-1A homologs of various eukaryotes including protists, fungi, and animalia confirmed a very high conservation of these four Tyr residues (Fig. S3), supporting the idea that these sites might be involved in the Tyr-mediated phosphorylation of this protein.

In a first attempt to identify the host kinase responsible for Coro-1A Tyr-phosphorylation upon *S. aureus* infection, we tested the spleen tyrosine kinase Syk on Mm_Coro-1A Tyr-phosphorylation, as Syk is highly expressed in RAW 264.7 cells (55), and implicated to play a pivotal role in macrophage-mediated inflammatory responses (56). Moreover, one of the human coronin homologs, coronin-1C (sharing 62% of identity to Hs_Coro-1A at the amino acid level), was previously identified as ligand of Syk in B-cells (57). However, we failed to detect any tyrosine phosphorylation signal on Coro-1A after co-incubation with Syk in our *in vitro* phosphorylation assays (data not shown), indicating that Syk is not the kinase responsible for Tyr-phosphorylation of Coro-1A.

Discussion

Our results provide the first interactor candidate identification of host partners of the secreted phosphatase PtpA, and its involvement in the process of infection and intracellular survival of *S. aureus.* The macrophage survival and infection data suggest an important role for PtpA during infection. Our co-immunoprecipitation studies indicate PtpA interacts with a number of host factors including Coro-1A, and revealed that this actin-binding protein can be phosphorylated at tyrosine residues. Together, these findings suggest a role for PtpA as modulator of the host immune response, particularly after uptake of *S. aureus* by macrophages. As the impact of Tyr-phosphorylation on Coro-1A function/activity was not studied yet, we can only hypothesize that phosphorylation of the Coro-1A Tyr residue(s) might affect the intracellular distribution of this actin-binding protein within macrophages, as it has been suggested for serine/threonine-mediated phosphorylation of coronin-1. Indeed, protein kinase C-dependent coronin-1 phosphorylation on Ser and Thr residues was identified as an important mechanism to modulate the intracellular distribution of this protein during phagolysosome maturation. Earlier work studying the phagocytosis of opsonized zymosan particles by HL-60 leukemia cells demonstrated that phosphorylation of coronin-1 by protein kinase C triggered the dissociation of the actin-binding protein from nascent phagosomes (51). Another study performed with mycobacteria-infected macrophages showed that coronin-1 accumulated on bacteria-loaded phagosomes, and

Table 2
Strains, plasmids, and primers used in this study

Strain or plasmid	Relevant genotype or characteristic(s)[a]	Ref. or source
S. aureus		
Newman	Laboratory strain (ATCC 25904), wildtype	
Newman Δ*ptpA*	Newman Δ*ptpA*::lox66-*aphA*III-lox71; Kan^r	34
Newman Δ*ptpA*::*ptpA*_Flag	Newman Δ*ptpA* derivative *cis*-complemented with pEC1_ptpA_Flag; Em^r	34
SA564	Clinical isolate, wildtype	60
SA564 Δ*ptpA*	SA564 Δ*ptpA*::lox66-*aphA*III-lox71; Kan^r	This study
SA564 Δ*ptpA*::*ptpA*	SA564 Δ*ptpA* derivative *cis*-complemented with pEC1_ptpAc; Em^r	This study
E. coli		
IM08B	SA08BΩP$_{N25}$-*hsdS* (CC8–1) (SAUSA300_0406) of NRS384 integrated between the *essQ* and *cspB* genes	62
D. discoideum		
DH1–10	Axenic, uracil auxotroph *D. discoideum* strain	64
Plasmids		
pDXA	*D. discoideum* vector	63
pFL1290	pDXA-3 × myc-Coronin-A corresponding to pDXA vector with a 1338-kb fragment covering the ORF for the *D. discoideum* coronin-A (*corA*; Gene ID: DDB_G0267382; NCBI reference sequence: XM_642251.1) fused to 3 × myc tag.	This study
pDXA_PtpA-C8S-Flag	pDXA_PtpA-C8S-FLAG corresponding to pDXA vector with a 0.8-kb fragment covering the ORF for the *S. aureus ptpA* gene fused to 3 × FLAG tag.	This study
pEC1	pUC19 derivative containing the 1.45-kb ClaI *erm*(B) fragment of Tn*551*	61
pEC1_ptpAc	pEC1 with a 0.8-kb fragment covering the C-terminal part of ORF RT87_RS09705 and the putative terminator region (NCBI reference sequence: NZ_CP010890.1).	This study
pETPhos_PtpA_C8S	Modified from pETPhos_PtpA (34)	This study
pCDFDuet GST Cter up1	Modified from Novagen pCDFDuet-1	This study
pCDFDuet-Coro-1A-GST	pCDFDuet-GST Cter Up1 with a 1.4-kb fragment covering the ORF for the human Coro-1A (NCBI reference sequence: NC_000016.10) optimized for bacterial expression and fused to GST.	This study
Primers		
OL989–5′-BamHIx2 (forward)	GGATCCGGATCCATGTCTAAAGTAGTCCGTAGTAGTAAA	This study
OL990–3′-XhoIx2-stop (reverse)	CTCGAGCTCGAGTTAGTTGGTGAGTTCTTTGATTTTGGC	This study
Nterm_PtpA_C8S	ATGGTAGATGTAGCATTTGTC**AGT**CTTGGCAATATATGTCG	This study
Nter_PtpA_flag_C8S_IF_Bam	GAATTCCCGGGGATCCATGGTAGATGTAGCATTTGTC**AGT**CTTGGCAATATATGTCG	This study
Cter_PtpAflag_Xho_pDXA	ATCTATCTCGAGTTATTTATCATCATCATCTTTGTA	This study
Nterm PtpA Bam_pGEX	TAT*GGATCC*ATGGTAGATGTAGCATTTGTCTGT	This study
Cterm PtpA Hind IF_pGEX	TATCATCGAT***AAGCTT***CTACCCCTCTTTCAAATTTGCATC	This study
MBH425	GCAATTAT***GAATTC***TTTCAATGTTGC	This study
MBH426	GCT***GGTACC***GAATTAAGAAAAGTTACTTACGCC	This study

[a] Em^r, Erythromycin resistant; Kan^r, kanamycin resistant.

that this protein was actively retained by viable mycobacteria residing inside phagosomes (45) suggesting that the retention of coronin-1 on mycobacteria-loaded phagosomes is responsible for suppressing phagosome-lysosome formation (58). More recent work demonstrated that trimerization of coronin-1 was essential for mycobacterial survival, and that the transition from the trimer to the monomer form was regulated by serine phosphorylation (59). In the light of the latter findings one may speculate that Tyr-phosphorylation of Coro-1A might also affect the spatial distribution of this protein in *S. aureus*-infected macrophages, and that the bacterium attempts to modulate this process via PtpA. However, it is unclear yet whether Tyr-phosphorylation of Coro-1A affects the trimerization of this protein, and further work is required to understand how PtpA recognizes Coro-1A and/or other putative interactors identified by our co-immunoprecipitation studies, and whether and how they participate in the establishment of *S. aureus* survival and virulence.

Experimental procedures

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 2. *Escherichia coli* strains were grown at 37 °C in LB medium supplemented with antibiotic when required. *S. aureus* isolates were plated on tryptic soy agar (BD Bioscience) supplemented with antibiotic when required, or grown in tryptic soy broth (TSB) (BD Bioscience) medium at 37 °C and 150 rpm. The Newman Δ*ptpA* mutant derivatives were previously constructed (34). The SA564 Δ*ptpA* mutant was obtained by phage transducing the *aphA*III-lox–tagged Δ*ptpA* mutation from Newman Δ*ptpA* into the low passage clinical isolate SA564 (60).

Construction of the S. aureus ptpA cis-complementation strain SA564 ΔptpA::ptpA

For *cis*-complementation of the *ptpA* mutation in strain SA564 Δ*ptpA*, a 0.8-kb fragment containing the C-terminal part of ORF RT87_RS09705 located downstream of *ptpA* (RT87_RS09695) was amplified by PCR from chromosomal DNA of *S. aureus* strain SA564 using the primer pair MBH425/MBH426 (Table 2). The resulting PCR product was digested with KpnI/EcoRI, and subsequently cloned into KpnI/EcoRI-digested vector pEC1 (61) to generate the suicide plasmid pEC1_ptpAc. *E. coli* IM08B (62)-derived plasmid pEC1_ptpAc was directly electroporated into *S. aureus* strain SA564. A SA564 derivative that integrated pEC1_ptpAc was subsequently used as a donor for transducing the *cis*-integrated pEC1_ptpAc into SA564 Δ*ptpA*, thereby replacing the *aph*-tagged *ptpA* deletion with the WT *ptpA* genome region.

Animal studies statement

Animal experiments were approved by the local State Review Boards of Saarland and conducted according to the regulations of German veterinary law.

Murine abscess model

Preparation of the bacterial inoculum and infection of the animals were carried out as described (37), with minor modifications. Briefly, 100-μl bacterial suspensions containing $\sim 10^7$ colony forming unit (cfu) were administered intravenously by retro-orbital injection into female, 8–10–week-old C57/BL6N mice that were anesthetized by isoflurane inhalation (5%; Baxter). Immediately after infection, mice were treated with a dose of caprofen (5 mg/kg; Pfizer), and at 4 days post-infection, mice were sacrificed, and livers were removed. The organs were weight adjusted and homogenized in PBS, and serial dilutions of the homogenates were plated on blood agar plates to enumerate the cfu.

Cloning and expression of PtpA in D. discoideum

For *S. aureus* PtpA-C8S overexpression in *D. discoideum*, the coding sequence of PtpA-C8S was amplified by PCR using the set of primer Nter_PtpA_flag_C8S_IF_Bam and Cter_PtpAflag_Xho_pDXA (Table 2). The amplified product was digested with BamHI and XhoI restriction enzymes and cloned into *D. discoideum* expression vector pDXA vector (63). The pDXA_PtpA-C8S-Flag plasmid was transfected in *D. discoideum* cells as described (64).

Cloning, expression, and purification of recombinant PtpA derivatives

GST-PtpA recombinant protein was obtained by cloning the *ptpA* fragment generated by PCR using *S. aureus* N315 genomic DNA as a template with the primers Nterm PtpA Bam_pGEX and Cterm PtpA Hind_pGEX (Table 2) into the BamHI-HindIII–digested pGEX vector. PtpA_C8S_His harboring cysteine to serine substitution was generated by using the QuikChange Site-directed Mutagenesis Kit (Agilent Technologies) on pETPhos_PtpA template (34) using the primer Nterm PtpA_C8S (Table 2) thus generating pETPhos_PtpA_C8S. PtpA-His and PtpA-GST derivatives were purified as previously described (34, 65).

Cloning, expression, and purification of coronin derivatives

The coding sequence of the *D. discoideum* CorA homolog was amplified by PCR from *D. discoideum* genomic DNA using the appropriate primers introducing BamHI and XhoI restriction sites, respectively (Table 2). The PCR product was digested with BamHI and XhoI, and ligated into the *D. discoideum* expression vector pDXA that was digested with the same restriction enzymes. The pDXA 3xmyc-CorA plasmid (pFL1290) was linearized with ScaI and transfected in *D. discoideum* strain DH1–10. Cells were grown at 22 °C in HL5 medium as previously described (64). Human coronin-1A (Hs-Coro-1A) (gi: 300934762, NP_001180262.1) was synthesized, codon optimized for bacterial production by GenScript, and cloned into pUC57 vector. The coding sequence was amplified by PCR from pUC57 vector, digested by NcoI-HindIII restriction enzymes, and ligated into pCDFDuet-GSTCter-up1 expression vector, thus generating pCDFDuet-Coro-1A-GST. The construct was verified by DNA sequencing. For pulldown assays, lysates of BL21 Star expressing GST-Hs-coronin-1A or GST alone were prepared as follows. Transformed *E. coli* BL21 Star cells with human coronin-1A codon optimized were grown at 16 °C in LB medium containing 1 g/liter of glucose and 50 μg/ml of spectinomycin and protein synthesis induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside overnight. Bacteria expressing GST-Hs-coronin-1A or GST alone were disrupted by sonication (Branson, digital sonifier) and centrifuged at 14,000 rpm for 25 min. Protein concentration was determined using BCA reagent (ThermoFisher Scientific, France).

Macrophage culture and infection

The murine macrophage cell line RAW 264.7 (mouse leukemic monocyte macrophage, ATCC TIB-71) was cultured in Dulbecco's modified Eagle's medium (ThermoFisher Scientific, France) supplemented with 10% fetal calf serum (ThermoFisher Scientific, France) in a humidified atmosphere at 5% CO_2 at 37 °C. For macrophage infection, *S. aureus* Newman and SA564 strains were grown to the midexponential growth phase (A_{600} = 0.7–0.9) in TSB medium. The bacteria were then collected by centrifugation at 10,000 rpm for 5 min and resuspended in sterile PBS. The RAW 264.7 cells (5×10^5 cells/ml, in 24-well plates) were inoculated with *S. aureus* at the m.o.i. of 20:1 (bacteria:cells) and incubated at 37 °C and 5% CO_2 for the indicated time. Subsequently, cells were washed once with PBS and the remaining extracellular bacteria were killed by incubation with gentamicin (100 μg/ml) for 30 min. After gentamicin treatment, macrophages were rinsed twice with PBS (T0), and then further incubated in Dulbecco's modified Eagle's medium containing 5 μg/ml of lysostaphin for 45 min and 22 h, respectively. Enumeration of intracellular bacteria was performed by lysing infected macrophages with 0.1% Triton X-100 in PBS. Macrophages lysates were serially diluted and plated on TSB agar plates that were subsequently cultivated at 37 °C for 16 h. The survival rate of bacteria was defined as follows: number of bacterial colonies at time post gentamicin/number of bacterial colonies at T0 $\times$ 100%.

Immunoprecipitation of PtpA from D. discoideum

For immunoprecipitations, 2×10^7 *D. discoideum* cells expressing flagged PtpA ($PtpA_{\_FLAG}$), were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.5% Nonidet P-40, protease inhibitors (Roche Applied Science), and cleared by centrifugation for 15 min at 14,000 rpm. Lysate supernatants were incubated overnight at 4 °C with a monoclonal anti-FLAG antibody coated on agarose beads (Genscript). The beads were then washed five times in wash buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% Nonidet P-40) and once in PBS. Bound proteins were migrated on SDS-PAGE and analyzed by immunoblotting or MS when required.

PtpA phosphatase activity assay

Phosphatase activity of PtpA-wt and -C8S His-tagged recombinant proteins was determined using *p*-nitrophenylphosphate as chromogenic substrate as previously published (34).

Immunoprecipitation of Coro-1A from infected macrophages

Murine macrophage coronin-1A (Mm_Coro-1A) was obtained from *S. aureus*-infected RAW 264.7 macrophage extracts

by immunoprecipitation with anti-Coro-1A antibody (5 μg, rabbit anti-coronin-1A; SAB4200078, Sigma). Briefly, the anti-Coro-1A antibody was first coupled to 50 μl of Dynabeads-Protein G (10003D, Life Technologies) in a rotator for 10 min at room temperature and then covalently cross-linked using 5 mM BS3 (21580, Thermo Fisher Scientific) for 30 min at room temperature. Infected macrophages (m.o.i. = 20) were lysed at different time points pGt in 0.1% Triton X-100 in PBS containing a protease inhibitor mixture (complete EDTA-free protease inhibitor mixture, Roche). Antibody-coupled beads were added to these lysates and incubated in a rotator for 1 h at room temperature. Thereafter, the beads were washed twice in PBS, 0.02% Tween 20 and 3 times in phosphatase buffer (Tris-HCl 20 mM, pH 7.5, $MgCl_2$ 5 mM, DTT 5 mM). Immunoprecipitated samples were washed 3 times in PBS, 0.02% Tween 20 followed by elution in Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting using the mouse anti-phosphotyrosine (clone 4G10, Millipore) or the rabbit anti-coronin-1A antibodies.

In vitro dephosphorylation of Mm_Coro-1A

Immunoprecipitated Mm_Coro-1A from infected macrophages was obtained as described above. To examine if phosphorylated Mm_Coro-1A is a suitable substrate of PtpA, equal amounts of beads were incubated with 2 μg of purified PtpA. After 30 min and 1 h at 37 °C at 650 rpm, respectively, the reaction was stopped by the addition of sample buffer and proteins bound to the beads were resolved in a 4–20% SDS-PAGE. Mm_Coro-1A contents and its phosphorylation status were revealed by immunoblotting using anti-Coro-1A and anti-phosphotyrosine antibodies on the same blot.

Pulldown assays

For GST pulldown assays, GST-PtpA fusion proteins were produced as described above and bound to GSH-Sepharose 4B beads according to manufacturer's instructions (GE Heathcare). To prepare cell lysates, *D. discoideum* cells, expressing 3xmyc-Dd_Coro-A (2×10^7) were incubated 15 min in lysis buffer (20 mM HEPES buffer, pH 7.0, 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100; complete protease inhibitor mixture, Roche) and centrifuged for 15 min at 14,000 rpm in a refrigerated microcentrifuge. GST beads were then incubated with cell lysates (800 μg) overnight at 4 °C on a wheel. After three washes in lysis buffer, beads were heated at 95 °C for 10 min. Bound proteins were separated by SDS-PAGE and transferred to nitrocellulose before incubation with an anti-myc antibody (clone 9E10, Sigma). Blots were revealed with the Odyssey Western Detection System (Bio-Rad).

For His-pulldown assays, His-tagged PtpA fusion proteins were produced as described (34). Equal amounts of $PtpA_{\_His}$ Ni-NTA beads were incubated with BL21 Star lysates expressing GST-Hs Coro-1A or GST alone for 30 min at 4 °C with gentle agitation in coupling buffer (HEPES 20 mM, NaCl 100 mM, $MgCl_2$ 5 mM, Nonidet P-40 0.5%, glycerol 10%, imidazole 10 mM, pH 7.4). In parallel, as a control for unspecific binding, the same amount of Ni-NTA without immobilized PtpA was incubated in the same buffer with both lysates. For each Coro-1A assay, 100 μl of the matrix with or without immobilized PtpA was incubated with 5 mg of BL21 Star lysates diluted to 1 mg/ml. The matrix was collected by low speed centrifugation and then washed three times with the coupling buffer containing 50 mM imidazole. Proteins bound to the beads were recovered by the treatment with 40 μl of Laemmli sample buffer at 95 °C for 5 min. Samples were then resolved on SDS-PAGE gel and subjected to immunoblotting with anti-GST and anti-Coro-1A antibodies, respectively.

Statistical analyses

Statistical significance was assessed using the Mann-Whitney *U* test. p values <0.05 were considered significant.

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Supplementary Material

PtpA, a secreted tyrosine phosphatase from *Staphylococcus aureus*, contributes to virulence and interacts with coronin-1A during infection

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Figure S1

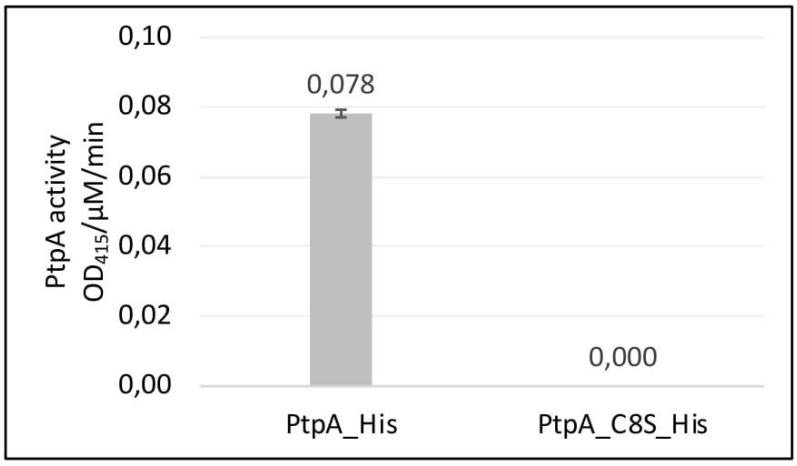

Figure S1: Phosphatase activity of PtpA and derivatives. PtpA$_{_His}$ or PtpA_C8S$_{_His}$ were used to test their phosphatase activity by using *p*-nitrophenylphosphate (pNPP) as a substrate as previously described by Brelle *et al.* (2016). The assay was performed in triplicate. The PtpA_C8S activity is null.

*Brelle, S., Baronian, G., Huc-Brandt, S., Zaki, L. G., Cohen-Gonsaud, M., Bischoff, M., and Molle, V. (2016) Phosphorylation-mediated regulation of the Staphylococcus aureus secreted tyrosine phosphatase PtpA. Biochem Biophys Res Commun **469**, 619-625*

Figure S2

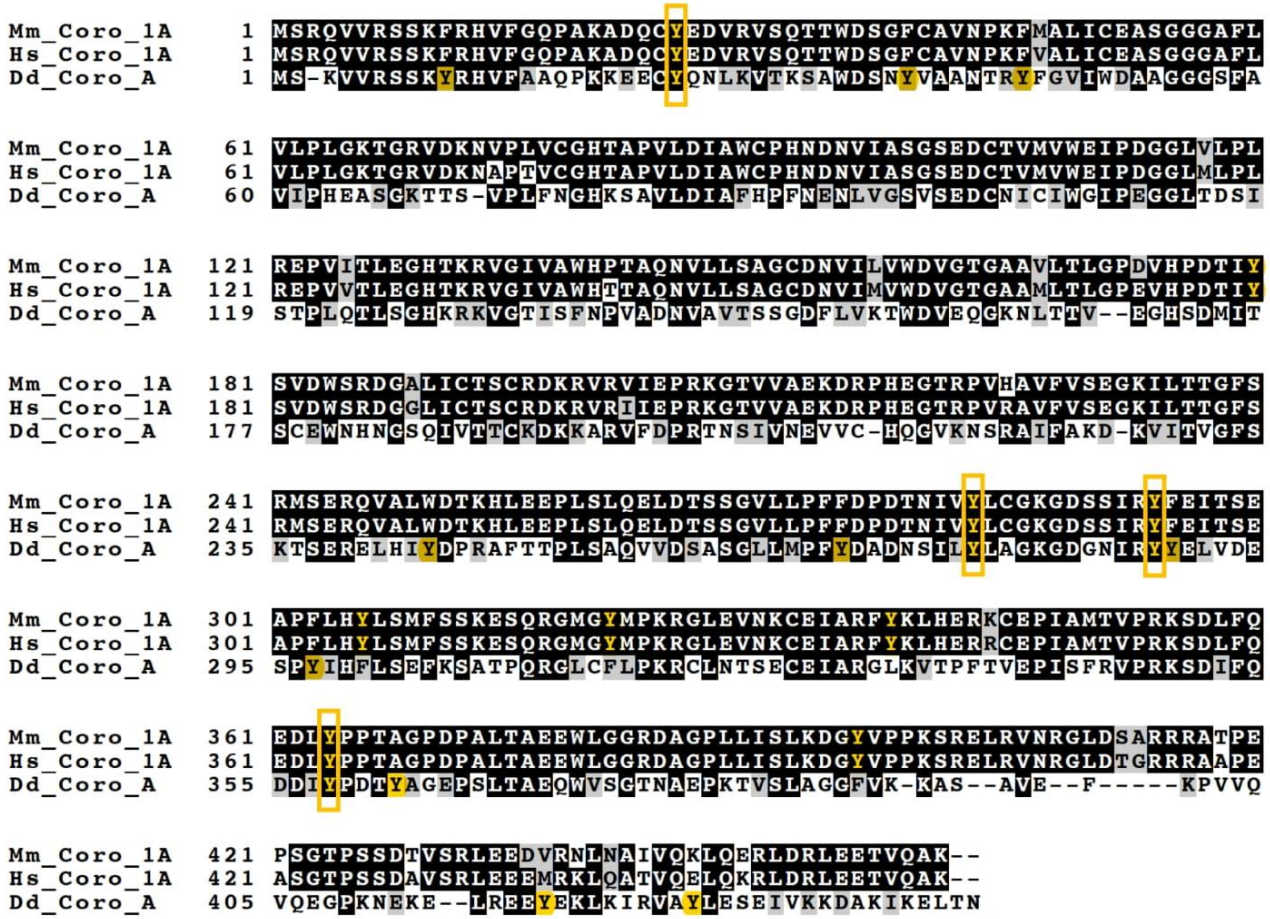

Figure S2: Sequence alignment of Coronin 1A proteins. Sequences were aligned using ClustalW program. Alignments are shaded using the BOXSHADE server. Sequence designations and NCBI GI numbers are as follows: Mm_Coro-1A from Murine macrophages, GI O89053; Hs_Coro-1A from Homo sapiens, GI P31146; and Dd_Coro-A from *Dictyostelium discoideum*, GI P27133. Black boxes represent identical residues and grey boxes indicate similar residues. Tyrosine residue (Y) are highligted in orange. Similar tyrosine residues in all three species are framed in orange boxes.

Figure S3

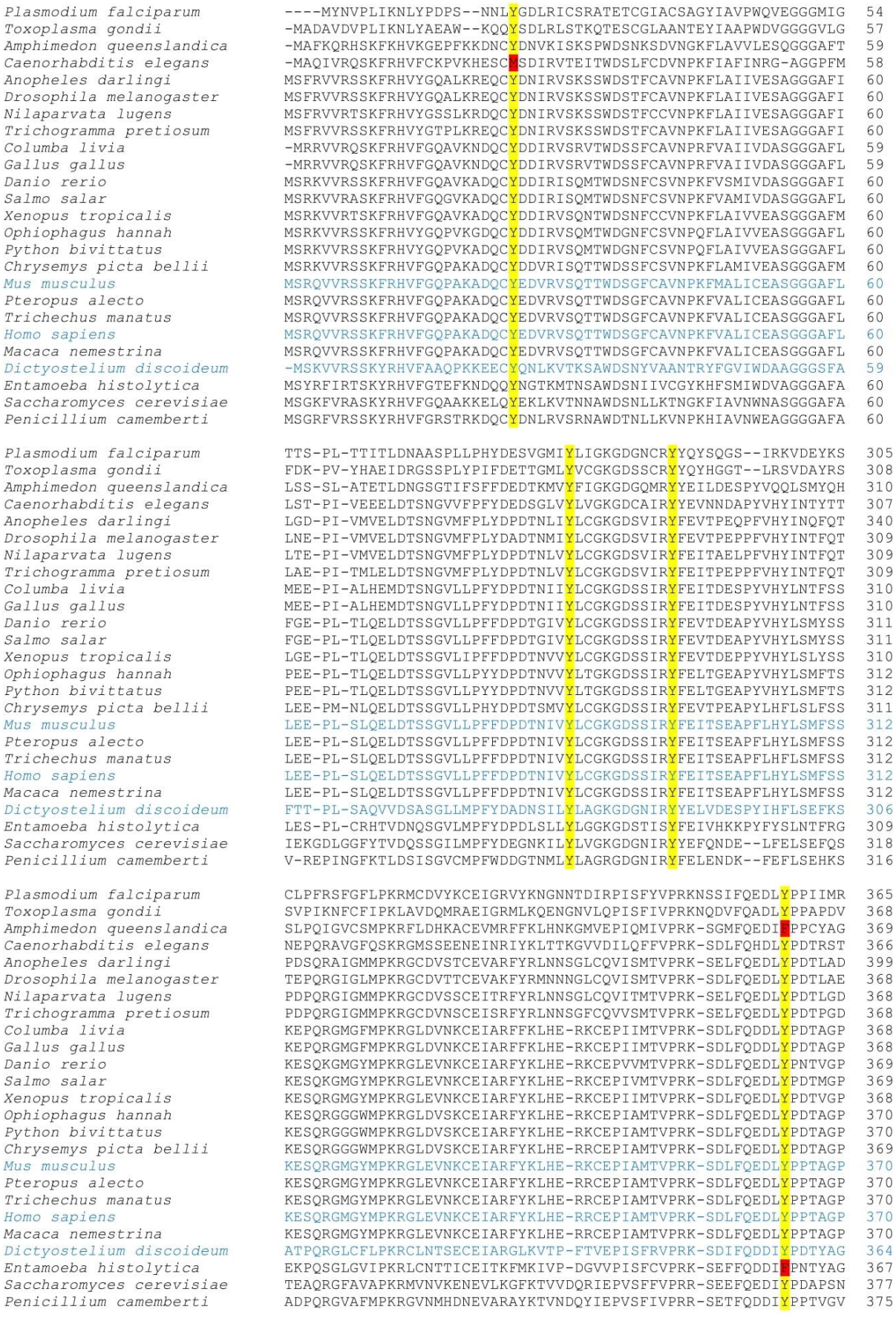
Plasmodium falciparum ----MYNVPLIKNLYPDPS--NNLYGDLRICSRATETCGIACSAGYIAVPWQVEGGGMIG 54
Toxoplasma gondii -MADAVDVPLIKNLYAEAW--KQQYSDLRLSTKQTESCGLAANTEYIAAPWDVGGGGVLG 57
Amphimedon queenslandica -MAFKQRHSKFKHVKGEPFKKDNCYDNVKISKSPWDSNKSDVNGKFLAVVLESQGGGAFT 59
Caenorhabditis elegans -MAQIVRQSKFRHVFCKPVKHESCRSDIRVTEITWDSLFCDVNPKFIAFINRG-AGGPFM 58
Anopheles darlingi MSFRVVRSSKFRHVYGQALKREQCYDNIRVSKSSWDSTFCAVNPKFLAIIVESAGGGAFI 60
Drosophila melanogaster MSFRVVRSSKFRHVYGQALKREQCYDNIRVSKSSWDSTFCAVNPKFLAIIVESAGGGAFI 60
Nilaparvata lugens MSFRVVRTSKFRHVYGSSLKRDQCYDNIRVSKSSWDSTFCCVNPKFLAIIVESAGGGAFI 60
Trichogramma pretiosum MSFRVVRSSKFRHVYGTPLKREQCYDNIRVSKSSWDSTFCAVNPKFLAIIVESAGGGAFI 60
Columba livia -MRRVVRQSKFRHVFGQAVKNDQCYDDIRVSRVTWDSSFCAVNPRFVAIIVDASGGGAFL 59
Gallus gallus -MRRVVRQSKFRHVFGQAVKNDQCYDDIRVSRVTWDSSFCAVNPRFVAIIVDASGGGAFL 59
Danio rerio MSRKVVRSSKFRHVFGQAVKADQCYDDIRISQMTWDSNFCSVNPKFVSMIVDASGGGAFI 60
Salmo salar MSRKVVRASKFRHVFGQGVKADQCYDDIRISQMTWDSNFCSVNPKFVAMIVDASGGGAFL 60
Xenopus tropicalis MSRKVVRTSKFRHVFGQAVKADQCYDDIRVSQNTWDSNFCCVNPKFLAIVVEASGGGAFM 60
Ophiophagus hannah MSRKVVRSSKFRHVYGQPVKGDQCYDDIRVSQMTWDGNFCSVNPQFLAIVVEASGGGAFL 60
Python bivittatus MSRKVVRSSKFRHVYGQPVKADQCYDDIRVSQMTWDGNFCSVNPQFLAIVVEASGGGAFL 60
Chrysemys picta bellii MSRKVVRSSKFRHVFGQPAKADQCYDDVRISQTTWDSSFCSVNPKFLAMIVEASGGGAFM 60
Mus musculus MSRQVVRSSKFRHVFGQPAKADQCYEDVRVSQTTWDSGFCAVNPKFMALICEASGGGAFL 60
Pteropus alecto MSRQVVRSSKFRHVFGQPAKADQCYEDVRVSQTTWDSGFCAVNPKFVALICEASGGGAFL 60
Trichechus manatus MSRQVVRSSKFRHVFGQPAKADQCYEDVRVSQTTWDSGFCAVNPKFVALICEASGGGAFL 60
Homo sapiens MSRQVVRSSKFRHVFGQPAKADQCYEDVRVSQTTWDSGFCAVNPKFVALICEASGGGAFL 60
Macaca nemestrina MSRQVVRSSKFRHVFGQPAKADQCYEDVRVSQTTWDSGFCAVNPKFVALICEASGGGAFL 60
Dictyostelium discoideum -MSKVVRSSKYRHVFAAQPKKEECYQNLKVTKSAWDSNYVAANTRYFGVIWDAAGGGSFA 59
Entamoeba histolytica MSYRFIRTSKYRHVFGTEFKNDQQYNGTKMTNSAWDSNIIVCGYKHFSMIWDVAGGGAFA 60
Saccharomyces cerevisiae MSGKFVRASKYRHVFGQAAKKELQYEKLKVTNNAWDSNLLKTNGKFIAVNWNASGGGAFA 60
Penicillium camemberti MSGRFVRSSKYRHVFGRSTRKDQCYDNLRVSRNAWDTNLLKVNPKHIAVNWEAGGGGAFA 60
Plasmodium falciparum TTS-PL-TTITLDNAASPLLPHYDESVGMIYLIGKGDGNCRYYQYSQGS--IRKVDEYKS 305
Toxoplasma gondii FDK-PV-YHAEIDRGSSPLYPIFDETTGMLYVCGKGDSSCRYYQYHGGT--LRSVDAYRS 308
Amphimedon queenslandica LSS-SL-ATETLDNGSGTIFSFFDEDTKMVYFIGKGDGQMRYYEILDESPYVQQLSMYQH 310
Caenorhabditis elegans LST-PI-VEEELDTSNGVVFPFYDEDSGLVYLVGKGDCAIRYYEVNNDAPYVHYINTYTT 307
Anopheles darlingi LGD-PI-VMVELDTSNGVMFPLYDPDTNLIYLCGKGDSVIRYFEVTPEQPFVHYINQFQT 340
Drosophila melanogaster LNE-PI-VMVELDTSNGVMFPLYDADTNMIYLCGKGDSVIRYFEVTPEPPFVHYINTFQT 309
Nilaparvata lugens LTE-PI-VMVELDTSNGVMFPLYDPDTNLVYLCGKGDSVIRYFEITAELPFVHYINTFQT 309
Trichogramma pretiosum LAE-PI-TMLELDTSNGVMFPLYDPDTNLVYLCGKGDSVIRYFEITPEPPFVHYINTFQT 309
Columba livia MEE-PI-ALHEMDTSNGVLLPFYDPDTNIIYLCGKGDSSIRYFEITDESPYVHYLNTFSS 310
Gallus gallus MEE-PI-ALHEMDTSNGVLLPFYDPDTNIIYLCGKGDSSIRYFEITDESPYVHYLNTFSS 310
Danio rerio FGE-PL-TLQELDTSSGVLLPFFDPDTGIVYLCGKGDSSIRYFEVTDEAPYVHYLSMYSS 311
Salmo salar FGE-PL-TLQELDTSSGVLLPFFDPDTGIVYLCGKGDSSIRYFEVTDEAPYVHYLSMYSS 311
Xenopus tropicalis LGE-PL-TLQELDTSSGVLIPFFDPDTNVVYLCGKGDSSIRYFEVTDEPPYVHYLSLYSS 310
Ophiophagus hannah PEE-PL-TLQELDTSSGVLLPYYDPDTNVVYLTGKGDSSIRYFELTGEAPYVHYLSMFTS 312
Python bivittatus PEE-PL-TLQELDTSSGVLLPYYDPDTNVVYLTGKGDSSIRYFELTGEAPYVHYLSMFTS 312
Chrysemys picta bellii LEE-PM-NLQELDTSSGVLLPHYDPDTSMVYLCGKGDSSIRYFEVTPEAPYLHFLSLFSS 311
Mus musculus LEE-PL-SLQELDTSSGVLLPFFDPDTNIVYLCGKGDSSIRYFEITSEAPFLHYLSMFSS 312
Pteropus alecto LEE-PL-SLQELDTSSGVLLPFFDPDTNIVYLCGKGDSSIRYFEITSEAPFLHYLSMFSS 312
Trichechus manatus LEE-PL-SLQELDTSSGVLLPFFDPDTNIVYLCGKGDSSIRYFEITSEAPFLHFLSMFSS 312
Homo sapiens LEE-PL-SLQELDTSSGVLLPFFDPDTNIVYLCGKGDSSIRYFEITSEAPFLHYLSMFSS 312
Macaca nemestrina LEE-PL-SLQELDTSSGVLLPFFDPDTNIVYLCGKGDSSIRYFEITSEAPFLHYLSMFSS 312
Dictyostelium discoideum FTT-PL-SAQVVDSASGLLMPFYDADNSILYLAGKGDGNIRYYELVDESPYIHFLSEFKS 306
Entamoeba histolytica LES-PL-CRHTVDNQSGVLMPFYDPDLSLLYLGGKGDSTISYFEIVHKKPYFYSLNTFRG 309
Saccharomyces cerevisiae IEKGDLGGFYTVDQSSGILMPFYDEGNKILYLVGKGDGNIRYYEFQNDE--LFELSEFQS 318
Penicillium camemberti V-REPINGFKTLDSISGVCMPFWDDGTNMLYLAGRGDGNIRYFELENDK--FEFLSEHKS 316
Plasmodium falciparum CLPFRSFGFLPKRMCDVYKCEIGRVYKNGNNTDIRPISFYVPRKNSSIFQEDLYPPIIMR 365
Toxoplasma gondii SVPIKNFCFIPKLAVDQMRAEIGRMLKQENGNVLQPISFIVPRKNQDVFQADLYPPAPDV 368
Amphimedon queenslandica SLPQIGVCSMPKRFLDHKACEVMRFFKLHNKGMVEPIQMIVPRK-SGMFQEDIFPPCYAG 369
Caenorhabditis elegans NEPQRAVGFQSKRGMSSEENEINRIYKLTTKGVVDILQFFVPRK-SDLFQHDLYPDTRST 366
Anopheles darlingi PDSQRAIGMMPKRGCDVSTCEVARFYRLNNSGLCQVISMTVPRK-SELFQEDLYPDTLAD 399
Drosophila melanogaster TEPQRGIGLMPKRGCDVTTCEVAKFYRMNNNGLCQVISMTVPRK-SDLFQEDLYPDTLAE 368
Nilaparvata lugens PDPQRGIGMMPKRGCDVSSCEITRFYRLNNSGLCQVITMTVPRK-SELFQEDLYPDTLGD 368
Trichogramma pretiosum PDPQRGIGMMPKRGCDVNSCEISRFYRLNNSGFCQVVSMTVPRK-SELFQEDLYPDTPGD 368
Columba livia KEPQRGMGFMPKRGLDVNKCEIARFFKLHE-RKCEPIIMTVPRK-SDLFQDDLYPDTAGP 368
Gallus gallus KEPQRGMGFMPKRGLDVNKCEIARFFKLHE-RKCEPIIMTVPRK-SDLFQDDLYPDTAGP 368
Danio rerio KESQKGMGYMPKRGLEVNKCEIARFYKLHE-RKCEPVVMTVPRK-SDLFQEDLYPNTVGP 369
Salmo salar KESQKGMGYMPKRGLEVNKCEIARFYKLHE-RKCEPIVMTVPRK-SDLFQEDLYPDTMGP 369
Xenopus tropicalis KESQRGMGYMPKRGLEVNKCEIARFYKLHE-RKCEPIIMTVPRK-SDLFQEDLYPDTVGP 368
Ophiophagus hannah KESQRGGGWMPKRGLDVSKCEIARFYKLHE-RKCEPIAMTVPRK-SDLFQEDLYPDTAGP 370
Python bivittatus KESQRGGGWMPKRGLDVSKCEIARFYKLHE-RKCEPIAMTVPRK-SDLFQEDLYPDTAGP 370
Chrysemys picta bellii KESQRGGGWMPKRGLDVSKCEIARFYKLHE-RRCEPIAMTVPRK-SDLFQEDLYPDTAGP 369
Mus musculus KESQRGMGYMPKRGLEVNKCEIARFYKLHE-RKCEPIAMTVPRK-SDLFQEDLYPPTAGP 370
Pteropus alecto KESQRGMGYMPKRGLEVNKCEIARFYKLHE-RRCEPIAMTVPRK-SDLFQEDLYPPTAGP 370
Trichechus manatus KESQRGMGYMPKRGLEVNKCEIARFYKLHE-RRCEPIAMTVPRK-SDLFQEDLYPPTAGP 370
Homo sapiens KESQRGMGYMPKRGLEVNKCEIARFYKLHE-RRCEPIAMTVPRK-SDLFQEDLYPPTAGP 370
Macaca nemestrina KESQRGMGYMPKRGLEVNKCEIARFYKLHE-RRCEPIAMTVPRK-SDLFQEDLYPPTAGP 370
Dictyostelium discoideum ATPQRGLCFLPKRCLNTSECEIARGLKVTP-FTVEPISFRVPRK-SDIFQDDIYPDTYAG 364
Entamoeba histolytica EKPQSGLGVIPKRLCNTTICEITKFMKIVP-DGVVPISFCVPRK-SEFFQDDIFPNTYAG 367
Saccharomyces cerevisiae TEAQRGFAVAPKRMVNVKENEVLKGFKTVVDQRIEPVSFFVPRR-SEEFQEDIYPDAPSN 377
Penicillium camemberti ADPQRGVAFMPKRGVNMHDNEVARAYKTVNDQYIEPVSFIVPRR-SETFQDDIYPPTVGV 375

Figure S3: Sequence alignment of conserved tyrosine residues harboring regions of Coronin 1 proteins. Sequences were aligned using Clustal Omega. Conserved tyrosine residues are labeled in yellow, mismatches are indicated in red. Sequences corresponding to the ones dipicted in Fig. S2 are labeld in blue. Sequences were obtained from the following NCBI entries: *Amphimedon queenslandica*, XP_003382924; *Anopheles darlingi*, GI 568254291; *Caenorhabditis elegans*, GI 25150742; *Chrysemys picta bellii*, XP_005290238; *Columba livia*, GI 1307741494; *Danio rerio*, GI 41055464; *Drosophila melanogaster*, GI 21685588; *Entamoeba histolytica*, GI 1033413111; *Gallus gallus*, GI 482661642; *Homo sapiens*, GI P31146; *Macaca nemestria*, GI 795657297; *Mus musculus*, GI O89053; *Nilaparvata lugens*, XP_022202333; *Ophiophagus hannah*, GI 565307310; *Penicillium camemberti*, GI 902279349; *Plasmodium falciparum*, GI 2808641; *Pteropus alecto*, GI 586561765; *Python bivittatus*, GI 602635008; *Saccharomyces cerevisiae*, GI 151940945; *Salmo salar*, GI 223647640; *Toxoplasma gondii*, GI 53801426; *Trichechus manatus*, GI 471410223;*Trichogramma pretiosum*, XP_014222528; *Xenopus tropicalis*, GI 54262238.

Article (II)

The phosphoarginine phosphatase PtpB from *Staphylococcus aureus* is involved in bacterial stress adaptation during infection

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Article

The Phosphoarginine Phosphatase PtpB from *Staphylococcus aureus* Is Involved in Bacterial Stress Adaptation during Infection

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Abstract: *Staphylococcus aureus* continues to be a public health threat, especially in hospital settings. Studies aimed at deciphering the molecular and cellular mechanisms that underlie pathogenesis, host adaptation, and virulence are required to develop effective treatment strategies. Numerous host-pathogen interactions were found to be dependent on phosphatases-mediated regulation. This study focused on the analysis of the role of the low-molecular weight phosphatase PtpB, in particular, during infection. Deletion of *ptpB* in *S. aureus* strain SA564 significantly reduced the capacity of the mutant to withstand intracellular killing by THP-1 macrophages. When injected into normoglycemic C57BL/6 mice, the SA564 Δ*ptpB* mutant displayed markedly reduced bacterial loads in liver and kidney tissues in a murine *S. aureus* abscess model when compared to the wild type. We also observed that PtpB phosphatase-activity was sensitive to oxidative stress. Our quantitative transcript analyses revealed that PtpB affects the transcription of various genes involved in oxidative stress adaptation and infectivity. Thus, this study disclosed first insights into the physiological role of PtpB during host interaction allowing us to link phosphatase-dependent regulation to oxidative bacterial stress adaptation during infection.

Keywords: *Staphylococcus aureus*; arginine phosphatase; infection; oxidative response

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1. Introduction

Staphylococcus aureus has emerged as a major human pathogen responsible for hospital and community-associated infections that can involve almost any organ system, including skin and soft tissue infections, necrotizing pneumonia, and infective endocarditis [1]. The increase in multi-resistant variants of this species, coupled with its increasing prevalence as a nosocomial pathogen, is of major concern [2]. The success of *S. aureus* as a pathogen and its ability to cause such a wide range of infections are the result of its large armamentarium of virulence factors that is controlled by a sophisticated network of regulatory molecules [3,4]. *S. aureus* has an exceptional ability to survive under unfavorable conditions, either by adapting to environmental factors or by defending against exogenous stresses [5]. Within the human host, the bacterium can infect and reside in a wide range of tissues, ranging from superficial surfaces like the skin to deeper tissues such as the gastrointestinal tract, heart and bones. In order to achieve this multifaceted lifestyle, *S. aureus* uses complex regulatory networks to sense diverse signals that enable it to adapt to different environments and modulate virulence [6].

To survive in changing environments, bacteria have developed exquisite systems that not only sense these stresses but also trigger appropriate responses, which allow survival and even propagation under these conditions. Basic research of staphylococcal protein regulation is therefore required to decipher molecular and cellular mechanisms that underlie pathogenesis, host adaptation and virulence. Thus, understanding how *S. aureus* regulates its virulence in response to host environments is important to devise effective treatment strategies.

Signal transduction is an essential and universal mechanism that allows all cells—from prokaryotes to eukaryotes—to translate environmental signals into adaptive changes. By this mechanism, extracellular inputs propagate through complex signaling networks whose activity is often regulated by reversible protein phosphorylation [7]. Protein kinases and phosphatases are the regulatory proteins that tend to amplify an external signal, thus making it crucial. Protein phosphorylation is carried out by a multitude of protein kinases that transfer the gamma-phosphate from ATP to specific amino acids on proteins, predominantly to the side chains of serine, threonine, and tyrosine residues [8,9]. An analogous number of protein phosphatases counteract these reactions by catalyzing the dephosphorylation of specific substrate proteins. Based on their sequence, structure, and function, protein phosphatases are grouped into three main classes. Phosphatases acting on phospho-serine/threonine (pSer, pThr) comprise the PPP (phospho-protein phosphatase) and PPM (Mg^{2+}/Mn^{2+}-dependent protein phosphatase) families, whereas enzymes acting on phospho-tyrosine (pTyr) constitute the protein tyrosine phosphatase (PTP) superfamily [8,10]. In addition, specialized protein phosphatases act on phospho-aspartate, phospho-histidine, and phospho-cysteine residues [11–14]. Recently, a member of the low-molecular weight protein tyrosine phosphatase (LMW-PTP) family was shown to efficiently target phospho-arginine [15]. Protein arginine phosphorylation represents a novel posttranslational modification (PTM) that alters protein function in vitro and in vivo [16,17]. While bacterial tyrosine phosphatases can be intimately involved in a number of cellular processes, one major theme has become apparent with the involvement of phosphatases as virulence factors [18]. Although a detailed picture is yet unavailable, a role of bacterial protein tyrosine phosphatases during host infection has been identified in different facultative and obligate intracellular pathogens, and the strategies employed by them are currently being elucidated [18,19].

A wealth of information has been gained from studies aimed at deciphering the pathophysiological events during *S. aureus*-macrophage infection [20], but the signaling pathways leading to these adaptations are still poorly understood. Protein phosphatases are suggested to be important regulatory enzymes in pathogenic bacteria, though the upstream signaling of these proteins needs to be further analyzed under specific environmental conditions, which may help in identifying their sensing mechanisms. *S. aureus* has been reported to produce two LMW-PTPs, PtpA and PtpB [21], with the corresponding genes being part of the *S. aureus* core genome [22]. In our previous work, we demonstrated the secretion of the *S. aureus* phosphatase PtpA [23], and its involvement in the process of infection and intracellular survival [24]. Furthermore, while PtpB was initially described as a tyrosine phosphatase [21], recent phosphoproteomic analysis indicated that PtpB functions as an arginine phosphatase [25] that is involved in oxidative stress response, amino acid metabolism, and virulence factor synthesis [26]. Yet, its role during infection remains to be investigated. In this study, we demonstrate that PtpB contributes to the intracellular survival capacity of *S. aureus* within macrophages and participates in the infectivity of this pathogen in a murine *S. aureus* abscess model. In vitro phosphatase activity assays revealed furthermore that PtpB is sensitive to oxidative stress. Additionally, we show that PtpB affects the transcription of various genes involved in oxidative stress adaptation and infectivity.

2. Materials and Methods

2.1. Bacterial Strains, Media, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All mutant strains and plasmids generated for this study were confirmed by sequencing of the affected region, and by assessing gene transcription by quantitative real-time reverse transcriptase PCR (qRT-PCR). *Escherichia coli* strains were grown at 37 °C in LB medium. *S. aureus* isolates were plated on Tryptic Soy Agar (TSA; BD, Heidelberg, Germany), or grown in Tryptic Soy Broth (TSB; BD) medium at 37 °C and 225 rpm with a culture to flask volume of 1:10. Antibiotics were only used for strain construction and phenotypic selection at the following concentrations: ampicillin, 100 µg/mL; tetracycline, 2.5 µg/mL; erythromycin, 2.5 µg/mL; and chloramphenicol, 10 µg/mL.

Table 1. Strains and plasmids used in this study.

Strain	Description [1]	Reference or Source
S. aureus		
SA564	*S. aureus* clinical isolate, wild type	[27]
SA564 Δ*ptpB*	SA564 Δ*ptpB*::lox66-*erm*(B)-lox71; Erm^R	This study
SA564 Δ*ptpB*::*ptpB*	*cis*-complemented SA564 Δ*ptpB* derivative	This study
E. coli		
BL21(DE3)Star	*E. coli* strain allowing a high-level recombinant protein expression. IPTG-inducible T7 RNA polymerase	Invitrogen
IM08B	*E. coli* DC10B derivative harboring *hsdS* of *S. aureus* strain NRS384, Δ*dcm*	[28]
TOP10	*E. coli* derivative ultra-competent cells used for general cloning	Invitrogen
Plasmids		
pBASE6	*E. coli–S. aureus* temperature-sensitive suicide shuttle vector, *secY* counterselection; *bla cat*	[29]
pBASE6 *ptpB* comp	pBASE6 derivative harboring the C-terminal region of NWMN_2020, *ptpB*, and the N-terminal region of *glyA*; *bla*, *cat*	This study
pBT	*S. aureus* suicide plasmid; *tet*(L)	[30]
pBT ptpB KO	pBT derivative harboring the genomic regions flanking *ptpB* and lox66-*erm*(B)-lox71; *tet*(L), *erm*(B)	This study
pET19b	*E. coli* vector for IPTG inducible protein expression; *bla*	Novagen
pET19b_PtpB	pET19b derivative used to express HAT-tagged fusion of *S. aureus* PtpB WT in *E. coli*; *bla*	This study
pET19b_PtpB_D111A	pET19b derivative used to express HAT-tagged fusion of *S. aureus* PtpB_D111A in *E. coli*; *bla*	This study
pET19b_PtpB_T11I	pET19b derivative used to express HAT-tagged fusion of *S. aureus* PtpB_T11I in *E. coli*; *bla*	This study
pRMC2	*E. coli–S. aureus* shuttle vector, Tetracycline-inducible expression; *bla*, *tet*	[31]
pRMC2_PtpA-Spot	pRMC2 derivative used to express C-terminal Spot-tagged fusion of *S. aureus* PtpA; *bla*, *tet*	This study
pRMC2_PtpB-Spot	pRMC2 derivative used to express C-terminal Spot-tagged fusion of *S. aureus* PtpB; *bla*, *tet*	This study
pRMC2_SecA-Spot	pRMC2 derivative used to express C-terminal Spot-tagged fusion of *S. aureus* SecA; *bla*, *tet*	This study

[1] Erm^R, erythromycin-resistant.

2.2. Cloning and Expression of PtpA, PtpB and SecA Spot-Tagged Proteins in S. aureus

The *ptpA*, *ptpB*, and *secA* genes were amplified by PCR using *S. aureus* Newman chromosomal DNA as a template, and the Spot fragment was amplified by PCR using the pSpot2 vector as a template (Chromotek, Planegg, Germany) with the primers listed in Table S1. The *ptpA*, *ptpB*, and *secA* plasmids were constructed using NEB Gibson Assembly kit (New England Biolabs, Ipswich, MA, USA). The *ptpA*, *ptpB* and *secA* purified PCR products were fused to the Spot-tag at the *C*-terminus and Gibson cloned into the KpnI/EcoRI digested pRMC2 vector [31], thus generating pRMC2_PtpA-Spot, pRMC2_PtpB-Spot and pRMC2_SecA-Spot, respectively. The plasmids were propagated in *E. coli* IM08B [28] and electroporated into *S. aureus* strain SA564 [27].

2.3. Cloning, Expression and Purification of Recombinant PtpB Proteins.

The *ptpB* derivatives plasmids were constructed using NEB Gibson Assembly kit (New England Biolabs). The *ptpB* gene was amplified by PCR using *S. aureus* Newman chromosomal DNA as a template with the primers listed in Table S1. The *ptpB* fragment was fused to Histidine Affinity Tag (HAT) [32] at the *C*-terminus and Gibson cloned into the NcoI/BamHI digested pET19b vector (Novagen, Madison, WI, USA), thus generating pET19b_PtpB. PtpB_T11I and PtpB_D111A derivatives harboring threonine to isoleucine or aspartic acid to alanine substitutions, respectively, were generated by Gibson assembly (Table S1). Transformed *E. coli* BL21 Star cells were grown at 16 °C in LB medium containing 1 mg/mL of glucose and 100 µg/mL of ampicillin, and protein synthesis was induced with 0.5 mM IPTG overnight. Bacteria were disrupted in a French pressure cell and centrifuged at 14,000 rpm for 25 min. Purifications of the HAT-tagged recombinants were performed using TALON® metal affinity resins (Clontech, Mountain View, CA, USA) accordingly to the manufacturer's instructions and eluted in 200 mM imidazole, 50 mM Tris-HCl [pH 7.4], 100 mM NaCl, and 10% [vol/vol] glycerol, to be stored at −20 °C.

2.4. Construction of the S. aureus ptpB Deletion and Cis-Complementation Strains

For the *S. aureus ptpB* deletion mutants, 0.95- and 1.1-kb fragments (nucleotides 2236272-2237225 and 2234785-2235883 of GenBank accession no. AP009351.1, respectively), containing the flanking regions of the *ptpB* open reading frame (ORF), were amplified by PCR from chromosomal DNA of *S. aureus* strain Newman using primer pairs MBH-510/MBH-511 and MBH-512/MBH-513, respectively (Table S1). The PCR products were digested with KpnI/XhoI and EcoRI/SacI, respectively, and cloned together with an XhoI/EcoRI digested lox66-*erm*(B)-lox71 fragment into suicide vector pBT [30] to generate plasmid pBT *ptpB* KO. Plasmid pBT *ptpB* KO was propagated in *E. coli* strain IM08B [28] and subsequently electroporated directly into *S. aureus* strain Newman to obtain strain Newman Δ*ptpB*, in which nucleotides 17 to 405 of the 420-bp spanning *ptpB* open reading frame were replaced by the lox66-*erm*(B)-lox71 cassette by allelic replacement. The deletion of *ptpB* in Newman Δ*ptpB* was confirmed by sequencing, and the strain was then used as a donor for transducing the lox66-*erm*(B)-lox71 tagged *ptpB* deletion into *S. aureus* strain SA564 [27].

For the *cis*-complementation of the Δ*ptpB-erm*(B) mutation in SA564 Δ*ptpB*, a 2.4-kb fragment (nucleotides 2234785-2237225 of GenBank accession no. AP009351.1) covering the C-terminal region of ORF NWMN_2020, *ptpB*, the annotated terminator region of the *NWMN-2020-ptpB* operon, and the N-terminal region of *glyA* was amplified by primers MBH-510/MBH-513 (Table S1), digested with KpnI/SacI, and cloned into KpnI/SacI-predigested plasmid pBASE6 [29] to generate plasmid pBASE6 *ptpB* comp. The plasmid was propagated in *E. coli* IM08B and electroporated into *S. aureus* strain SA564 Δ*ptpB*. Replacement of the *erm*(B)-tagged *ptpB* deletion by the functional *NWMN_2020-ptpB* locus was done as previously described [33].

2.5. Murine Abscess Model

Animal experiments were performed with approval of the local State Review Board of Saarland, Germany, and conducted following the national and European guidelines for the ethical and human treatment of animals. Preparation of the bacterial inoculum and infection of the animals were carried out as described [34], with minor modifications. Briefly, 100 µL bacterial suspensions containing ~10^7 colony forming units (CFU) were administered intravenously by retro-orbital injection into female, 8- to 10-week-old C57BL/6N mice (Charles River, Sulzfeld, Germany) that were anesthetized by isoflurane inhalation (3.5%; Baxter, Unterschleißheim, Germany). Immediately after infection, mice were treated with a dose of carprofen (5 mg/kg; Zoetis, Berlin, Germany), and at four days post infection, mice were sacrificed, and livers and kidneys were removed. The organs were weight adjusted and homogenized in PBS (Thermo Fisher, Dreieich, Germany), and serial dilutions of the homogenates were plated on blood agar plates to enumerate the CFU rates in the organs.

2.6. Macrophage Culture and Infection

Cells of the human leukemia monocytic cell line THP-1 [35] were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ no. ACC 16). THP-1 cells were cultured and differentiated into macrophages as described in [36]. For macrophage infection, *S. aureus* cells were grown to the mid-exponential growth phase (i.e., 2 h) in TSB medium. The bacteria were collected by centrifugation at 4,000 g for 10 min and resuspended in PBS. The differentiated THP-1 macrophages (5×10^5 cells/well) were inoculated with *S. aureus* at the multiplicity of infection (MOI) of 20:1 (bacteria to cells) and incubated in 10% fetal calf serum (FCS; PAA, Pasching, Germany)-supplemented RPMI-1640 (Thermo Fisher) at 37 °C and 5% CO_2 for 1 h. Subsequently, cells were washed with PBS to remove unbound bacteria, and the remaining extracellular bacteria were killed by incubation with 100 μg/mL gentamicin (Merck, Darmstadt, Germany) and 20 μg/mL lysostaphin (Genmedics, Reutlingen, Germany) for 30 min. After gentamicin/lysostaphin treatment, macrophages were rinsed twice with PBS (time point T0), and then further incubated in FCS (10%) and gentamicin (100 μg/mL)-supplemented RPMI-1640 for 45 min (time point T45). Lysis of macrophages and enumeration of intracellular bacteria at time points T0 and T45 was performed as described in [24]. The intracellular survival rate was determined by dividing the CFU rate seen at T45 by the corresponding CFU rates seen at T0.

2.7. PtpB Phosphatase Activity Assay

The phosphatase activity of PtpB was assayed in vitro by using a method based on the detection of p-nitrophenol (PNP) formed from p-nitrophenyl phosphate (PNPP) cleavage. Tests were performed in buffer containing 100 mM sodium citrate, 40 mM PNPP, and 3 mM dithiothreitol (DTT) when required. The reaction was initiated by the addition of 0.5 μM PtpB phosphatase or derivatives followed by incubation in 96-well microplates at 25 °C. To test the effect of oxidation, DTT was omitted and H_2O_2 (500 μM final concentration) was added, respectively. The phosphatase reaction was monitored at 405 nm (absorption maximum of the generated PNP) using a Spark 20M fluorimeter (Tecan, Lyon, France) microplate reader. All experiments were performed at least in triplicate.

2.8. H_2O_2 Susceptibility Assays

Minimal inhibitory concentrations of SA564 and its Δ*ptpB* derivative for H_2O_2 (Sigma, Saint-Quentin-Fallavier, France) were determined by broth microdilution assays according to CLSI standard M07 [37], however, by using TSB instead of cation-adjusted Mueller-Hinton broth. In a second assay, bacterial cells were cultured in TSB at 37 °C and 225 rpm for 2 h, and subsequently challenged with H_2O_2 (50 mM final concentration) for 1 h. At the end of the incubation time, CFU rates of the cultures were determined by plate counting.

2.9. NO Susceptibility Assay

SA564 and its isogenic Δ*ptpB* mutant were grown overnight on TSA, and colonies were picked on the next morning and suspended in fresh TSB to a McFarland of 0.5. Cell suspensions were diluted 1:100 in TSB and subsequently mixed 1:1 with TSB supplemented with different concentrations (0, 6.25 and 12.5 μM final concentration) of the $NO^{\cdot}$ donor diethylamine NONOate diethylammonium salt (DEA NONOate, Sigma). Growth of the cultures was monitored for 12 h using digital time-lapse microscopy with the oCelloScope instrument (BioSense Solutions, Farum, Denmark) under static conditions at 37 °C and 5% CO_2. Growth kinetics of the cultures were determined using the Background Corrected Absorption (BCA) algorithm of the UniExplorer software (BioSense Solutions, version 9.0).

2.10. pH 5.5 Survival Assay

Exponential growth phase cells (i.e., 2 h) of SA564 and its isogenic Δ*ptpB* mutant were washed with PBS (pH 7.4) and suspended in PBS (pH 5.5) to an OD_{600} of 1. PBS washed cells resuspended in PBS (pH 7.4) served as unchallenged controls. Cell suspensions were cultured for up to 3 h at 37 °C, and OD_{600} values of acid stressed cell suspensions were

determined every 30 min. The relative survival rates were determined by dividing the OD_{600} readings of the cell suspensions through the values recorded at time point 0. At 1 h post inoculation, aliquots of acid stress challenged cell suspensions and controls were removed, and CFU rates of the cultures were determined by plate counting.

2.11. Measurement of Gene Expression by qRT-PCR

RNA isolation, cDNA synthesis and qRT-PCR were carried out as previously described [38], using the primer pairs listed in Table S1. Transcripts were quantified in reference to the transcription of gyrase B (in copies per copy of *gyrB*).

2.12. Statistical analyses

The statistical significance of changes between groups was assessed by the Mann-Whitney *U* test for experiments containing ≥ 4 biological replicates using the GraphPad Prism 6.01 software package (GraphPad, San Diego, CA, USA). p values < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. PtpB Does Not Affect the In Vitro Growth of S. aureus SA564 but Promotes Survival in Macrophages

Earlier work demonstrated that *S. aureus* survives readily in macrophages [39]. Given the impact of Ptp homologues on survival within macrophages [24,40], we wondered whether PtpB might fulfill similar functions in *S. aureus*. For this purpose, a *ptpB* deletion mutant in *S. aureus* strain SA564, a clinical isolate of clonal complex 5 (CC5), was generated (SA564 Δ*ptpB*), as well as the *cis*-complementation derivative SA564 Δ*ptpB::ptpB*. In vitro growth curves performed with the wild type, the mutant and the complemented strain demonstrated that the deletion of *ptpB* in *S. aureus* SA564 did not markedly affect the bacterial growth in TSB (Figure 1a), which is in line with earlier observations made with *S. aureus ptpB* mutants cultured in full media [26,41]. Next, survival rates of *S. aureus* SA564, the Δ*ptpB* mutant, and the *cis*-complemented strain were determined within THP-1 macrophages at 45 min post Gentamicin treatment (pGt). Already after this short period, a significantly smaller proportion of intracellular surviving cells were observed in THP-1 cells infected with the Δ*ptpB* mutant. *Cis*-complementation of the *ptpB* mutant with a functional *ptpB* locus reverted the intracellular survival rates to levels comparable to the wild type strain (Figure 1b). These data suggest that PtpB is involved in the intracellular survival capacity of *S. aureus* within macrophages.

3.2. PtpB Contributes to Infectivity of S. aureus in a Murine Abscess Model

As PtpB is involved in the survival capacity of *S. aureus* within macrophages (Figure 1b), we wondered whether and how PtpB might affect the infectivity of the bacterium in vivo. To address this, we assessed the ability of the strain triplet SA564/SA564 Δ*ptpB*/SA564 Δ*ptpB::ptpB* to cause disease in a murine abscess model [34]. Coherent with our intra-macrophage survival results (Figure 1b), a significant decrease (about 3-*log*) in the bacterial loads in liver and kidney was detected in mice infected with the Δ*ptpB* mutant when compared to mice challenged with the wild type strain (Figure 2).

Mice infected with the *cis*-complemented derivative SA564 Δ*ptpB::ptpB* displayed comparable bacterial loads in liver as wild type infected mice. In kidneys, SA564 Δ*ptpB::ptpB* infected mice produced CFU rates that were about 1-*log* lower than the CFU rates determined in wild type infected mice (Figure 2a). However, SA564 Δ*ptpB::ptpB* infected mice displayed about 100-fold higher bacterial loads in kidneys than the Δ*ptpB* mutant challenged mice, indicating that the decreases in bacterial loads seen in livers and kidneys of Δ*ptpB* mutant challenged mice were due to the deletion of *ptpB* and not caused by polar mutations. Taken together, these findings suggest that PtpB is a major contributor to abscess formation of *S. aureus* in mice. Remarkably, inactivation of *ptpB* in *S. aureus* decreased abscess formation in both, liver and kidneys, which is not the rule for other

mutations affecting liver and renal abscess formation of *S. aureus*, such as *ccpA*, *nor*, *nos*, and *sea* that altered the abscess formation only in one of the two organs upon infection via the blood system [42–44].

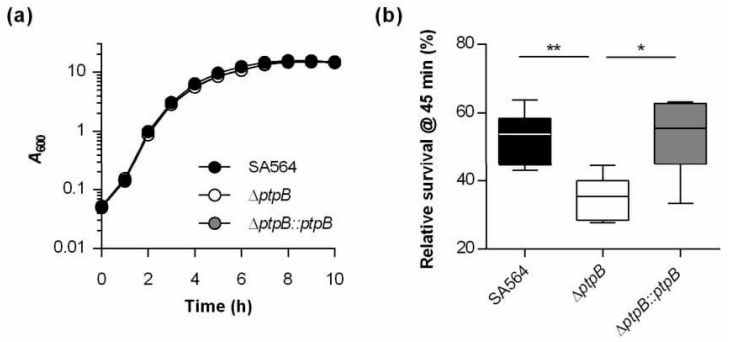

Figure 1. PtpB does not affect growth of *S. aureus* SA564 in TSB but promotes survival in macrophages. (**a**) Growth kinetics of *S. aureus* strains SA564 (black symbols), SA564 Δ*ptpB* (white symbols), and SA564 Δ*ptpB::ptpB* (gray symbols) in TSB. Cells were cultured at 37 °C and 225 rpm at a culture to flask volume of 1:10. Data represent the mean A_{600} readings ± SD at the time points indicated ($n = 3$). (**b**) *S. aureus* short-term survival in infected macrophages. Survival rates are given in relationship to the intracellular bacterial cell numbers seen immediately after the gentamicin/lysostaphin treatment, which was set to 100%. The data are presented as box and whisker plot showing the interquartile range (25–75%, box), the median (horizontal line) and the standard deviation (bars) of six independent experiments. * $p < 0.05$; ** $p < 0.01$ (Mann-Whitney *U* test).

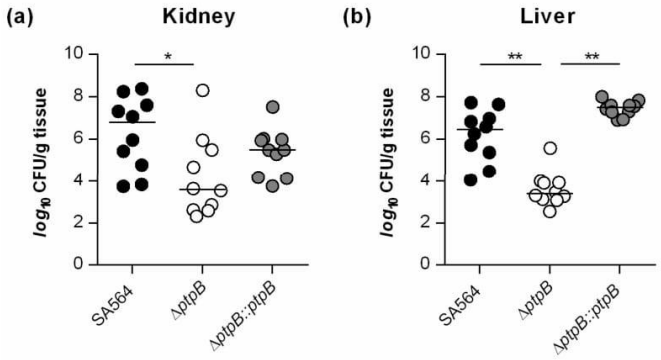

Figure 2. PtpB contributes to infectivity of *S. aureus* SA564 in a murine abscess model. C57BL/6N mice were infected via retroorbital injection with 1×10^7 cells of *S. aureus* strain SA564 (black symbols), SA564 Δ*ptpB* (white symbols), and the *cis*-complemented derivative SA564 Δ*ptpB::ptpB* (gray symbols), respectively ($n = 10$ per group). Mice were euthanized 4 days post-infection, livers and kidneys were removed and homogenized in PBS, and serial dilutions of the homogenates plated on sheep blood agar plates to determine the bacterial loads in kidney (**a**) and liver (**b**) organs. Each symbol represents an individual mouse. Horizontal bars indicate the median of all observations. * $p < 0.05$; ** $p < 0.01$ (Mann-Whitney *U* test).

3.3. PtpB Is Not Secreted by S. aureus during In Vitro Growth and Upon Ingestion by Macrophages

Various pathogenic bacteria use the secretion of bacterial signaling proteins into target host cells to modulate the phosphorylation status of host signaling networks [45–47]. We have recently shown that *S. aureus* PtpA is secreted during in vitro growth or within macrophages [23,24]. Additionally, PtpA has been identified in the secretome of this bacterium [48], despite of the fact that PtpA does not exhibit a general export pathway

signal sequence at its *N*-terminus. In order to determine whether PtpB might be secreted by *S. aureus* and interact directly with signaling networks of the host, we created a *C*-terminal Spot-tag *ptpB* translational fusion construct and introduced this construct in *trans* into the SA564 Δ*ptpB* derivative to avoid the expression of endogenous PtpB, generating strain SA564 Δ*ptpB* + pRMC2_PtpB-Spot. The plasmid is expected to drive high levels of expression of a PtpB-Spot fusion protein upon anhydrotetracycline induction [31]. Cultures expressing the PtpB-Spot fusion protein were induced and grown in liquid media for only two hours in order to minimize cell death and lysis. Presence of PtpB-Spot in supernatants and bacterial cells was determined by Western-blot analyses using anti-Spot antibodies. SA564 derivatives expressing Spot-tagged versions of PtpA and SecA served as positive and negative secretion control, respectively [24,49] (Figure 3).

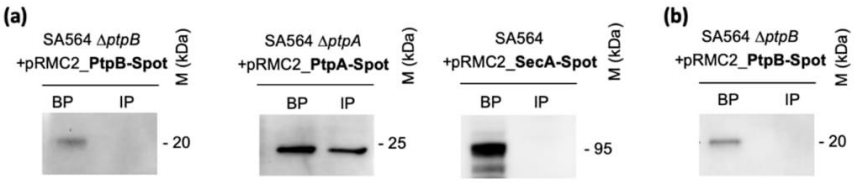

Figure 3. PtpB is not secreted by *S. aureus*. (**a**) Cultures were induced with 0,2 µg/mL of anhydrotetracycline for 2 h and supernatants from *S. aureus* SA564 Δ*ptpB* + pRMC2_PtpB-Spot, SA564 Δ*ptpA* + pRMC2_PtpA-Spot (positive secretion control), and SA564 + pRMC2_SecA-Spot (negative secretion control) were filtered and immunoprecipitated with anti-Spot magnetic beads, whereas bacterial pellets were resuspended and lysed in PBS with protease inhibitor cocktail, lysostaphin, and DNAaseI. Immunoprecipitated proteins (IP) from supernatants and bacterial pellets (BP) were resolved on SDS-PAGEs, transferred to PVDF membranes, and subjected to Western-blot analyses using an anti-Spot antibody as primary antibody (Chromotek) and a HRP-coupled goat-anti-lama antibody as secondary antibody (Bethyl). Data are representative of three independent experiments. (**b**) RAW 264.7 macrophages (5×10^5 cells/mL) were incubated in presence of anhydrotetracycline (0.1 µg/mL) to induce PtpB expression after phagosomal uptake of the bacteria for 2 h with *S. aureus* SA564 Δ*ptpB* + pRMC2_PtpB-Spot at a MOI of 20, and non-phagocytosed bacteria were subsequently removed by gentamicin/lysostaphin treatment. At 3 h pGt, infected macrophages were lysed in 0.1%Triton X-100, and centrifuged at 14 000 g. The obtained supernatants corresponding to macrophage lysates were immunoprecipitated with anti-Spot magnetic beads (Chromotek), whereas the pellets containing intracellular bacteria were resuspended in an equal amount of lysis buffer. Immunoprecipitated proteins and bacterial pellets were resolved on SDS-PAGE and detected with an anti-Spot antibody as described in (**a**). Data are representative of three independent experiments (M kDa: molecular markers).

Cultures expressing the PtpB-Spot fusion construct allowed the identification of the fusion protein within the bacterial pellet but did not indicate the presence of PtpB-Spot in immunoprecipitated culture supernatants (Figure 3a). As a positive secreted control, we used our recently constructed *C*-terminal Spot-tag translational fusion to the *S. aureus ptpA* gene in the pRMC2 vector [24], and introduced this construct into strain SA564 Δ*ptpA*. A *C*-terminal Spot-tag translational fusion to the *S. aureus secA* gene cloned into pRMC2 served as negative control to monitor the potential release of cytosolic proteins by cell lysis. In line with our earlier observations [23,24], we observed clear signals for PtpA-Spot in the bacterial pellets and in immunoprecipitated supernatant fractions, while SecA-Spot was exclusively found in bacterial pellets (Figure 3a), indicating that PtpA is secreted by *S. aureus* during exponential growth, while PtpB is kept within the bacterial cell under these growth conditions. The lack of a SecA-Spot signal in the IP culture supernatant fractions indicates furthermore a negligible release of intracellular proteins via cell lysis under the growth conditions used in this assay, supporting the idea that PtpA is actively secreted by *S. aureus* into the extracellular milieu by a yet unidentified mechanism [24]. Moreover, to test whether PtpB might be secreted by *S. aureus* under infection mimicking

conditions, we next infected THP-1 macrophages with the SA564 Δ*ptpB* + pRMC2_PtpB-Spot derivative, induced the expression of PtpB-Spot by addition of anhydrotetracycline to the infected macrophages, and tested the presence of PtpB-Spot in lysed macrophage supernatants and intracellular persisting bacteria three hours post infection. Similar to our observations made with the in vitro cultured cells (Figure 3a), we failed to detect a PtpB-Spot signal in macrophage lysates upon cell infection, while a clear signal could be detected in bacterial cell pellets (Figure 3b), demonstrating that PtpB-Spot was produced by THP-1 ingested *S. aureus* cells. Taken together, these findings indicate that PtpB is not actively secreted by *S. aureus* to modulate host cell signaling, suggesting that the phospho-arginine phosphatase affects the macrophage survival capacity and infectivity of *S. aureus* via intracellular pathways.

3.4. S. aureus PtpB Phosphatase Activity Depends on a Highly Specific Threonine Residue in Its Catalytic Loop

PtpB from *S. aureus* was originally characterized in vitro as an acid low-molecular-mass phosphotyrosine protein phosphatases [21,50], although recent in vitro studies reported substrate specific activity of PtpB to release inorganic phosphate from arginine phosphate containing substrates [25,26]. However, the phosphotyrosine phosphatase activity of *S. aureus* PtpB was confirmed by structural motifs typical for LMW-PTPases, which are highly conserved among different staphylococcal strains and even species, and known to play pivotal roles in the catalytic cleavage mechanism [50]. All LMW-PTPs have a specifically shaped binding pocket to distinguish pTyr from other phosphorylated residues. Given this conserved architecture, it is surprising that an annotated LMW-PTP is able to target a different phospho-residue, but this was firstly reported for the LMW-PTP YwlE from *Bacillus subtilis*, which possesses a highly specific phospho-arginine phosphatase activity [15]. YwlE belongs to the LMW-PTP family which contains a conserved active site signature motif, C(X)4CR(S/T) (Supplementary Figure S1), corresponding to the active-site loop (P-loop) region that comprises a conserved cysteine and arginine residue. This P-loop is critical for binding the phosphate group of the incoming substrate and subsequently, to form a transient enzyme-substrate phosphothioester adduct [51]. Overall, YwlE adopts the typical LMW-PTP fold consisting of four β-strands forming a central, highly twisted parallel β-sheet that is flanked by α-helices H1, H2, H5, and H6 on one side, and H3 and H4 on the other side (Fuhrmann et al., 2013a). The P-loop encompassing the C7XXXXXR13 motif connects strand S1 and a helix H1 and constitutes the base of the active-site pocket. As a result, residues Cys7 and Asp118 are properly arranged to dephosphorylate the incoming substrate in a concerted reaction [52]. Although all LMW-PTPs exhibit a similar active-site architecture, in which residues lining the substrate-binding cleft are particularly well conserved, structural comparison of YwlE with the related *S. aureus* PtpA and PtpB phosphatase revealed a remarkable difference regarding position 5 within the CXXXX5XR P-loop (the PX5 residue: Thr in YwlE and PtpB; Ile in PtpA) (Supplementary Figure S1). Furhmann et al. [15] demonstrated already that position PX5 of the CXXXX5XR P loop is critical to direct substrate selectivity either to pArg (PX5 = Thr) or pTyr (PX5 = Ile), as a Thr to Ile exchange increase the pTyr phosphatase activity of YwlE, while reducing its pArg phosphatase activity. We hypothesized that the *S. aureus* PtpB specificity towards pArg is mainly driven by the Thr residue at position PX5, as has been seen with its orthologue YwlE [15]. To assess the role of the critical residues, Thr11 and Asp111, on PtpB phosphatase activity, we constructed single mutants where Thr11 and Asp111 (the catalytically active Asp residue of the conserved D-P-Y triad) were mutated to isoleucine and alanine, respectively. Proteins were overexpressed in *E. coli* and purified as recombinant proteins fused to a HAT-tag. The purified tagged PtpB protein derivatives were then assayed for phosphatase activity in presence of the universal phosphate donor PNPP and the reducing agent DTT (Figure 4, black bars).

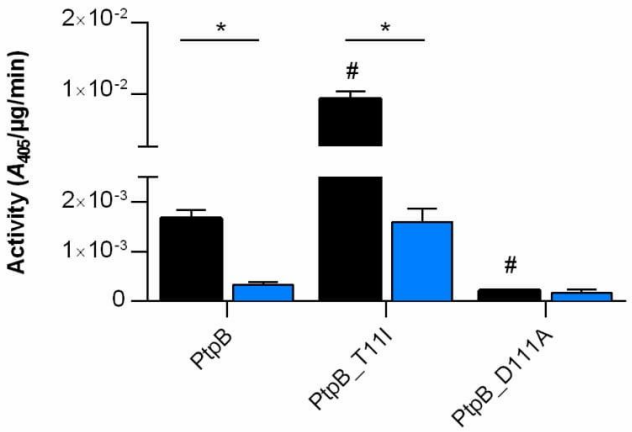

Figure 4. *S. aureus* PtpB phosphatase activity. Recombinant expressed versions of PtpB, PtpB_T11I, and PtpB_D111A were used to test their phosphatase activity on PNPP in presence (black bars) and absence of DTT (blue bars), respectively. Data are presented as mean + SD of four biological replicates. $^{*,\#}p < 0.05$ ($^{\#}$ Mann-Whitney *U* test between PtpB and its derivatives in presence of DTT; * Mann-Whitney *U* test between + DTT and –DTT samples).

The D111A exchange in PtpB strongly decreased the PNPP hydrolysis capacity by a factor of ~7-fold, in line with earlier findings indicating that this Asp residue acts as the general acid catalyst in the transition stage for the phosphate group release [50]. Mutating residue Thr11, on the other hand, had an opposing effect. The T11I exchange in PtpB resulted in a significant increase in PNPP hydrolysis (~6-fold). Therefore, substitution of a single hydroxyl group (threonine) by an ethyl group (isoleucine) led to a drastic change in the phosphatase activity of PtpB as previously described for YwlE from *B. subtilis* [15].

The C(X)4 CR(S/T) motif possessed by LMW-PTPs is crucial for catalysis and redox regulation of their activity. In fact, the dephosphorylation mechanism occurs in a two-step process and involves the formation of a covalent phosphothioester reaction intermediate that is generated by nucleophilic attack of the active site cysteine on the incoming phosphoarginine residue. Studies with other LMW-PTPs suggest that the active site cysteine exists as the negatively charged thiolate anion at physiological pH [53]. In this form, the cysteine residue acts as a strong nucleophile. However, in the latter state, the cysteine residue is also particularly vulnerable to oxidation via reactive oxygen species (e.g., hydrogen peroxide, H_2O_2). To further explore the redox-based putative regulation of PtpB, we performed phosphatase assays with our PtpB derivatives in absence of DTT and in presence of H_2O_2, respectively. The results of these studies revealed that both the wild-type PtpB and the T11I mutant activities were decreased in absence of the reducing agent by a factor > 5, while the PNPP hydrolase activity of the D111A mutant was not markedly changed (Figure 4, blue bars). No PNPP hydrolase activities were detectable for all three PtpB derivatives in presence of H_2O_2 (data not shown), in line with observations obtained with other LMW-PTPs [54–56]. Thus, it can be inferred that the redox status of the cell is likely to affect PtpB activity in *S. aureus*.

3.5. PtpB Affects the Capacity of S. aureus to Adapt to Oxidative Stress

S. aureus cells phagocytosed by macrophages are likely to end up in phagolysosomes in which the bacterial cells are challenged among others by reactive oxygen species (ROS) and low pH [57]. Based on our observations that *S. aureus* cells lacking a functional *ptpB* locus have a reduced capacity to survive within THP-1 macrophages (Figure 1b) and that PtpB activity is modulated by oxidative treatment (Figure 4), we wondered whether PtpB

might affect the ability of *S. aureus* to cope with oxidative stress. To test this hypothesis, we first determined the minimal inhibitory concentration (MIC) of SA564 and its Δ*ptpB* derivative for H_2O_2 using a broth microdilution assay. Unexpectedly, cells of the wild type and the *ptpB* deletion mutant displayed an equal MIC of 0.25 mM. However, when grown in TSB for 2 h and subsequently challenged with 50 mM of H_2O_2, SA564 and its Δ*ptpB* derivative produced clearly differing CFU rates at 1 h post H_2O_2 challenge (Figure 5).

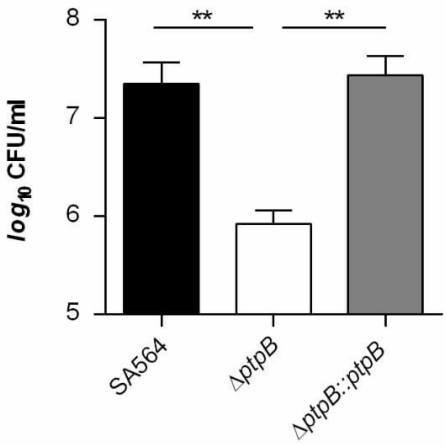

Figure 5. Effect of a *ptpB* deletion on the H_2O_2 resistance of *S. aureus* SA564. Cells of *S. aureus* strain SA564, its isogenic Δ*ptpB* mutant, and the *cis*-complemented derivative were cultured in TSB at 37 °C and 225 rpm for 2 h, challenged with 50 mM H_2O_2, and CFU rates of the cultures determined at 1 h post H_2O_2 challenge. The data presented are the mean + SD of five biological experiments. ** $p < 0.01$ (Mann-Whitney *U* test).

Treatment of exponential growth phase cultures of the Δ*ptpB* mutant with H_2O_2 significantly reduced the number of CFU, when compared to the CFU rates obtained with H_2O_2 challenged cultures of the wild type and the *cis*-complemented derivative, respectively, in line with findings made by Junker et al. [26] for *S. aureus* strain COL. These findings suggest that PtpB does not alter the ability of *S. aureus* SA564 to cope with H_2O_2 stress *per se*, but does affect the ability of the pathogen to adapt to oxidative stress during growth.

3.6. PtpB Promotes the Capacity of S. aureus to Withstand NO˙ Stress.

In order to test whether PtpB might also affect the capacity of *S. aureus* to cope with NO˙ stress, cells of SA564 and its Δ*ptpB* mutant were challenged with NO˙ donor diethylamine NONOate (DEA NONOate), and growth of the cultures monitored over time (Figure 6).

Addition of the NO donor at a concentration of 12.5 µM delayed the growth of wild type and Δ*ptpB* mutant cultures for about 2 h, but had only little effect on the growth rate of the wild type after 12 h of growth (Figure 6a,b). In contrast, a clear decrease in growth rates was observed when cells of the Δ*ptpB* mutant were challenged with the NO˙ donor, which reached about 75% of the growth rates seen with the untreated control after 12 h of cultivation (Figure 6b). These findings suggest that PtpB also contributes positively to the capacity of *S. aureus* to recover from nitrosative stress.

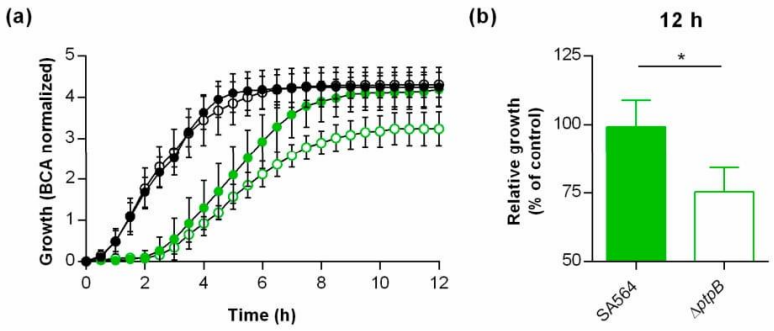

Figure 6. Effect of a *ptpB* deletion on the NO· resistance of *S. aureus* SA564. Cells of *S. aureus* strain SA564 (filled symbols) and its isogenic Δ*ptpB* mutant (opened symbols) were cultured in presence and absence of 12.5 µM DEA NONOate in TSB under static conditions at 37 °C and 5% CO_2 for 12 h. Growth was measured by optical density using the oCelloScope BCA algorithm. (**a**) Growth kinetics of DEA NONOate treated (green symbols) and untreated (black symbols) cell suspensions. The graph represents the average growth values ± SD of four biological experiments. (**b**) Relative growth rates of DEA NONOate treated cells in relation to the growth rates recorded for the untreated controls at 12 h of growth. * $p < 0.05$ (Mann-Whitney *U* test).

3.7. PtpB Alters the Transcription of Genes Encoding Factors Involved in the Detoxification of ROS

The adaptive capacity of staphylococcal cells to oxidative stress is of major impact for host infection [58], and our findings show that the lack of *ptpB* decreased the capacity of *S. aureus* to protect itself from H_2O_2 and NO· induced oxidative stress (Figures 5 and 6). Thus, we wondered whether PtpB might affect the transcription of genes whose products are involved in the detoxification of ROS, specifically of H_2O_2. The detoxification of H_2O_2 in *S. aureus* is thought to be accomplished mainly by catalase (encoded by *katA*) and the peroxiredoxin alkyl hydroperoxide reductase (encoded by *ahpC*), which convert H_2O_2 to water and oxygen [59]. At first, we analyzed the impact of the *ptpB* deletion on *katA* and *ahpC* transcription of *S. aureus* during growth in TSB (Figure 7a). In line with earlier findings [59], we observed a growth phase-dependent transcription of both genes in wild type cells, with a maximum expression in the post-exponential growth phase. Notably, when the transcript rates of *katA* and *ahpC* were determined in cultures of the isogenic Δ*ptpB* mutant along with growth, rather similar growth phase-dependent kinetics were observed. However, when compared in relation to the transcript rates seen for *katA* and *ahpC* in wild type cells at a given time point, Δ*ptpB* mutant cells produced significantly reduced amounts of *katA* and *ahpC* at all three time points analyzed (Figure 7a), suggesting that PtpB is involved in the expression of KatA and AhpC in *S. aureus*.

Another mechanism utilized by *S. aureus* to cope with extracellular H_2O_2 stress is the production of the yellow to orange pigment staphyloxanthin [60], which is synthesized from the enzymes coded within the *crtOPQMN* operon [61], and known to be of importance for the resistance of *S. aureus* to phagocytotic killing [60,62,63]. However, we neither observed clear differences in pigment production between the Δ*ptpB* mutant and wild type cells of SA564 during in vitro growth (data not shown), nor in the transcription of *crtM* (Figure 7b), suggesting that PtpB does not affect intramacrophage survival via the modulation of staphyloxanthin biosynthesis.

Encouraged by our findings that PtpB promotes the transcription of *katA* and *ahpC* (Figure 7a), we additionally tested the impact of the *ptpB* deletion on the transcription of the superoxide dismutases encoding genes *sodA* and *sodM* [64,65]. Both gene products are involved in maintaining cell viability during exogenous O_2^- stress by catalyzing the dismutation of O_2^- to oxygen and H_2O_2 [58]. While no clear differences in *sodM* transcription between Δ*ptpB* mutant cells and wild type cells were noticed in our qRT-PCR analyses, we observed significantly decreased *sodA* transcript rates in Δ*ptpB* mutant cells for the later time points analyzed (Figure 7c).

Detoxification of nitric oxide in *S. aureus* is mediated mainly by the flavohemoglobin Hmp [66]. To test whether PtpB might also affect the expression of Hmp, we also analyzed the transcription of the respective gene (Figure 7d). Here, no clear differences in *hmp* transcription between Δ*ptpB* mutant cells and wild type cells were noticed, suggesting that PtpB does not modulate the nitrosative stress response of *S. aureus* via transcription of *hmp*, at least under uninduced conditions. Taken together, these data suggest that PtpB is likely to contribute to intramacrophage survival and abscess formation of *S. aureus* via the upregulation of ROS detoxifying enzymes such as AhpC, KatA, and SodA.

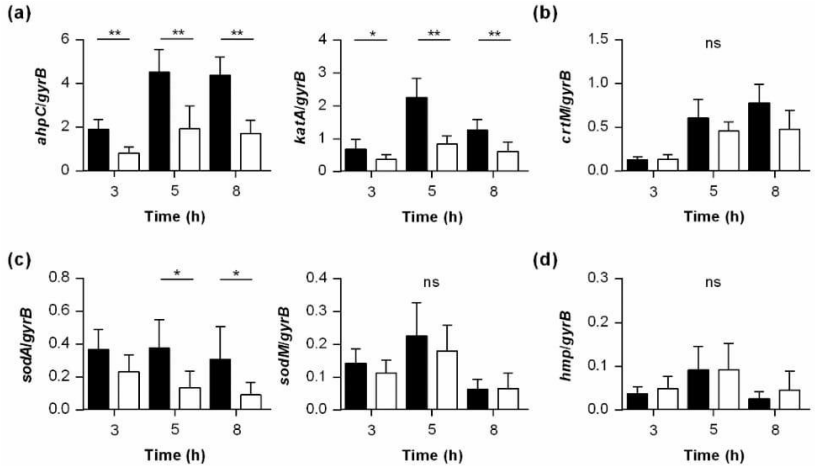

Figure 7. Effect of a *ptpB* deletion on the transcription of genes encoding enzymes involved in the detoxification of ROS in *S. aureus*. Quantitative transcript analyses of *ahpC* and *katA* (**a**), *crtM* (**b**), *sodA* and *sodM* (**c**), and *hmp* (**d**) by qRT-PCR in SA564 (black bars) and SA564 Δ*ptpB* (white bars) cells grown in TSB at 37 °C and 225 rpm to the time points indicated. Transcripts were quantified in reference to the transcription of gyrase B (in copies per copy of *gyrB*). Data are presented as mean + SD of five biological replicates. * $p < 0.05$; ** $p < 0.01$ (Mann-Whitney *U* test between WT and mutant at a given time point).

3.8. PtpB Contributes to the Survival Capacity of S. aureus Under Low pH.

S. aureus cells ingested by macrophages are challenged among others in the phagolysosome by low pH [67]. To evaluate whether PtpB might also affect the capacity of *S. aureus* to cope with acidic stress, we next tested the survival capacity of wild type and Δ*ptpB* mutant cells in PBS at pH 5.5, an average pH found in phagolysosomes of macrophages loaded with viable *S. aureus* cells [67]. To closer resemble conditions seen in the macrophage phagolysosome, exponential growth phase cells of the wild type and the Δ*ptpB* mutant were challenged with the low pH in PBS and not the culture medium (i.e., TSB). When compared to wild type cells incubated in PBS at pH 5.5, the OD_{600} values of the Δ*ptpB* mutant cell suspensions dropped faster at all time points analyzed (Figure 8a).

Similarly, cell suspensions of the Δ*ptpB* mutant produced significantly smaller relative CFU rates after 1 h of cultivation than wild type cultures (Figure 8b), suggesting that PtpB contributes to the ability of *S. aureus* to survive under pH conditions encountered by the bacterial cell in the macrophage phagolysosome. As urease was recently reported to represent an essential component of the acid response network of *S. aureus* [68], we next tested whether PtpB might affect the transcription of the corresponding operon, *ureABCEFGD*. Our qRT-PCR analyses revealed that the *ptpB* deletion indeed strongly affected the transcription of *ureA* in SA564 (Figure 8c), however, unlike expected. When cultured in TSB, cells of the Δ*ptpB* mutant produced significantly increased transcript rates of *ureA* than wild type cells at all time points analyzed, suggesting that PtpB represses the transcription of the *ureABCEFGD* operon during in vitro growth. The enhanced level

of *ureA* transcripts seen in Δ*ptpB* mutant cells is probably due to a reduced production of the transcriptional regulator CodY, a known repressor of urease gene transcription in gram-positive bacteria [68], as Junker et al. [26] reported significantly lower amounts of CodY in Δ*ptpB* mutant cells when compared to wild type cells. However, if and how a reduction of urease affects the ability of *S. aureus* to survive within the macrophage phagolysosome remains subject to further analysis.

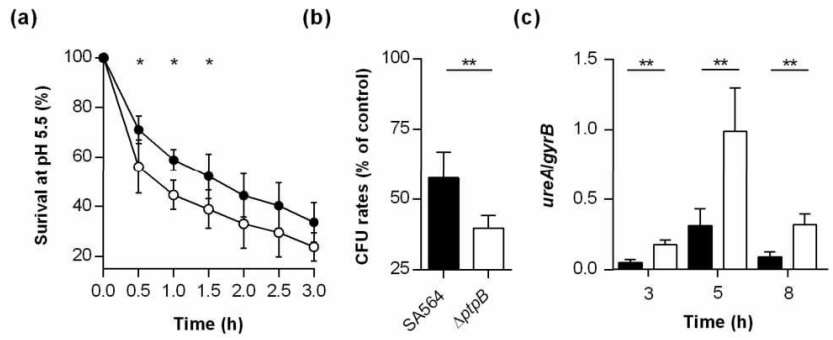

Figure 8. Effect of a *ptpB* deletion on the survival capacity of *S. aureus* at pH 5.5. Exponential growth phase cells of SA564 (black symbols) and its SA564 Δ*ptpB* mutant (white symbols) were inoculated into PBS (pH 5.5) at an OD_{600} of 1 and cultured for 3 h at 37 °C. (**a**) Survival rates over time. Data are presented as mean ± SD of five biological replicates. (**b**) Relative CFU rates of the cultures after 1 h of incubation in PBS at pH 5.5. CFU rates are given in relation to the CFU rates determined in cell suspensions cultured for 1 h in PBS at pH 7.4. Data are presented as mean + SD of five biological replicates. (**c**) Quantitative transcript analyses of *ureA* in *S. aureus* cells grown in TSB at 37 °C and 225 rpm to the time points indicated. Transcripts were quantified in reference to the transcription of gyrase B (in copies per copy of *gyrB*). Data are presented as mean + SD of five biological replicates. * $p < 0.05$; ** $p < 0.01$ (Mann-Whitney *UI* test between WT and mutant at a given time point).

4. Conclusions

Phosphatases expressed by pathogenic bacteria can act as key regulators of important microbial processes, especially during environmental adaptation encountered upon host infection [18,19]. Our macrophage survival-, mice infection-, and transcription data suggest a similar role for PtpB in *S. aureus*. Interestingly, *S. aureus* PtpB presents important differences compared to its LMW-PTPs orthologues. In particular, we provide evidence that PtpB phosphatase activity could be influenced by a single Thr residue in its catalytic loop. Our observations that PtpB activity is affected by its redox status suggest a putative role during the oxidative stress response, as has been seen for the PtpB homolog YlwE in bacilli [56,69]. In line with this assumption, we observed a decreased capacity of the *ptpB* deletion mutant to withstand H_2O_2, $NO^{\cdot}$, and low pH stresses, skills that are probably very important for *S. aureus* to survive inside macrophages [70]. Professional phagocytes such as macrophages express a multifaceted antimicrobial arsenal, which includes but is not limited to the production of reactive oxygen or nitrogen species (ROS, RNS), and antimicrobial peptides in an acidified environment [71]. Nevertheless, *S. aureus* can withstand these mechanisms and conditions [70]. It is thus tempting to speculate that PtpB redox regulation might participate in the adaptation processes initiated by *S. aureus* to survive and replicate within the phagolysosome. However, further work will be needed to decipher the molecular mechanisms utilized by PtpB that allow *S. aureus* to adapt to oxidative stresses encountered in macrophages and during infection.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4409/10/3/645/s1, Table S1: Primers used in this study; Supplementary Figure S1: Alignment of LMW-PTPs.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Supplementary Material

The phosphoarginine phosphatase PtpB from *Staphylococcus aureus* is involved in bacterial stress adaptation during infection

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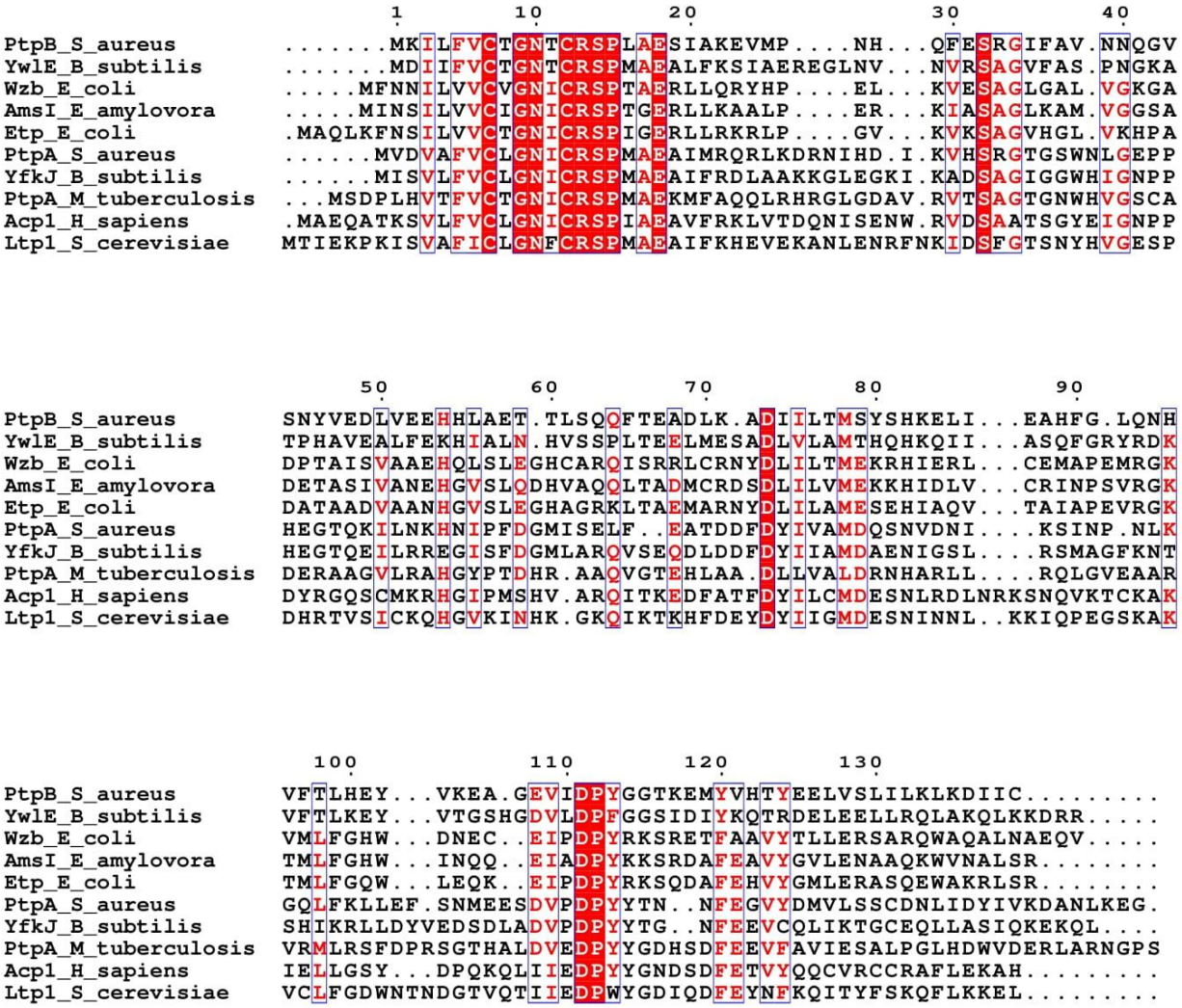
Supplementary Table 1: Primers used in this study.

Primer	Direction	Sequence (5′-3′) [1]
Cloning primer		
MBH510	For.	gtcggTACCAACTGAAACAGTTTATGGAC
MBH511	Rev.	gtgctcgagACGAATAAAATCTTCATAGTTCACATCC
MBH512	For.	GATATTATTTGCTAGTAGGAATTCTG
MBH513	Rev.	gtcgagCTCCATATTCAACGAAATTGTAG
VM11_PtpA_pRMC2	For.	tatGGTACCGGGATATAACTACATAGT
VM11(1)_ PtpA-spot-Ct_Gib	Rev.	CACGAACACGATCTGGCCCTTCTTTCAAATTTGC
VM11(2)_ PtpA-spot-Ct_Gib	For.	GCAAATTTGAAAGAAGGGCCAGATCGTGTTCGTG
VM26_PtpB_pRMC2	For.	TAAGCTTGATGGTACATTATTAAAGGATGTGAACT
VM27_PtpB-spot-Ct_Gib	Rev.	ACGATCTGGTGACCCGCAAATAATATCTTTTAATT
VM28_PtpB-Spot-Ct_Gib	For.	AAAGATATTATTTGCGGGTCACCAGATCGTGTTCG
VM29_Spot-Ct_pRMC2	Rev.	GACGGCCAGTGAATTCTAAGAACTCCAATGTGATA
VM49_SecA_pRMC2	For.	TAAGCTTGATGGTACAACAGTTTTTTAGCTAAAGGAGC
VM50_SecA-spot-Ct_Gib	Rev.	ACGATCTGGTGACCCTTTTCCATGGCAATTTTTGAAT
VM51_SecA-spot-Ct_Gib	For.	AATTGCCATGGAAAAGGGTCACCAGATCGTGTTCG
qRT-PCR primer		
ahpC	For.	TCCAACTGAATTAGAAGACT
ahpC	Rev.	GAGAATACATTTACGCCTAAT
crtM	For.	ACAGTAGGTGAAGTATTGAC
crtM	Rev.	ATCGTATGTCTGATGTGTTT
gyrB	For.	GACTGATGCCGATGTGGA
gyrB	Rev.	AACGGTGGCTGTGCAATA
hla	For.	AACCCGGTATATGGCAATCAACT
hla	Rev.	CTGCTGCTTTCATAGAGCCATTT
katA	For.	AATGGACAATGTATATTCAAGT
katA	Rev.	ATCAAATGGATTATCTTTATGGT
sodA	For.	ACCAAGATAATCCATTAACTGA
sodA	Rev.	ATTTTAGGTAATAAGCGTGTTC
sodM	For.	CCAAGATAATCCATTAACAGAA
sodM	Rev.	CCAAACATCAAATAGTAAGATTG

[1] Small letters represent nucleotides that do not fit with the target sequence. Restriction sites used for cloning are underlined.

Supplementary Figure 1



Supplementary Figure 1: Alignment of LMW-PTPs. Sequence alignment of LMW-PTPs from various species (the sequence names consist of the protein name followed by the source organism). Conserved residues are highlighted in red. The characters in red represent the conservative residues. This figure was created using the ESPript server (Robert & Gouet, 2014).

Article (III)

The low-molecular weight protein arginine phosphatase PtpB affects nuclease production, cell wall integrity, and uptake rates of *Staphylococcus aureus* by polymorphonuclear leukocytes

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Article

The Low-Molecular Weight Protein Arginine Phosphatase PtpB Affects Nuclease Production, Cell Wall Integrity, and Uptake Rates of *Staphylococcus aureus* by Polymorphonuclear Leukocytes

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Abstract: The epidemiological success of *Staphylococcus aureus* as a versatile pathogen in mammals is largely attributed to its virulence factor repertoire and the sophisticated regulatory network controlling this virulon. Here we demonstrate that the low-molecular-weight protein arginine phosphatase PtpB contributes to this regulatory network by affecting the growth phase-dependent transcription of the virulence factor encoding genes/operons *aur*, *nuc*, and *psmα*, and that of the small regulatory RNA *RNAIII*. Inactivation of *ptpB* in *S. aureus* SA564 also significantly decreased the capacity of the mutant to degrade extracellular DNA, to hydrolyze proteins in the extracellular milieu, and to withstand Triton X-100 induced autolysis. SA564 Δ*ptpB* mutant cells were additionally ingested faster by polymorphonuclear leukocytes in a whole blood phagocytosis assay, suggesting that PtpB contributes by several ways positively to the ability of *S. aureus* to evade host innate immunity.

Keywords: *Staphylococcus aureus*; low-molecular-weight protein arginine phosphatase; PtpB; nuclease; whole blood phagocytosis assay; cell wall integrity; gene transcription; Triton X-100 induced autolysis; innate immunity

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1. Introduction

Staphylococcus aureus is a major bacterial pathogen in humans and animals [1]. The ability of this Gram-positive bacterium to respond accurately to changing environments is one of the prerequisites for its success as a versatile pathogen in mammals [2]. The opportunistic pathogen is equipped with an armamentarium of virulence factors and a sophisticated network of regulatory molecules allowing it to control/fine-tune the expression of its virulon in a way to rapidly respond to changing conditions [3]. While virulence factor synthesis in *S. aureus* is mainly driven by one component systems such as the Sar family of DNA binding proteins and two-component system (TCS) response regulators [4], phosphatases are also known to contribute to this network. Earlier work demonstrated, for instance, that the serine/threonine phosphatase Stp1 directly contributes to virulence factor synthesis and infectivity of *S. aureus* by promoting the transcription of the α-hemolysin encoding gene *hla* [5]. More recent data indicate that the low-molecular-weight protein arginine phosphatase (LMW-PAP) PtpB might also contribute to staphylococcal pathophysiology by modulating the arginine phosphorylation states of regulators such as the stress response regulator CtsR and the global regulator MgrA, particularly in response to oxidative stress [6,7]. A deletion of *ptpB* in the *S. aureus* clinical isolate SA564 was additionally shown lately to reduce the capacity of the mutant to survive inside of macrophages and to cause infection in a murine-based *S. aureus* abscess model [8]. PtpB might thereby

form a regulatory circuit with McsB, the presumed protein arginine kinase of *S. aureus*, which is part of the *clpC* operon and known to affect the hemolytic and proteolytic activity of this bacterium [9]. The McsB homolog in *Bacillus subtilis* was shown to modulate the activity of McsB-targeted regulators by phosphorylating arginine residues within the DNA-binding domains and by marking proteins and aberrant polypeptide molecules for degradation [10,11]; similar functions are assumed for McsB in *S. aureus* [6]. Thus, by counteracting McsB-dependent arginine phosphorylation of the aforementioned global regulators, PtpB might affect the activity and stability of these transcription factors and thus the expression of stress response genes and virulence factors that are directly and/or indirectly controlled by these regulatory molecules. Additionally, PtpB might interfere with the degradation process of proteins phosphorylated at arginine residues. Notably, a functional classification of arginine phosphorylated proteins found in in vitro cultured *S. aureus* cells carried out by Junker et al. [6] revealed that nearly half (47%) of the identified arginine phosphoproteins in the *ptpB* mutant could be associated with virulence functions, supporting the idea that PtpB contributes to the regulatory network controlling the virulence factor synthesis in this bacterial pathogen.

In this study, we further strengthen this assumption by demonstrating that inactivation of *ptpB* in *S. aureus* strain SA564—a whole genome sequenced clinical human isolate of the worldwide distributed clonal complex 5 that harbors functional *agr* and *sigB* operons and a $saeS^{L}$ allele [12,13]—affects the transcription of the virulence factor encoding genes *aur* (encoding the zinc-metalloprotease aureolysin), *nuc* (encoding thermonuclease) and *psmα* (encoding the phenol-soluble modulins α1-4), and of the small regulatory RNA *RNAIII*, one of the master regulators controlling exoprotein synthesis in this bacterium [14], which also encodes a protein, δ-hemolysin (Hld). Deletion of *ptpB* also impedes the capacity of *S. aureus* to evade phagocytosis by polymorphonuclear leukocytes (PMNs), to withstand Triton X-100 induced autolysis or lysostaphin mediated lysis, and decreases the exonuclease- and exoprotease activities of this bacterium.

2. Results and Discussion

2.1. PtpB Affects the Ability of S. aureus to Evade Phagocytosis by PMNs

Recent work demonstrated that PtpB contributes to the ability of *S. aureus* to survive inside macrophages [8]. However, it remains unknown whether PtpB also supports the capacity of the pathogen to evade innate immunity. In order to address this issue, we first investigated the impact of a *ptpB* deletion on attachment/phagocytosis of *S. aureus* SA564 by PMNs in whole blood (Figure 1).

When human blood was supplemented with fluorescent-labeled bacteria at a multiplicity of infection (MOI) of 50 per PMNs, a clear difference in uptake rates of *ptpB* negative and positive *S. aureus* SA564 cells by human PMNs was observed (Figure 1). After 30 min of coincubation in human whole blood, CFSE-stained SA564 isolates harboring a functional *ptpB* (wild-type and *cis*-complemented derivative) demonstrated significantly decreased rates of attachment/phagocytosis by PMNs compared to the CFSE-stained SA564 derivative lacking *ptpB* (SA564 Δ*ptpB*). These findings suggest that PtpB mediates a relevant protective effect against phagocytosis by PMNs in blood.

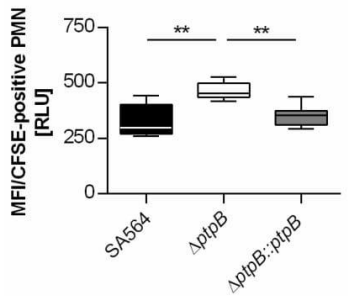

Figure 1. Effect of PtpB on phagocytosis of *S. aureus* SA564 by polymorphonuclear leukocytes (PMNs). Carboxy fluorescein diacetate succinimidyl ester (CFSE)-stained cells of *S. aureus* SA564 (black boxes), SA564 Δ*ptpB* (white boxes), and SA564 Δ*ptpB::ptpB* (gray boxes) were added to lithium heparin anticoagulated fresh human blood at a multiplicity of infection of 50 per PMN, and incubated for 30 min at 37 °C and 1000 rpm. Attachment/uptake of bacteria by PMNs was analyzed by flow cytometry as outlined in Material and Methods. The data are representative of three biological replicates carried out in triplicate. Data are presented as box and whisker plots showing the interquartile range (25–75%; box), median (horizontal line), and whiskers (bars; min/max). ** $p < 0.01$ (Kruskal–Wallis test followed by Dunn's post hoc test).

2.2. PtpB Promotes the Transcription and Secretion of Nuclease in S. aureus

Neutrophils are the main pathogen-fighting immune cells in our blood system [15]. Besides their capacity to ingest circulating pathogens, activated neutrophils exert a number of cytotoxic functions such as the production of reactive oxygen and nitrogen species, the release of antimicrobial peptides, and the formation of extracellular DNA nets called neutrophil extracellular traps (NETs) [15]. The latter bacteria-capturing and killing mechanism is counteracted by *S. aureus*, among others, via the secretion of nucleases and the extracellular adherence protein (Eap), which degrade and aggregate NETs, respectively [16,17]. We have recently shown that PtpB supports the capacity of *S. aureus* to cope with oxidative and nitrosative stress [8]. To test whether PtpB might also interfere with other neutrophil-derived cytotoxic activities, we next tested whether the LMW-PAP might modulate the capacity of *S. aureus* to degrade extracellular DNA (Figure 2).

When cell suspensions of SA564 and the *cis*-complemented Δ*ptpB::ptpB* derivative were spotted on DNase test agar plates and incubated at 37 °C for 24 h, clearly visible lytic areas around the bacterial growth areas were observed that were in comparable ranges (Figure 2a,b). However, when equal volumes of SA564 Δ*ptpB* cell suspensions were spotted onto the DNase test agar plates, significantly smaller lytic areas surrounding the bacterial growth zones were observed, suggesting that the Δ*ptpB* mutant produces and/or secretes lower amounts of nucleases into the extracellular milieu.

To elucidate whether the reduced nuclease activity observed with the Δ*ptpB* mutant might be due to PtpB-induced changes in *nuc* transcription, we next assayed the transcription rates of *nuc* in SA564 and its Δ*ptpB* derivative upon growth in tryptic soy broth (TSB) over time. Specifically, total RNAs were obtained from cell populations grown for 3 h (exponential growth phase), 5 h (transition phase), and 8 h (early stationary phase), respectively (Figure 2c). In line with our DNase activity findings, we observed a significantly reduced transcription of *nuc* in SA564 Δ*ptpB* cells at two of the three growth stages analyzed, if compared to the wild-type transcript level, while cells of the *cis*-complemented derivative produced *nuc* transcript level that were comparable to the wild-type. Taken together, these findings suggest that PtpB contributes positively to the exonuclease activity of *S. aureus* by supporting *nuc* transcription.

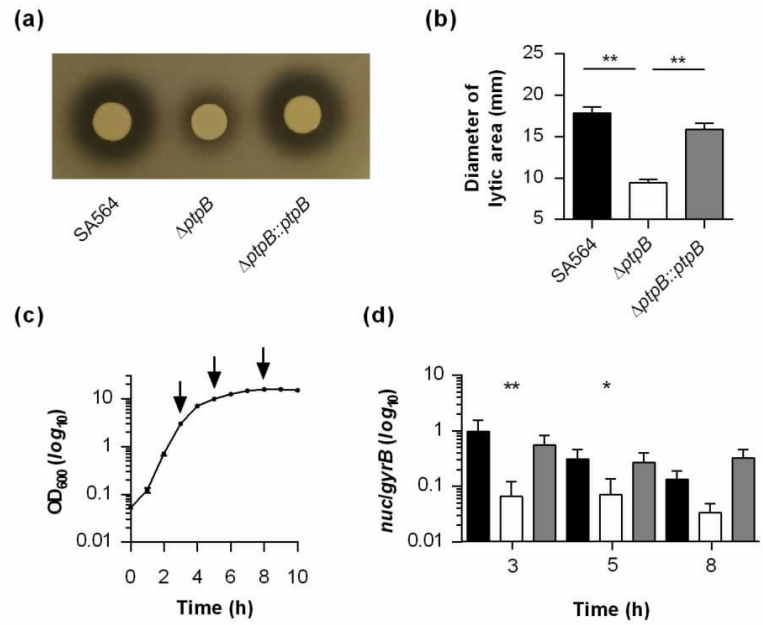

Figure 2. Effect of PtpB on extracellular DNase activity and *nuc* transcription of *S. aureus* SA564. (**a**,**b**) Impact of PtpB on extracellular DNase activity. Overnight cultures of *S. aureus* strain SA564 (black bars), its isogenic Δ*ptpB* mutant (white bars), and the *cis*-complemented derivative (gray bars) were normalized with fresh tryptic soy broth (TSB) medium to an optical density at 600 nm (OD_{600}) of 12, and 5 µL of the suspensions spotted on DNase-Test-Agar plates. Inoculated plates were cultured for 24 h at 37 °C, and lytic areas were determined. (**a**) Representative image of one experiment. (**b**) Diameter of lytic areas. The data presented are the mean + SD of three biological experiments done in duplicate. ** $p < 0.01$ (Kruskal–Wallis test followed by Dunn's post hoc test). (**c**) Growth kinetics of *S. aureus* strain SA564 in TSB. Cells were cultured at 37 °C and 225 rpm at a culture to flask volume of 1:10. Data represent the mean OD_{600} readings ± SD at the time points indicated ($n = 3$). Time points of sampling for the transcriptional analyses are indicated by arrows. (**d**) Impact of PtpB on transcription of the thermonuclease encoding *nuc* gene. Quantitative transcript analyses of *nuc* by qRT-PCR in SA564 (black bars), SA564 Δ*ptpB* (white bars), and SA564 Δ*ptpB::ptpB* (gray bars) cells grown to the time points indicated. Transcript rates were quantified in reference to the transcription of gyrase B (in copies per copy of *gyrB*). Data are presented as mean + SD of five biological replicates. * $p < 0.05$; ** $p < 0.01$ (Kruskal–Wallis test followed by Dunn's post hoc test between wild-type and mutants at a given time point).

2.3. PtpB Promotes the Transcription of the Aureolysin Encoding Gene aur in S. aureus

Another major virulence determinant interfering with innate host immunity and supporting the survival of *S. aureus* in blood is the zinc-metalloprotease aureolysin, which cleaves, among others, various factors of the human complement system [18]. To test whether PtpB might aid the immune evasion of *S. aureus* by modulating aureolysin expression, we tested the transcription of *aur* during growth in TSB (Figure 3a). This transcriptional analysis revealed a significantly decreased number of *aur* transcripts in exponential growth phase cells of the *ptpB* deletion mutant when compared with cells of the wild-type and the *cis*-complemented derivative harvested at the same growth stage, respectively. However, *aur* transcript rates were rather comparable between wild-type, Δ*ptpB* mutant, and *cis*-complemented cells obtained from later growth stages (i.e., 5 and 8 h of growth), suggesting that PtpB contributes to *aur* expression primarily in *S. aureus* exponential growth phase cells.

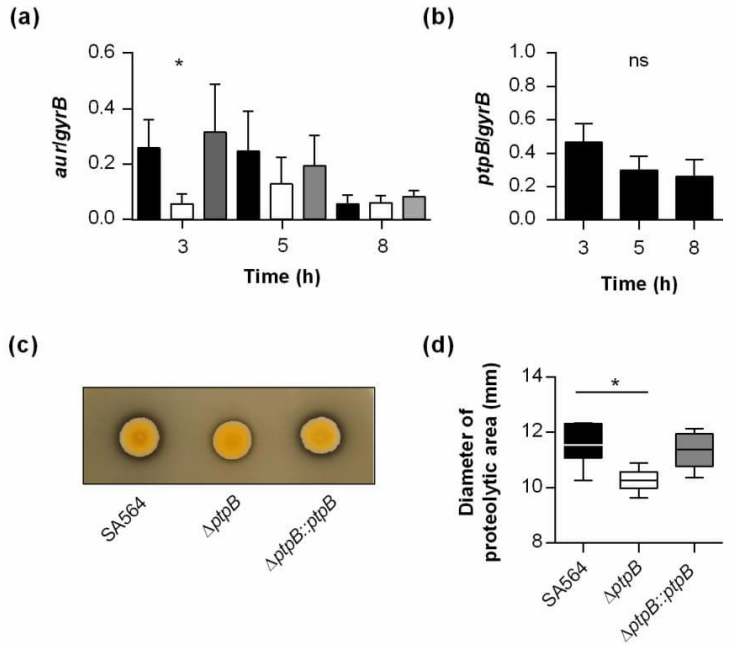

Figure 3. Effect of a *ptpB* deletion on the transcription of *aur* and the proteolytic activity of *S. aureus* SA564. (**a**) Quantitative transcript analyses of *aur* by qRT-PCR in SA564 (black bars), SA564 Δ*ptpB* (white bars), and SA564 Δ*ptpB::ptpB* (gray bars) cells grown in tryptic soy broth (TSB) at 37 °C and 225 rpm to the time points indicated. Transcript rates were quantified in reference to the transcription of gyrase B (in copies per copy of *gyrB*). Data are presented as mean + SD of four to five biological replicates. * $p < 0.05$ (Kruskal–Wallis test followed by Dunn's post hoc test between wild-type and mutants at a given time point). (**b**) Quantitative transcript analyses of *ptpB* by qRT-PCR in SA564 cells grown in TSB. Sampling and transcription rate quantifications were done as described above. Data are presented as mean + SD of five biological replicates. (**c**,**d**) Impact of PtpB on extracellular protease activity. Overnight cultures of *S. aureus* strain SA564 (black bars), its isogenic Δ*ptpB* mutant (white bars), and the *cis*-complemented SA564 Δ*ptpB::ptpB* derivative (gray bars) were normalized with fresh TSB medium to an OD_{600} of 12, and 2 µL of the suspensions spotted on TSA plates supplemented with 10% skim milk. Inoculated plates were cultured for 48 h at 37 °C, and proteolytic areas were determined. (**c**) Representative image of one experiment. (**d**) Diameter of proteolytic areas. The data presented are the mean + SD of three biological experiments done in duplicate. ns, not significant; * $p < 0.05$ (Kruskal–Wallis test followed by Dunn's post hoc test).

To get an idea about whether the impact of PtpB on *aur* transcription might correlate with the expression of the LMW-PAP, we determined the transcription rates of *ptpB* in SA564 during growth in TSB (Figure 3b). These analyses revealed that *ptpB* is transcribed on a rather constant level throughout growth (fold changes in relative transcription rates between growth stages < 2), suggesting that the growth phase-dependent effect of PtpB on *aur* transcription is not likely to be determined by the expression rates of the LMW-PAP during growth.

Besides its complement factors degrading functions [18], aureolysin is also known for its capacity to process and thus activate serine protease SspA (syn. V8 protease), another major exoprotease produced by *S. aureus*, which in turn cleaves the propeptide from the SspB zymogen to create the active cysteine protease SspB (syn. staphopain), a process also known as staphylococcal proteolytic cascade [19].

Wondering whether PtpB might also affect the exoproteolytic capacity of *S. aureus*, we conducted a series of skim milk agar-based protease assays (Figure 3c,d). Stationary phase cell suspensions of SA564 normalized to an OD_{600} of 12 spotted onto the skim

milk supplemented tryptic soy agar (TSA) plates produced only very small proteolytic areas surrounding the bacterial growth zones after 48 h of incubation at 37 °C (Figure 3c). However, when normalized stationary phase cell suspensions of SA564 Δ*ptpB* were spotted onto the skim milk TSA plates, significantly smaller proteolytic areas were observed, while cell suspensions of the *cis*-complemented SA564 Δ*ptpB::ptpB* produced proteolytic areas on skimmed milk TSA plates that were in a comparable range to those seen with the wild-type cultures (Figure 3c,d). These findings suggest that PtpB affects the proteolytic capacity of *S. aureus*, potentially via the modulation of *aur* transcription.

2.4. PtpB Reduces the Autolytic Activity of S. aureus

Activated neutrophils secrete, among others, antimicrobial peptides (e.g., defensins) which exert potent in vitro microbicidal activity against *S. aureus* through mechanisms involving cell membrane destabilization, interference with intracellular targets, and activation of autolysins [20]. To test whether and how PtpB might influence the autolytic behavior of *S. aureus*, we next studied the impact of the *ptpB* deletion on the autolytic behavior of SA564 in Triton X-100- and lysostaphin-induced lysis assays, respectively (Figure 4).

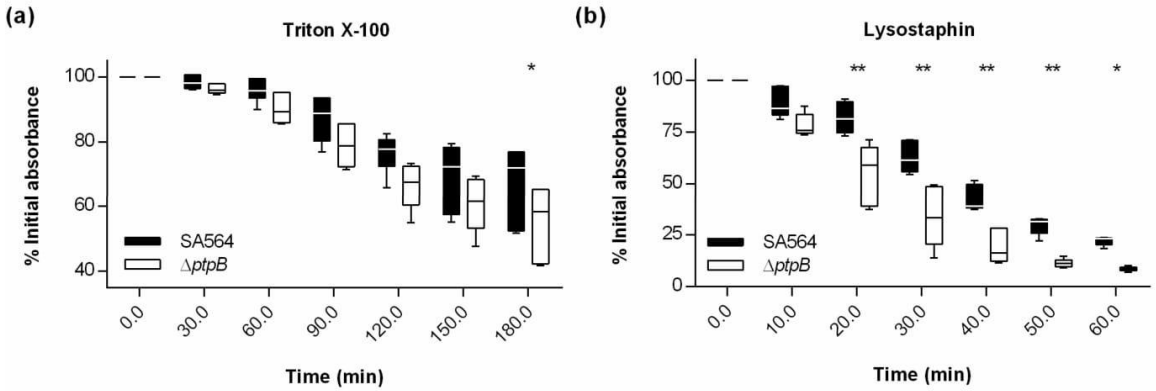

Figure 4. Effect of a *ptpB* deletion on the Triton X-100 induced autolysis and lysostaphin-mediated lysis of *S. aureus* SA564. (**a**) Cells of *S. aureus* strain SA564 (black symbols) and its isogenic Δ*ptpB* mutant (white symbols) were cultured in TSB at 37 °C and 225 rpm to an OD_{600} of 0.7, washed twice in ice-cold water, and resuspended in 0.05 M Tris-HCl (pH 7.2) containing 0.01% (vol/vol) Triton X-100. Triton X-100 induced autolysis was measured as the decline of optical density at 600 nm (OD_{600}) versus time and is expressed as the percent of the initial optical density ($n = 6$ biological replicates). (**b**) Effect of a *ptpB* deletion on the lysostaphin-mediated lysis. Cells of *S. aureus* strain SA564 (black symbols) and its isogenic Δ*ptpB* mutant (white symbols) were cultured in TSB at 37 °C and 225 rpm to an OD_{600} of 3, washed twice in PBS, and resuspended in PBS to an OD_{600} of 1. The PBS cell suspensions were supplemented with 250 ng/mL lysostaphin and incubated under static conditions at 30 °C for 60 min. Lysostaphin-mediated lysis was measured as the decline of OD_{600} versus time and is expressed as the percent of the initial optical density ($n = 5$ biological replicates). Data are presented as box and whisker plots showing the interquartile range (25–75%; box), median (horizontal line), and whiskers (bars; min/max). * $p < 0.05$; ** $p < 0.01$ (Ordinary two-way ANOVA followed by Holm–Sidak's multiple comparison test).

When washed exponential growth phase cells of SA564 and its Δ*ptpB* mutant were challenged with a low dose of Triton X-100, respectively, cells of the *ptpB* deletion mutant autolyzed to a significantly larger extent after 3 h of coincubation with the detergent than wild-type cells (Figure 4a). A similar behavior was observed when SA564 and its Δ*ptpB* mutant were challenged with the glycyl-glycyl endopeptidase lysostaphin, a *S. aureus* pentaglycine cross-bridges cleaving exoenzyme from *Staphylococcus simulans* bv. *staphylolyticus* [21]. Washed and PBS resuspended late exponential growth phase cells of the *ptpB* deletion mutant lysed again significantly faster than the wild-type cells (Figure 4b), indicating that PtpB is likely to affect the cell wall composition of *S. aureus*. One possible explanation for both observations is that PtpB might interfere with the production and/or

activation of autolysins (e.g., endogenous murein hydrolases), as has been suggested for the lysostaphin resistance of some glycopeptide-intermediate-resistant *S. aureus* (GISA) [22].

As previous studies have already demonstrated that the global regulator MgrA acts as a repressor of autolysins in *S. aureus* [23,24], and that MgrA serves as a substrate for PtpB [6,7], it was tempting to speculate that PtpB might affect the autolytic behavior of *S. aureus* via modulation of MgrA activity. In order to address this hypothesis, we first studied the impact of the *ptpB* deletion on the transcription of MgrA regulated genes [25,26], focusing on genes whose products are involved in the autolytic behavior of *S. aureus* (Figure 5). Specifically, the transcription of *atlR* (encoding a MarR family transcriptional regulator repressing *atl* transcription), *fmtB* (encoding a cell wall-anchored protein involved in methicillin resistance and cell wall biosynthesis), *lrgA* (encoding the holin-like murein hydrolase regulator LrgA), and *lytN* (encoding the cell-wall hydrolase LytN) was analyzed, all of which were reported to directly or indirectly affect the autolytic behavior of *S. aureus* [27–29].

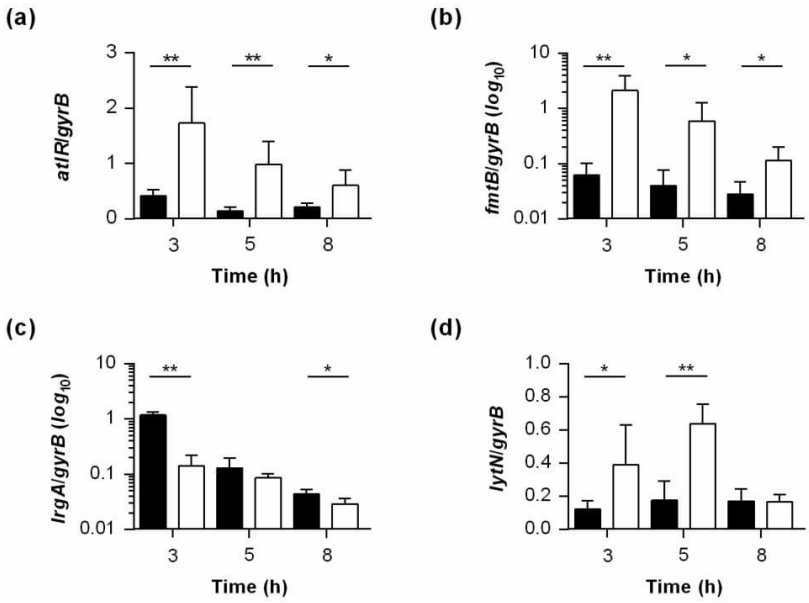

Figure 5. Effect of a *ptpB* deletion on the transcription of MgrA-regulated genes affecting autolysis of *S. aureus*. Quantitative transcript analyses of MgrA regulated genes by qRT-PCR in SA564 (black bars) and SA564 Δ*ptpB* (white bars) cells grown in TSB at 37 °C and 225 rpm to the time points indicated. Growth-phase-dependent transcript rates of MgrA-regulated genes *atlR* (**a**), *ftmB* (**b**), *lrgA* (**c**), and *lytN* (**d**). Transcript rates were quantified in reference to the transcription of gyrase B (in copies per copy of *gyrB*). Data are presented as mean + SD of five biological replicates. * $p < 0.05$; ** $p < 0.01$ (Mann–Whitney-U test between wild-type and mutant at a given time point).

Notably, all four MgrA-regulated genes were transcribed in a significantly different manner in SA564 Δ*ptpB* cells than in wild-type cells. Importantly, genes reported to be repressed by MgrA (i.e., *atlR*, *fmtB*, and *lytN*) were all transcribed on higher levels in the *ptpB* deletion mutant, while transcription of *lrgA*, which is positively affected by MgrA activity [25], was increased in wild-type cells throughout growth. However, while transcription of *atlR*, *fmtB*, and *lrgA* was affected by the *ptpB* deletion basically at all three growth stages (Figure 5a–c), this was not the case with *lytN*. Transcription of the cell-wall hydrolase encoding gene was only affected by the *ptpB* deletion during exponential growth phase and transition phase, but not in the early stationary phase (Figure 5d), suggesting that *lytN* expression in the stationary phase cells is dominated by PtpB/MgrA-independent mechanisms. Taken together, these data indicate that the PtpB-driven dephosphorylation

of MgrA phosphoarginine residues observed by Junker et al. [6] is likely to affect MgrA activity, and supports our hypothesis that PtpB interferes with autolysis of *S. aureus* via modulation of MgrA activity.

2.5. PtpB Does Not Alter MgrA Activity per Se

Given that MgrA also promotes the transcription of *aur* and *nuc* [25], which were also found to be transcribed in significantly higher levels in wild-type cells when compared to Δ*ptpB* mutant cells (Figures 2c and 3), we wondered whether PtpB might affect the transcription of the whole MgrA regulon. In order to address this hypothesis, we additionally tested the transcription of two MgrA-regulated surface factor encoding genes, *ebh* (encoding extracellular matrix-binding protein Ebh) and *spa* (encoding immunoglobulin G binding protein A), both of which are known to be strongly repressed by MgrA activity on the transcriptional level [26]. In contrast to findings obtained for the MgrA-regulated genes affecting autolysis (Figure 5), we surprisingly observed no clear differences in transcription rates of *ebh* and *spa* between the wild-type and the *ptpB* deletion mutant at any time point analyzed (Figure 6).

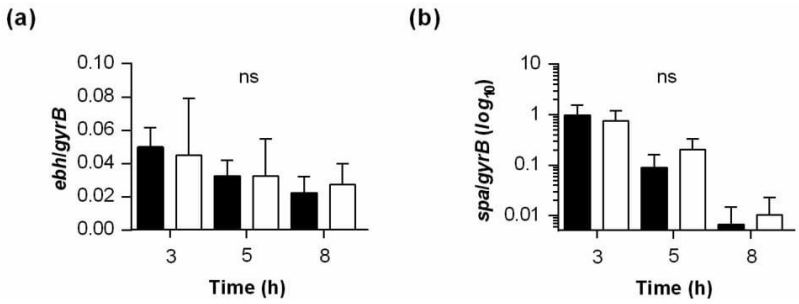

Figure 6. Effect of a *ptpB* deletion on the transcription of MgrA-repressed surface factors encoding genes. Quantitative transcript analyses of MgrA-regulated genes *ebh* (**a**) and *spa* (**b**) by qRT-PCR in SA564 (black bars) and SA564 Δ*ptpB* (white bars) cells grown in TSB at 37 °C and 225 rpm to the time points indicated. Transcript rates were quantified in reference to the transcription of gyrase B (in copies per copy of *gyrB*). Data are presented as mean + SD of five biological replicates. ns, not significant (Mann–Whitney-U test between wild-type and mutant at a given time point).

As MgrA directly represses *ebh* transcription [26], our findings presented here indicate that PtpB-driven dephosphorylation of MgrA does not alter the activity of the transcription factor towards its regulon per se, but might be important for the expression of a subset of MgrA-regulated factors, particularly of those involved in autolysis. One explanation for our observations might be that PtpB-mediated dephosphorylation of MgrA arginine residues influences the activity of the regulator by differentiating the binding specificity among target gene promoters, depending on the phosphorylation status at arginine residues. In such a scenario, target genes with a high binding affinity for MgrA would not be affected in their expression by the arginine phosphorylation status of the regulator and thus would not display a change in transcription depending on the PtpB status of the cell.

2.6. PtpB Alters the Transcription of Some but Not All SarA Regulated Genes

Transcription of *aur*, *lrgA*, and *nuc* is also reported to be affected by SarA [30–32], suggesting that PtpB might also interfere with the transcription of the aforementioned genes via modulation of SarA activity, although SarA was not identified as a direct substrate for McsB/PtpB [6,7]. To test whether and how PtpB affects the transcription of the SarA regulon in SA564, we assayed the transcription rates of three additional SarA target genes, *hla* (encoding α-hemolysin), *hld* (encoding δ-hemolysin), and *splB* (encoding serine protease SplB; Figure 7) [32–34], which all contain a putative Sar box within the gene/operon's promoter region [32]. Similar to the situation seen with MgrA-regulated genes, we found

significant changes in transcription rates in *ptpB* lacking cells for some but not all SarA-regulated genes. While transcription of the *hld* encoding *RNAIII* was affected by the *ptpB* deletion during the exponential growth phase and early stationary phase (Figure 7b), this was neither the case with *hla* nor *splB* (Figure 7a,c). Notably, exponential growth phase cells of the *ptpB* mutant produced lower *RNAIII* transcript rates than wild-type cells, while the opposite was encountered with early stationary phase cells, suggesting PtpB to modulate the transcription of the *agr* locus in SA564 by several means.

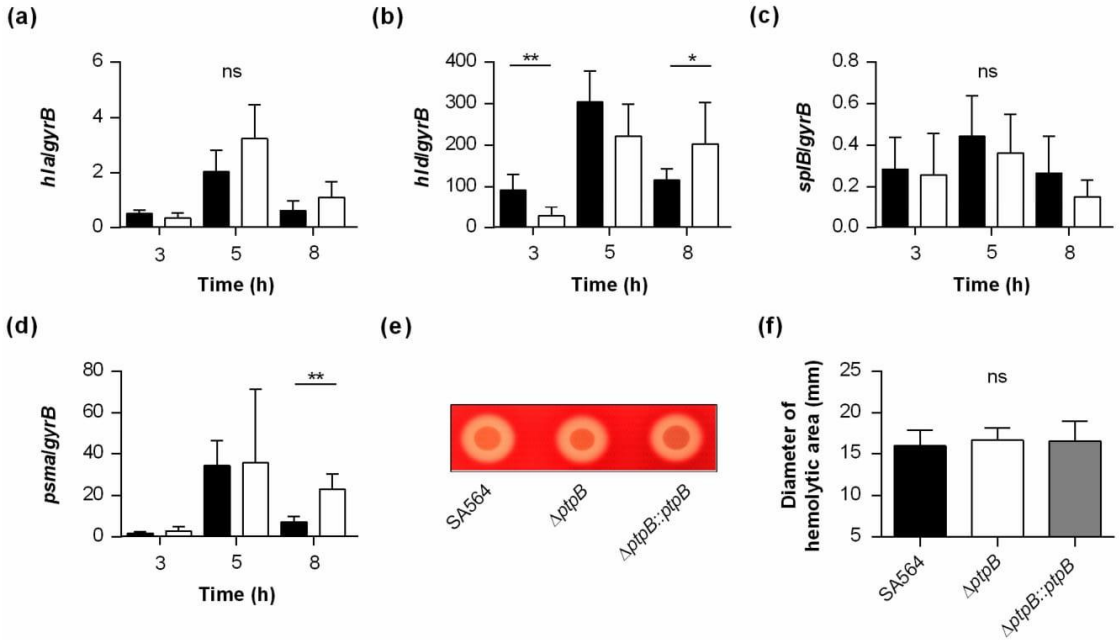

Figure 7. Effect of a *ptpB* deletion on the transcription of SarA- and/or AgrA-regulated genes in *S. aureus* SA564. (**a**–**d**) Quantitative transcript analyses of SarA- and/or AgrA-regulated genes by qRT-PCR in SA564 (black bars) and SA564 Δ*ptpB* (white bars) cells grown in TSB at 37 °C and 225 rpm to the time points indicated. Growth phase-dependent transcript rates of Sar box containing genes *hla* (**a**), *hld* (**b**), and *splB* (**c**), and of the AgrA-driven *psma* cluster (**d**). Transcript rates were quantified in reference to the transcription of gyrase B (in copies per copy of *gyrB*). Data are presented as mean + SD of five biological replicates. * $p < 0.05$; ** $p < 0.01$; ns, not significant (Mann–Whitney-U test between wild-type and mutant at a given time point). (**e**,**f**) Overnight cultures of *S. aureus* strain SA564 (black bar), its isogenic Δ*ptpB* mutant (white bar), and the *cis*-complemented SA564 Δ*ptpB::ptpB* derivative (gray bar) were normalized with fresh TSB medium to an OD_{600} of 12, and 5 µL of the suspensions spotted on sheep blood agar plates. Inoculated plates were cultured for 24 h at 37 °C, and hemolytic areas were determined. (**e**) Representative image of one experiment. (**f**) Diameter of hemolytic areas. The data presented are the mean + SD of three biological experiments done in duplicate. ns, not significant (Kruskal–Wallis test followed by Dunn's post hoc test).

One potential factor contributing to this phenomenon could be AgrA, the response regulator of the *agr* locus, and the main transcription factor driving *RNAIII* transcription [35]. Since AgrA is also known to control the expression of the phenol-soluble modulin operons *psmα* and *psmβ* [36], we additionally tested whether *psmα* transcription in SA564 is altered by the *ptpB* deletion (Figure 7d). In line with our *RNAIII* transcription data (Figure 7b), we observed significantly increased *psmα* transcript rates in early stationary phase cells of the Δ*ptpB* mutant, suggesting that PtpB might modulate *RNAIII* and *psmα* transcription in stationary phase cells via AgrA.

To support our transcriptional findings indicating that PtpB does not markedly alter the expression of α-hemolysin in SA564, we tested the hemolytic activity of our strain triplet on sheep blood agar plates (Figure 7e,f). In line with our *hla* transcription findings,

we did not encounter clear differences in the hemolytic areas surrounding the growth zones for all three SA564 derivatives.

Together with the observations that SarA was not identified to be phosphorylated at arginine residues [6,7], our transcriptional findings made for the Sar box-containing genes *hla*, *spa*, and *splB* suggest that PtpB is not likely to directly modulate SarA activity, albeit of the fact that the transcription rates of other Sar box containing genes such as *aur*, *hld*, and *nuc* (Figures 2c, 3 and 7b) were markedly affected by the *ptpB* deletion. The differences in transcription rates observed for the latter genes between the wild-type and the *ptpB* deletion mutant might be attributed to other direct PtpB mediated effects on regulatory factors such as MgrA and AgrA, and/or indirect PtpB effects on regulators such as ArlR, CodY, SaeR, SarR, SarV, SarX, and SarZ, which all were found to be expressed to different extents in wild-type and *ptpB* deletion mutant cells of *S. aureus* strain COL under non-stress conditions [6].

3. Conclusions

Protein posttranslational modifications such as reversible phosphorylation by kinases/phosphatases is a common mechanism employed by bacteria and eukaryotes to modulate the activity of enzymes and regulatory molecules, which is also utilized by *S. aureus* to adjust central metabolic pathways and virulence factor synthesis [37]. The serine/threonine protein kinase-phosphatase pair Stk1-Stp1 is, for instance, known for its ability to modulate the activities of the SarA family transcription factors MgrA, SarA, and SarZ [38], with Stp1 promoting infectivity, while Stk1 attenuates infectivity [5,38]. Stk1-driven phosphorylation of the catabolite control protein A was additionally shown to inhibit the DNA-binding capacity of the master regulator of carbon catabolite repression in *S. aureus*, thereby modulating the expression of metabolic and virulence genes [39], and infectivity [40]. Our findings, presented here and elsewhere [8], demonstrate that the deletion of *ptpB* in the clinical *S. aureus* isolate SA564 alters the transcription of various genes/operons whose products are involved in stress adaptation and infectivity, suggesting that PtpB-driven removal of phosphates from arginine phosphosites is another posttranscriptional mechanism utilized by this pathogen to fine-tune the expression and activity of its virulon, in order to successfully adapt to the diverse host environmental conditions encountered by the bacterium during infection. Given the clear impact of PtpB on the transcription of specific virulence determinants shown here, its impact on *S. aureus* to cause disease in mice [8], and the fact that several low molecular weight protein tyrosine phosphatase inhibitors are currently in development to combat diseases such as cancer, diabetes/obesity, and bacterial infections [41–45], PtpB might constitute an additional interesting target for drug development against this notorious human nosocomial pathogen.

4. Materials and Methods

4.1. Bacterial Strains, Media, and Growth Conditions

The bacterial strains used in this study are listed in Table 1. *S. aureus* isolates were plated on Tryptic Soy Agar (TSA; BD, Heidelberg, Germany), or grown in Tryptic Soy Broth (TSB; BD) medium at 37 °C and 225 rpm with a culture to flask volume of 1:10.

Table 1. Strains used in this study.

Strain	Description [1]	Reference
SA564	*S. aureus* clinical isolate, wild type	[12]
SA564 Δ*ptpB*	SA564 Δ*ptpB*::lox66-*erm*(B)-lox71; Erm^R	[8]
SA564 Δ*ptpB*::*ptpB*	*cis*-complemented SA564 Δ*ptpB* derivative	[8]

[1] Erm^R, erythromycin-resistant.

4.2. Human Whole Blood Phagocytosis Assay

The uptake of *S. aureus* cells by PMNs in whole blood was performed essentially as described in [46]. Overnight cultures of *S. aureus* strains SA564, SA564 Δ*ptpB*, and SA564 Δ*ptpB::ptpB* were inoculated into fresh TSB to an optical density at 600 nm (OD_{600}) of 0.05 and grown at 37 °C and 225 rpm to mid-exponential growth-phase (i.e., 2 h). Cultures were centrifuged at 10,000× *g* for 5 min, bacterial pellets washed three times with phosphate buffered saline (PBS; Thermo Fisher, Dreieich, Germany), and subsequently stained with a 50 µM carboxy fluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Darmstadt, Germany)-PBS solution for 15 min at 37 °C and 1000 rpm. CFSE-stained bacterial cells were afterwards washed again three times with PBS to remove unbound dye, and adjusted to an OD_{600} of 1. Fresh human whole blood was withdrawn from healthy donors and anticoagulated with lithium heparin (S-Monovette; Sarstedt, Nümbrecht, Germany). The PMN contents of the blood samples were determined using the RAL DIFF-QUICK kit (RAL Diagnostics, Martillac, France) according to the manufacturer's recommendations, and blood samples were substituted with fluorescent-labeled bacteria at an MOI of 50 per PMN. Infected blood samples were cultured in the dark at 37 °C and 1000 rpm for 30 min and subsequently placed into 5 mL round bottom polystyrene tubes (BD). Erythrocytes were lysed by adding FACS lysis solution (BD), and lysed cell debris was removed by centrifugation at 450× *g* for 5 min. The cell pellets were resuspended in PBS supplemented with 2% fetal calf serum (PAA, Pasching, Germany) and 0.05% sodium azide, and subjected to flow cytometry using a FACSCalibur (BD) system. PMNs were gated using the CellQuest Pro Software version 4.02 (BD), and the mean fluorescence intensity (MFI) per PMN was recorded, indicating the number of bacteria that were attached to or ingested by the leukocyte.

4.3. Extracellular DNase-, Hemolytic- and Proteolytic Activity Assays

Overnight cultures of the *S. aureus* SA564 strain triplet were adjusted for all three assays with fresh TSB to an OD_{600} of 12. For extracellular DNase activity measurements, 5 µL of the adjusted bacterial suspensions were spotted on DNase-Test-Agar plates (Carl Roth, Karlsruhe, Germany) and incubated for 24 h at 37 °C. Lytic zones were visualized by overlaying the agar with 1N HCl to precipitate undigested DNA. The hemolytic activities of the bacterial cell suspensions were tested by spotting 5 µL aliquots of the bacterial suspensions on TSA II plates supplemented with 5% Sheep Blood (BD), and diameters of hemolytic zones were determined after 24 h of incubation at 37 °C. The proteolytic activity of the bacterial cell suspensions was determined by spotting 2 µL aliquots of the bacterial suspensions on TSA plates supplemented with 10% skim milk (BD). Variations in zones of proteolysis were evaluated after incubating the plates for 48 h at 37 °C.

4.4. Triton X-100 Induced Autolysis Assay

Triton X-100 induced autolysis of *S. aureus* was assayed using a modified version of the protocol described in [47]. Cells of *S. aureus* SA564 and its *ptpB* deletion mutant were cultured in TSB at 37 °C and 225 rpm to an OD_{600} of 0.7, washed twice in ice-cold water, and resuspended in 0.05 M Tris-HCl (pH 7.2) containing 0.01% (vol/vol) Triton X-100 (Merck, Darmstadt, Germany). The cell suspensions were incubated at 30 °C and 225 rpm and the OD_{600} measured every 30 min. Triton X-100 induced autolysis was determined as the decline of optical density versus time and is expressed as the percent of the initial optical density.

4.5. Lysostaphin Induced Autolysis Assay

The lysostaphin-induced autolysis of *S. aureus* was assayed as described in [48]. Cells of *S. aureus* SA564 and SA564 Δ*ptpB* were cultured in TSB at 37 °C and 225 rpm to an OD_{600} of 3, washed twice in PBS, and resuspended in PBS to an OD_{600} of 1. Cell suspensions were supplemented with 250 ng/mL of lysostaphin (Dr. Petry Genmedics GMBH, Reutlingen, Germany) and incubated under static conditions at 30 °C for 60 min. The OD_{600} was

measured every 10 min. Lysostaphin-induced autolysis was determined as the decline of optical density versus time and is expressed as the percent of the initial optical density.

4.6. qRT-PCR Analyses

RNA isolation, cDNA synthesis, and qRT-PCR were carried out as previously described [49], using the primer pairs listed in Table 2. Transcripts were quantified in reference to the transcription of gyrase B using the $2^{-\Delta Ct}$ method [50].

Table 2. qRT-PCR primer used in this study.

Gene Target	Primer	Sequence (5′-3′)
atlR	forward reverse	AACTTATTACACTGACTAACAATG TGTCCAAATCTTCTATTCACTAA
aur	forward reverse	AATAGTATTGACGGTGGATTT AATGCTGATAATTTACCTTGATG
ebh	forward reverse	GTAATAATGAACAGACTGAGAATC AGCGGATAATGATTGACTATT
fmtB	forward reverse	GATGCTTCAAGAATTACAACAA ATCCTGAGAATAGACCTACAT
gyrB	forward reverse	GACTGATGCCGATGTGGA AACGGTGGCTGTGCAATA
hla	forward reverse	AACCCGGTATATGGCAATCAACT CTGCTGCTTTCATAGAGCCATTT
hld	forward reverse	AGGAGTGATTTCAATGGCACAAG TGTGTCGATAATCCATTTTACTAAGTCA
lrgA	forward reverse	GCCGGTATCTCAGTTGTTAACTCTT AAATGGTGCTTGGCTAATGACAC
lytN	forward reverse	CTATTGTCTTAAATGGTGATTATG ATCTAAACTTTGGAACTTCATTA
nuc	forward reverse	TAGCTCAGCAAATGCATCACAA GAACCACTTCTATTTACGCCATTATCT
psma	forward reverse	ATCAACAACTCATCACTATGTTAAATCAAC GCCATCGTTTTGTCCTCCTGT
ptpB	forward reverse	AGCCCATTAGCGGAAAGTATTG AAATTGATGATTTGGCATAACCTCT
spa	forward reverse	TACTTATATCTGGTGGCGTAA GGTCGTCTTTAAGACTTTGA
splB	forward reverse	AAGGTAATGGTGGTATTTATTC GAATGACTGATACATCTTCTTTA

4.7. Statistical Analyses

The statistical significance of changes between groups was determined using the GraphPad software package Prism 6.01. p values < 0.05 were considered statistically significant.

Author Contributions: Conceptualization, V.M. and M.B.; methodology, M.I.E. and M.B.; formal analysis, M.I.E. and M.B.; investigation, M.I.E.; resources, M.B. and S.L.B.; writing—original draft preparation, M.B.; writing—review and editing, M.I.E., V.M., S.L.B. and M.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Medical Association of Saarland (code number 39/20, approved 25 February 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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6. Discussion and conclusion

6.1. Discussion

PtpA, a secreted tyrosine phosphatase from *Staphylococcus aureus*, contributes to virulence and interacts with coronin-1A during infection (article I).

In this study, the impact of PtpA on the pathogenicity of *S. aureus* was investigated. I started by constructing a *ptpA* deletion mutant within the clinical *S. aureus* strain SA564. I used the advantage of the availability of a Δ*ptpA* mutant in the *S. aureus* strain Newman, which was constructed in our laboratory and already used in a previously published study (Brelle *et al.,* 2016). I transferred the resistance cassette marked deletion region into the *S. aureus* strain SA564 via phage transduction to construct the strain SA564 Δ*ptpA*. In order to confirm that potential phenotypes observed with the *ptpA* deletion mutant were indeed caused by the deletion of *ptpA* in this *S. aureus* background, the *cis*-complemented derivative harboring the wild-type *ptpA* locus was constructed using the previously described pEC1 plasmid (Brückner, 1997). These two *S. aureus* backgrounds, *S. aureus* SA564 and Newman, were used in this study, because they represent genetically and phenotypically distinct lineages (Duthie, 1952; Somerville *et al.,* 2002). The *S. aureus* strain SA564 is a low-passage human clinical strain of the CC5 isolated from a patient with TSS in USA in 2007 (Somerville *et al.,* 2002). This *S. aureus* strain is worldwide distributed and especially prominent in the western hemisphere (Challagundla *et al.,* 2018). The *S. aureus* strain Newman (CC8) is a genetically amenable strain and extensively used in staphylococcal research including the phenotypic characterization of *S. aureus* in murine infection models (Baba *et al.,* 2008).

In a first set of experiments in this study, the effect of a *ptpA* deletion on the bacterial growth of *S. aureus* was investigated. When *S. aureus* strains Newman and its Δ*ptpA* mutant were cultivated in a rich medium (e.g. TSB) under aerobic conditions, both strains grew in a similar way, which is in line with earlier findings (Vega *et al.,* 2011) and suggests that PtpA is not involved in regulating metabolic pathways of this bacterium. Given the impact of the *Mtb* Ptp homologues on the infectivity of this intracellular pathogen, and the fact that MPtpA and MPtpB

are effectively secreted by *Mtb* without harboring a clear export signal sequence (Bach *et al.,* 2008; Wong *et al.,* 2011), it was then tempting to speculate that the *S. aureus* Ptp homologues may fulfill similar functions. In line with this assumption, the deletion of *ptpA* in *S. aureus* was found to decrease the capacity of this pathogen to survive inside the mouse-derived RAW 264.7 macrophages, and similar findings were reported with the THP-1 derived human macrophages. The overall ability of *S. aureus* to invade and survive inside macrophages has been described before (Kubica *et al.,* 2008; Grosz *et al.,* 2014; Lacoma *et al.,* 2013; Tranchemontagne *et al.,* 2016). With the observations made in study I, we could define PtpA as an additional factor contributing to the internal survival of *S. aureus* in this important immune cell type. Moreover, when a *S. aureus*-based murine abscess model was investigated, a more than 3-*log* reduction in bacterial loads obtained from the liver of mice infected with the Δ*ptpA* deletion mutant was noticed. Collectively, these findings indicate that the LMW-PTP homologue PtpA is of major importance for the infectivity of *S. aureus*, presumably by promoting its survival in macrophages.

To test whether *S. aureus* secretes PtpA not only under *in vitro* conditions (Brelle *et al.,* 2016), but also under infection-mimicking conditions, a number of immunoblotting experiments were carried out. In these experiments, it became clear that PtpA was also secreted by *S. aureus* when bacterial cells were internalized by macrophages, suggesting that PtpA secretion is also functional under *in vivo* conditions. Immunoprecipitation assays further identified putative intracellular binding partners for PtpA inside macrophages such as Coronin-1A, which was also found to be phosphorylated on tyrosine residues upon *S. aureus* infection. Besides its function in regulating the actin cytoskeleton assembly, Coronin-1A is thought to be involved also in a variety of cellular processes including signal transduction (Punwani *et al.,* 2015). Mutations of the Coronin-1A encoding gene (*CORO1A*) were reported to increase the infection susceptibility and modulate the immune response in mice and humans (Shiow *et al,* 2008; Moshous *et al.,* 2013). This protein accumulates in the phagocytic cups and on phagosomal membranes during phagocytosis hindering the fusion of phagosomes with lysosomes (Itoh *et al.,* 2002). Furthermore, Oku and colleagues (Oku *et al.,* 2021) reported that this protein is phosphorylated on the Thr412 residue in a process required for the full activity of phagocytes. Based on these observations, we propose that PtpA from *S. aureus* also modulates the host immune response, presumably by interacting with Coronin-1A inside macrophages. Although the impact of the phosphorylation of Coronin-1A on tyrosine residues in phagocytic cells infected with *S. aureus* is not known yet, one may hypothesize that such phosphorylation might be linked to alterations in the distribution of this protein inside macrophages during infection. This assumption is in line with a previous observation that macrophages infected with *Mtb* showed a retention of Coronin-1A on the phagosomal membrane. These phagosomes were

also found to contain viable and not killed bacteria, suggesting that the retention of Coronin-1A on phagosomes enhances the intracellular survival capacity of *Mtb* inside macrophages by blocking the delivery of bacterial cells to lysosomes (Ferrari *et al.,* 1999; Jayachandran *et al.,* 2007). Another study also observed that oligomerization of Coronin-1A is an important key determinant of *Mtb*-macrophage interaction, in which the trimerization of Coronin-1A was reported to enhance the survival of mycobacterial cells in macrophages (BoseDasgupta and Pieters, 2014).

The phosphoarginine phosphatase PtpB from *Staphylococcus aureus* is involved in bacterial stress adaptation during infection (article II).

As our first set of data indicated that PtpA plays an important role in host immune evasion and infectivity of *S. aureus* in mice, we hypothesized that the other closely related phosphatase, PtpB, might have similar effects. In this study, the Δ*ptpB* mutant strain was constructed using the phage transduction strategy and its *cis*-complemented derivative was then constructed using the shuttle vector pBASE6 in double-crossover mechanism (Geiger *et al.,* 2012). Firstly, i studied the survival capacity of a *S. aureus* SA564 *ptpB* deletion mutant in THP-1 derived macrophages, which were firstly described by Tsuchiya and colleagues (Tsuchiya *et al.,* 1980), and since then extensively used as a model to explore macrophage activity (Qin, 2012, Starr *et al.,* 2018). Infection of THP-1 macrophages with the *S. aureus* strain SA564 Δ*ptpB* cells revealed a significant reduction in the number of viable bacterial cells recovered from the infected macrophages, similar to our findings made with the *S. aureus ptpA* deletion mutant. Additionally, this finding agrees with a previous study in which the mycobacterial PtpB homologue was also found to promote intracellular survival of *Mtb* inside macrophages via the modulation of several macrophage proteins (Singh *et al.,* 2003). In addition, Zhou and colleagues (Zhou *et al.,* 2010) showed that mycobacterial PtpB interferes with the interferon gamma (IFN-γ) mediated signaling pathway, which in turn results in inhibition of IL-6 production. IL-6 is a very effective cytokine activating microbicidal activities of macrophages (Van der Poll *et al.,* 1997). By using our murine abscess model, we also showed that the deletion of *ptpB* in *S. aureus* is associated with decreased bacterial loads in liver and kidneys of the infected mice, a phenotype that also fits with observations made for the *Mtb* homologue showing that a deletion of *ptpB* in *Mtb* results in a severe reduction in bacterial loads in a guinea pig model of infection (Singh *et al.,* 2003). However, unlike the PtpB homolog of *Mtb* (Dhamija *et al.,* 2019), we failed to identify a relevant secretion of PtpB into the extracellular

milieu by *S. aureus*, suggesting that PtpB, unlike PtpA, is not secreted by *S. aureus,* and indicating that this protein is likely to affect the pathogenicity of *S. aureus* by different means.

Part of the success of *S. aureus* as a human pathogen originates from its ability to quickly adapt to adverse conditions encountered within the host during infection (Cole *et al.,* 2014; Dastghey and Otto, 2015). The diminished capacity of *S. aureus* strain SA564 Δ*ptpB* to survive intracellularly within THP-1 derived macrophages hit us with the idea to mimic relevant stresses encountered by *S. aureus* within macrophages in order to identify potential mechanisms exerted by PtpB to enhance the intracellular survival in this immune cell type. Phagocytosis of *S. aureus* by professional phagocytic cells is followed by activation of diverse killing mechanisms including the release of ROS, reactive nitrosative species (RNS), and a low pH milieu (Peyrusson *et al.,* 2020; Pidwill *et al.,* 2021). All of these stresses usually work together in a coordinated way to destroy the engulfed bacterial cells (Viola *et al.,* 2019). On the other side, the ability of bacteria to sense and respond to such stresses is considered a key signaling strategy used by these pathogens to evade the hostile immune response (Marshall *et al.,* 2000; Mongkolsuk and Helmann, 2002; Chen *et al.,* 2011). Challenging *S. aureus* strain SA564 Δ*ptpB* with 50 mM of H_2O_2 as a final concentration uncovered a reduced capacity of the mutant cells to resist oxidative stress induced by H_2O_2 compared to that of the wild-type. This finding is in a line with a previous study in which deletion of *ptpB* in the *S. aureus* strain COL resulted in a reduction in bacterial ability to survive in H_2O_2-supplemented media (Junker *et al.,* 2019). *S. aureus* exploits various mechanisms to counteract the effects of oxidative stress by increasing the expression of detoxification enzymes (Gaupp *et al.,* 2012). Moreover, PTMs of bacterial proteins are considered mechanisms used by bacterial cells to sense and adapt to such oxidative stress (Marshall *et al.,* 200). Based on these observations, we assumed that PtpB might affect the transcription of genes required to counteract this stress in *S. aureus*. To test this assumption, i performed qRT-PCR analyses of genes whose products are involved in detoxification of H_2O_2 in *S. aureus* which revealed decreased transcription rates for *ahpC*, *katA* and *sodA*. These rates of reduced expression explains the retarded capacity of the Δ*ptpB* cells to withstand the H_2O_2-induced stress and agree with a study in which deletion of *ahpC* and *katA* resulted in a severe decline in the ability of *S. aureus* to overcome deleterious effects of this oxidative stress (Cosgrove *et al.,* 2007). In accordance, inactivation of NADPH oxidase, a key component of oxidative burst in macrophages, results in a dramatic decrease in the killing ability of macrophages. This diminished killing capabilities of macrophages were reflected by increases in numbers of *S. aureus* cells (20 to 30-fold) surviving inside NADPH oxidase-defected macrophages (Surewaard *et al.,* 2016). These observations, together with another study (Krause *et al.,* 2019), indicate that macrophages

use high levels of ROS to restrict *S. aureus* during infections, a mechanism that seems to be counteracted by the bacterium via PtpB-driven modulation of ROS inactivating factors.

Activated macrophages also synthesize nitric oxide (NO˙) in a micromolar range together with other RNS to clear internalized *S. aureus* cells (Weinberg, 1998; Vazquez-Torres *et al.,* 2008). In fact, NO˙ is a main component of the bactericidal activity of macrophages (Fang und Vazquez-Torres 2002; Chakravortty und Hensel 2003). After engulfing pathogens, activated macrophages are stimulated to form NO˙ and oxygen by activation of iNOS (Stuehr, 1991; Webb *et al.,* 2001). RNS are then produced in the presence of oxygen to inhibit bacterial respiration (Fang, 2004; Pacher *et al.,* 2007). Thus, macrophages use RNS together with ROS to induce synergistic antimicrobial capabilities (Pacelli *et al.,* 1995). To investigate the impact of PtpB on the capacity of *S. aureus* to deal with NO˙ stress, we used the NO˙ donor dimethylamine NONOate (DEA/NONOate). This family of NO˙ donor compounds was used because of its established ability to release NO˙ in aqueous solutions with known decay rates (Ramamurthi and Lewis, 1997). Interestingly, we observed a reduced capacity of the Δ*ptpB* mutant cells to survive the NO˙ stress induced by DEA/NONOate challenge, suggesting that PtpB contributes to the intracellular survival of *S. aureus* inside immune cells by increasing the capacity of the bacterium to withstand NO˙-induced killing. This assumption is in line with findings made by Urbano and colleagues (Urbano *et al.,* 2018) who demonstrated that internalized *S. aureus* cells promote their survival inside macrophages by counteracting the produced NO˙. Other studies also linked the intracellular survival capacity of *S. aureus* to its adaptability to RNS produced by phagocytic cells (Hochgräfe *et al.,* 2008). Similarly, Kinkel *et al.* (Kinkel *et al.,* 2013) concluded that being resistant to NO˙ is a prerequisite for survival of *S. aureus* inside host cells. However, my qRT-PCR analysis of *hmp* transcription, a flavohemoglobin gene encoded in all sequenced *S. aureus* genomes and required for detoxification of nitrosative stress (Gonçalves *et al.,* 2006; Lewis *et al.,* 2015), revealed no clear differences between the wild-type and Δ*ptpB* mutant cells, which might be attributed to the lack of NO˙ challenge under the *in vitro* growth conditions used. This assumption is consistent with this of Kinkel *et al.* (Kinkel *et al.,* 2013) who reported that *hmp* is tightly regulated by the SrrAB two-component system and its expression in the absence of NO˙ could intensify the oxidative stress. This finding highlights the importance of the NO˙ challenge to induce the expression of *hmp* in *S. aureus*.

In addition to the above-mentioned mechanisms, acidification of the phagolysosome is another major mechanism employed by macrophages to kill the ingested *S. aureus* cells (Ip *et al.,* 2010). This bactericidal acidification step is generated by protons imported via a proton transporting ATPase (Westman and Grinstein, 2021). The resulting decline in pH generates acidic and hydrolytic phagolysosomes which are detrimental for the survival of intracellular *S.*

aureus cells (Korting *et al.,* 1992). However, the permeability of the phagosomal membrane and the rate of proton leakage are two important factors resulting in variations of the acidic pH value generated inside macrophages (Canton *et al.,* 2004). In this regard, the lumen of early phagosomes is a little acidic with a pH value of 6.1-6.5 (Kinchen and Ravichandran, 2008), however it becomes more acidic due to translocation more protons into the lumen of phagosomes forming the so-called late phagosomes with a pH value of 5.5-6 (Lee *et al.,* 2020). This pH value further declines to 5-5.5 when the phagolysosomes are formed (Marshansky and Futai, 2008). In order to mimic a similar *in vitro* effect, i used PBS adjusted to pH of 5.5 to estimate the survival capacity of *S. aureus* strain SA564 Δ*ptpB* under such a condition. My determinations of the CFU values together with the drop in the OD_{600} values indicated that PtpB is involved in enhancing the capacity of *S. aureus* to resist the low pH stress that is usually encountered within macrophages. Earlier work demonstrated that *S. aureus* alters its phospoproteome to adapt to acidic pH conditions (Junker *et al.,* 2019). Specifically, exposing *S. aureus* cells to acidic conditions resulted in an increased serine/theronine phosphorylation of proteins (Huemer *et al.,* 2021). Likewise, McsB was reported to be of importance for *S. aureus* to resist acidic stress (Wozniak *et al.,* 2012), further supporting the idea that the McsB/PtpB phosphorylation/dephosphorylation system is of relevance for this pathogen to adapt to acidic conditions. Nevertheless, additional analyses of the staphylococcal proteome are still required in order to identify potential specific targets of McsB and PtpB.

The low-molecular weight protein arginine phosphatase PtpB affects nuclease production, cell wall integrity, and uptake rates of *Staphylococcus aureus* by polymorphonuclear leukocytes (article III).

Within my third study, i investigated whether PtpB might also contribute to the pathogenicity of *S. aureus* toward the host innate immunity. PMNs represent another integral part of the innate immune response, which is particularly effective for the engulfment and clearance of *S. aureus* (Lu *et al.,* 2014). Activation of PMNs during *S. aureus* infections results in the production of proteases and AMPs in concert with a variety of ROS, which all aim at destroying the invading *S. aureus* cells (Guerra *et al.,* 2017; Kobayashi *et al.,* 2018). Despite this, *S. aureus* is able to evade clearance by PMNs in a process required for establishment and maintenance of infections (DuMont *et al.,* 2013). Upon the encounter with PMNs, *S. aureus* secretes several proteases to either inhibit uptake by these immune cells or even induce lysis

after being engulfed (Rigby and DeLeo, 2012, Spaan *et al.,* 2013; van Kessel *et al.,* 2014; Prajsnar *et al.,* 2020). In order to address the effect of *ptpB* deletion on the uptake of *S. aureus* by PMNs, i employed a whole blood flow cytometric assay (Ballhausen *et al.,* 2014; Jung *et al.,* 2017). This assay enables to quantitatively analyze phagocytosis of fluorescent labelled *S. aureus* cells by PMNs in whole human blood. Analysis of the obtained MFI together with the associated histograms (population comparisons) showed that cells of the SA564 Δ*ptpB* mutant were taken up by PMNs in significantly higher rates than cells of the wild-type and *cis*-complemented strains. These findings indicate that PtpB is of importance for *S. aureus* to evade phagocytosis by neutrophils in the human blood, although it cannot be excluded that cells of the *ptpB* deletion mutant adhered in larger quantities to PMNs than the wild-type and *cis*-complemented cells. In fact, the inability to differentiate between bacterial cells which are already phagocytosed and those being only attached to the PMNs is a major limitation of this assay (Boero *et al.,* 2021).

In order to gain a better understanding of the contribution of PtpB to the interactions of neutrophils with *S. aureus*, i tested whether PtpB might be crucial for *S. aureus* to inhibit cytotoxic activities of these immune cells. As mentioned earlier in this thesis, NETs are neutrophil-derived bactericidal determinants of the immune response and consist mainly of large quantities of eDNA decorated with granular proteins (Papayannopoulos and Zchlinsky, 2009). *S. aureus* is known to significantly reduce the activity of NETs mainly by the production of exonucleases (Speziale and Pietrocola, 2021). Thus, i tested the effect of the *ptpB* deletion on the ability of *S. aureus* to degrade eDNA. Here, i found that cells of the *ptpB* deletion mutant produced significantly smaller lytic areas surrounding the bacterial growth zones than the wild-type and *cis*-complemented cells when cultured on DNase agar test plates. To substantiate this finding, i further assessed the transcription of *nuc,* encoding for the major exonucleases of *S. aureus*. In line with the DNase assay, a significant reduction in transcription rates of *nuc* was observed in the Δ*ptpB* mutant cells in three time points representing different growth phases (exponential, transition and early stationary phases). Together, these findings suggest that PtpB promotes the expression of nucleases and thus positively contributes to the exonuclease activity of *S. aureus*, which is considered as a major pathogenicity factor of *S. aureus* to resist killing by NETs (Schilcher *et al.,* 2014) and to even persist within suppurative lesions during infection (Chen *et al.,* 2009).

Inactivation of the host complement system is another mechanism used by *S. aureus* to evade the host innate immunity (Sakiniene *et al.,* 1999). The complement system is a central part of the human immune response against pathogens (Dunkelberger and Song, 2010). During bacterial infections, activation of the complement system results in cleaving of the C3 into two proteins, C3a and C3b in a process required for opsonization of *S. aureus* (Harboe *et al.,*

2004). However, *S. aureus* is capable of counteracting this process by secreting the zinc-dependent metalloprotease Aur (Shaw *et al.,* 2004). Aur is synthesized by *S. aureus* throughout the growth cycle and effectively cleaves C3b, thus interfering with the phagocytic activity of phagocytes and providing opportunities for *S. aureus* to disseminate from the blood stream into different organs (Kubica *et al.,* 2008; Jusko *et al.,* 2014). In addition, Sieprawska-Lupa *et al.* (Sieprawska-Lupa *et al.,* 2004) reported that Aur secreted by *S. aureus* contributes to the inactivation of Cathelicidin LL-37, one of the main human AMPs with a potent antistaphylococcal activity. These findings, together with the information that Aur attenuates phagocytosis of *S. aureus* by neutrophils (Laarman *et al.,* 2011), indicate that Aur contributes to the protection *S. aureus* against phagocytosis (Kubica *et al.,* 2008). Based on these observations, i determined the transcription rate of *aur* (encoding aureolysin) when *ptpB* was deleted in comparison to the wild-type cells. My data showed that deletion of *ptpB* results in a significant reduction in *aur* transcription rates, suggesting that PtpB promotes immune evasion of *S. aureus* by increasing the expression of Aur. Regarding its other targets, aureolysin also activates V8 protease and cleaves staphylococcal surface-associated proteins such as FNBPs, SpA and ClfB, thus contributing to the proteolytic activity in *S. aureus* (McGavin *et al.,* 1997; McAleese *et al.,* 2001; Dubin, 2002; Shaw *et al.,* 2004). In this study, i also observed that deletion of *ptpB* reduced the overall proteolytic activity of *S. aureus* spotted on skimmed milk tryptic soy agar plates. In a similar way, Wozniak *et al.* (Wozniak *et al.,* 2012) reported that deletion of McsB was associated with a decreased proteolytic activity in *S. aureus* mainly by decreasing the expression of the *clpC* operon (Frees *et al.,* 2003).

Another major mechanism used by neutrophils during their battle with bacterial pathogens is to destabilize bacterial cytoplasmic membranes via the release of large amounts of AMPs (Oppenheim *et al.,* 2003). Defensins are the principal AMPs secreted by neutrophils, accounting for 50% of their azurophilic granules (Lundy *et al.,* 2008). Exposure of *S. aureus* to defensins is known to induce structural changes of the cell wall, even when low concentration is used (Shimoda *et al.,* 1995). To test whether PtpB contributes to the capacity of *S. aureus* to withstand such a kind of stress, i tested the capability of the wild-type and Δ*ptpB* mutant cells to cope with cell wall-damaging substances in a series of Triton X-100- and lysostaphin-induced lysis assays. Triton X-100 is a non-ionic detergent known to stimulate the activity of autolysins in *S. aureus* (Raychaudhuri and Chatterjee, 1985). Lysostaphin is zinc metalloproteinase degrading the cell wall of *S. aureus* by targeting the pentaglycine interpeptide bridges of the PG (Schindler and Schuhardt, 1964). Notably, when cells of the *ptpB* deletion mutant were exposed to Triton X-100 and lysostaphin, respectively, a significantly faster lysis was observed than with cells of the wild-type strain. These findings, in contrary to the previous findings of Brelle *et al.* (Brelle *et al.,* 2011), suggest a potential role of

PtpB in maintaining the integrity of the cell wall in *S. aureus*. Maintenance of the cell wall integrity is achieved either by affecting the cell wall synthesis processes or the cell wall lytic activities (Sutton *et al.,* 2021). Based on a previous study of Junker *et al.* (Junker *et al.,* 2018; Junker *et al.,* 2019) that MgrA is phosphorylated on Arg when *ptpB* was deleted and also the fact that MgrA controls the autolysis in *S. aureus* (Ingavale *et al.,* 2003), we postulated that PtpB might affect the autolysis in *S. aureus* by modulating the activity of MgrA. In *S. aureus*, autolysis is mediated by autolytic enzymes (autolysins or murein hydrolases) that cleave covalent bonds in the cross-linked PGN chains (Antignac *et al.,* 2007). Thus, i investigated the transcription rates of major genes affecting the staphylococcal autolytic activities such as *atlR*, *fmtB*, *lrgA* and *LytN* which are all regulated by MgrA (Komatsuzawa *et al.,* 2000; Luong *et al.,* 2006). AS expected, deletion of *ptpB* affected the transcription of all these genes in the S. aureus strain SA564.

The findings of Junker *et al.* (Junker *et al.,* 2018; Junker *et al.,* 2019) that MgrA is a substrate of PtpB in *S. aureus* and my findings that deletion of *ptpB* affected the transcription of a set of MgrA-regulated genes especially those controlling the autolysis in *S. aureus* supported the possibility that PtpB might affect the whole MgrA operon. Interestingly, my qRT-PCR analysis of the transcription of *ebh* and *spa* which are also controlled by MgrA showed that PtpB does not alter the activity of MgrA *per se*. In fact, interruption of the regulation process of MgrA induced by the lack of Arg phosphorylation was previously reported by Junker *et al.* (Junker *et al.,* 2018). Furthermore, changing of the phosphorylation status of MgrA induced by the Ser/Thr kinase (StK1) was also reported to change its binding capacity to promoters of the MgrA-targeted genes *norA* and *norB* (Truong-Bolduc *et al.,* 2008; Truong-Bolduc and Hooper, 2010). Thus, we postulated that Arg dephosphorylation of MgrA induced by PtpB results in differential promoter-binding reactions on a number of MgrA-regulated genes, and thus a differential transcription rate of these genes. Similarly, investigating the transcription of a number of genes regulated by the global transcriptional regulator SarA revealed that PtpB affects the transcription of some but not all genes controlled by this regulator. In this regard, PtpB enhanced the expression of *nuc*, *aur*, *lrgA* and *hld* but did not affect the expression of *hla* and *splB*. This finding agrees with previous studies of Junker *et al.*, (Junket *et al.,* 2018; Junker *et al.,* 2019) in which phosphorylation of SarA on Arg residues was not reported. Of importance, SarA phosphorylated by StK1 was found to interact with its promoters in a different way to the nonphosphorylated SarA (Didier *et al.,* 2010) Furthermore, my qRT-PCR data together with the hemolytic assay performed on blood agar plates showed a relation of PtpB to the activity of the accessory gene regulator locus (*agr*) locus presumably via the response regulator AgrA which is regarded to mainly control the transcription of the *agr* operon and stimulate the transcription of RNAIII (Dunman *et al.,* 2001). This assumption is supported

by the findings *ptpB* deletion induced a higher transcription rate of *hld* (encodes RNAIII) in the early stationary phase of growth. Similarly, transcription of *psmα,* which is also known to be regulated by the AgrA regulator (Tan *et al.,* 2018) was also found to be increased by the deletion of *ptpB*. Interestingly, i reported no difference in the hemolytic activity and in the transcription of *hla* when *ptpB* was deleted, supporting the hypothesis that PtpB does not modulate the SarA-activity in a direct way.

6.2. Conclusion

While the contribution of PtpA and PtpB homologues to the infectivity of other bacterial pathogen has been described, their biological significance in *S. aureus* remained unknown. The significance of the findings of the current thesis is to indicate the direct relation between these LMW-PPs and the virulence of *S. aureus*.

The major finding of the present study is to indicate for the first time that PtpA is secreted inside macrophages upon *S. aureus* infection and it interacts with potential intracellular binding proteins including coronin-1A. Deletion of *ptpA* was also associated with a decline in the *S. aureus* infectivity in a murine model of infection in which a significant reduction in the bacterial loads retrieved from the liver of mice infected with the *ptpA*-deletion mutant compared to those infected with the wild-type and *cis*-complemented strains was reported. Together, these results suggest that PtpA is secreted by *S. aureus* inside macrophages during infection to interfere with the phospho-dependent cellular signal transduction cascades within the host cells.

In a similar way, findings of this study highlight the positive contribution of PtpB to the ability of *S. aureus* to survive inside human macrophages. PtpB, unlike PtpA, is not secreted by *S. aureus*, indicating that this phosphatase contributes to the infectivity of *S. aureus* in another method away from interfering with the signal transduction cascades of the host cells. Further analysis showed that PtpB is required for *S. aureus* to resist against the oxidative-, nitrosative- and low pH stresses generated inside macrophages to degrade the phagocytosed bacterial cells. In addition, PtpB enhances the protection of *S. aureus* against being ingested by PMN. This finding is attributed to the decreased secretion of nucleases and the diminished proteolytic activities of the *ptpB* mutant cells. Interestingly, PtpB also contributes to the autolytic behavior of *S. aureus* and when deleted, the bacterial cells were autolyzed in a fast way in the presence of Triton X-100 or lysostaphin. This study also uncovers a modulatory effect of PtpB on the regulatory networks in *S. aureus*. Deletion of *ptpB* affected the transcription of the MgrA-regulated genes: *atlR*, *fmtB*, *lrgA* and *lytN*. However, this is only one

part of the story, as the findings presented here also showed that PtpB does not affect the MgrA activity *per se*. As a result, a direct link is indicated here between the Agr phosphorylation of MgrA and the virulence in *S. aureus* which was a missed point in a number of previous studies. In a similar way, PtpB also affects the *agr* locus and number of SarA-regulated genes in *S. aureus* including *nuc*, *aur*, *lrgA* and *hld*.

Collectively, the findings of this thesis (articles I, II and III) provide more knowledge about molecular determinants affecting the survival of *S. aureus* inside macrophages. Specifically, PtpA and PtpB could be defined as two protein phosphatases affecting the infectivity of *S. aureus*, and thus could be targeted to develop alternative anti-infectious therapies to combat infections caused by this pathogen.

7. References

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8. Publications and conferences

Publications (in peer-reviewed journals)

- **Elhawy, M.I., Molle, V., Becker, S.L., Bischoff, M.,** 2021. The Low-Molecular Weight Protein Arginine Phosphatase PtpB Affects Nuclease Production, Cell Wall Integrity, and Uptake Rates of Staphylococcus aureus by Polymorphonuclear Leukocytes. International journal of molecular sciences: IJMS 22, 5342.
- **Elhawy, M.I., Huc-Brandt, S., Pätzold, L., Gannoun-Zaki, L., Abdrabou, A.M.M., Bischoff, M., Molle, V.,** 2021. The Phosphoarginine Phosphatase PtpB from Staphylococcus aureus Is Involved in Bacterial Stress Adaptation during Infection. Cells 10, 645.
- **Shi, L., Muthukumar, V., Stachon, T., Latta, L., Elhawy, M.I., Gunaratnam, G., Orosz, E., Seitz, B., Kiderlen, A.F., Bischoff, M., Szentmáry, N.,** 2020. The Effect of Anti-Amoebic Agents and Ce6-PDT on Acanthamoeba castellanii Trophozoites and Cysts, In Vitro. Translational vision science & technology 9, 29.
- **Shi, L., Stachon, T., Latta, L., Elhawy, M.I., Gunaratnam, G., Orosz, E., Kiderlen, A.F., Seitz, B., Bischoff, M., Szentmáry, N.,** 2019. Comparison of in vitro assays to study the effectiveness of antiparasitics against Acanthamoeba castellani trophozoites and cysts. Acta Microbiologica et Immunologica Hungarica 67, 23-32.
- **Gunaratnam, G., Tuchscherr, L., Elhawy, M.I., Bertram, R., Eisenbeis, J., Spengler, C., Tschernig, T., Löffler, B., Somerville, G.A., Jacobs, K., Herrmann, M., Bischoff, M.,** 2019. ClpC affects the intracellular survival capacity of Staphylococcus aureus in non-professional phagocytic cells. Scientific reports 9, 16267.
- **Gannoun-Zaki, L., Pätzold, L., Huc-Brandt, S., Baronian, G., Elhawy, M.I., Gaupp, R., Martin, M., Blanc-Potard, A.B., Letourneur, F., Bischoff, M., Molle, V.,** 2018. PtpA, a secreted tyrosine phosphatase from Staphylococcus aureus, contributes to virulence and interacts with coronin-1A during infection. Journal of biological chemistry 293, 15569-15580.

Conferences (Poster-presentations)

- **Elhawy, M.I., Pätzold, L., Gannoun-Zaki, L., Molle, V., Becker, S., Bischoff, M.** The low molecular weight protein tyrosine phosphatase PtpB of Staphylococcus aureus affects intramacrophage survival and infectivity. The 72th Annual Conference of the German Society for Hygiene and Microbiology (DGHM). 8-11 March 2020, Leipzig, Germany.
- **Benthien, H., Jonas, B., Pätzold, L., Elhawy, M.I., Honecker, A., Beisswenger, C., Becker, S.L., Bischoff, M.** Impact of the transcription factor SpoVG on biofilm formation of Staphylococcus epidermidis in vitro and in vivo. The 72th Annual Conference of the German Society for Hygiene and Microbiology (DGHM). 8-11 March 2020, Leipzig, Germany.
- **Gunaratnam, G., Finke, M., Elhawy, M.I., Spengler, C., Eisenbeis, J., Jakobs, K., Tscherning, T., Herrmann, M., Bischoff, M.** The heat shock protein ClpC affects the intracellular survival capacity of Staphylococcus aureus in endothelial cells. The “Cell Physics 2019” Conference. 9-11 October 2019, Saarbrücken, Germany.
- **Shi, L., Stachon, T., Latta, L., Elhawy, M.I., Gunaratnam, G., Bischoff, M., Seitz, B., Szentmáry, N.** Comparison of in vitro assays to study the effectiveness of antiparasitics against Acanthamoeba castellani trophozoites and cysts. The 117th Congress of the DOG (Deutsche Ophthalmologist Gesellschaft). 26-29 September 2019, Berlin, Germany.

9. Curriculum Vitae

The curriculum vitae of the author was removed from the electronic version of the doctoral thesis for reasons of data protection.

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