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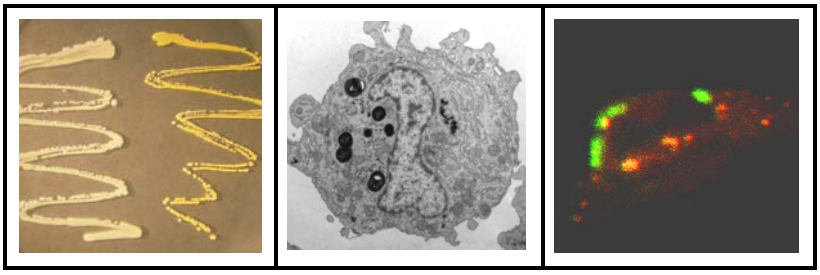
Ecole doctorale de biologie cellulaire et moléculaire, biochimie

INTRACELLULAR FATE OF *STAPHYLOCOCCUS AUREUS* IN HUMAN PHAGOCYTIC AND NON PHAGOCYTIC CELLS AND ROLE OF VIRULENCE FACTORS



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Lorsque les sciences dévoilent les secrets de la nature,
ce que celle-ci perd de mystérieux elle le gagne en merveilleux.

Paul Carvel

ABSTRACT

The intracellular survival of *S. aureus* is believed to contribute to the recurrence of some staphylococcal infections such as endocarditis or osteomyelitis. Previous publications reported the ability of this pathogen to colonize multiple cell types. However the precise fate of intracellular *S. aureus* is still poorly understood.

Here we examine the intracellular development of *S. aureus* in two human cell types, the THP-1 macrophages and HUVEC endothelial cells. We compare the internalization, intracellular growth and intracellular location in both cell types after 24h of infection as well as the activity of antibiotics against intracellular bacteria. *S. aureus* expresses a wide range of virulence factors; some of them are most probably required for intracellular survival. Hence, we studied the implication of phosphatase rsbU and the alternative transcription factor sigmaB involved in stress response and investigated the role of three membrane-damaging toxins (alpha-, beta- and gamma-hemolysins) on the development of intracellular infection.

Internalization was more efficient in THP-1 but, once inside the cells, *S. aureus* proliferated and reached similar intracellular growth in both cells types. After 24h of infection in THP-1, *S. aureus* was confined in phagolysosomes but was able to multiply actively in this acidic environment. In HUVEC bacteria were localized in acidic compartments and in the cytoplasm. Oxacillin, gentamicin, vancomycin and oritavancin showed a high activity in broth but the first three drugs had a poor activity against intracellular *S. aureus* which was similar in both cell types. Only oritavancin displayed a high intracellular activity close to 3 log decrease from initial inoculum. The alpha-, beta-, and gamma-hemolysins did not seem to contribute to the development of intracellular infection. In contrast, the presence of a functional phosphatase rsbU and a subsequent efficient regulation of the transcription factor sigmaB confered an advantage in terms of internalization, intracellular growth and resistance to hydrogen peroxide. This higher intracellular growth and resistance to H_2O_2 is related to the important production of the golden pigment staphyloxanthin, which seems to confer a significant advantage for intracellular survival.

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Abbreviations list

Agr :	Accessory gene regulator
AIP :	Autoinducing peptide
Arl :	Autolysis-related locus
B cells :	B lymphocyte, a type of white blood cells
Bp :	Base pairs
CA-MRSA :	Community-acquired methicillin resistant *S. aureus*
CGD	Chronic granulomatous disease
CFU :	Colony forming unit
ClfA – ClfB :	Clumping factor A – Clumping factor B
Cna :	Collagen-binding protein
Coa :	Coagulase
C_{static} :	Static concentration
DNA :	Deoxyribonucleic acid
EC_{50} :	Drug concentration causing a reduction of the inoculum half-way between the effect in absence of drug (E_0) and the maximal effect (E_{max})
E_{max} :	Maximal effect
EbpS :	Elastin-binding protein
ETs :	Exfoliative toxins
ETA – ETD :	Exfoliative toxin A - Exfoliative toxin D
Fc region :	Fragment crystallisable region (of imunoglobulins)
FITC :	Fluorescein isothiocyanate
FnBPs :	Fibronectin-binding proteins
FnBPA – FnBPB :	Fibronectin-binding protein A - Fibronectin-binding protein B
GM-CSF :	Granulocyte macrophage colony-stimulating factor

H_2O_2 :	Hydrogen peroxide
HA-MRSA :	Hospital-acquired methicillin resistant *S. aureus*
Hla :	Alpha-hemolysin
Hlb :	Beta-hemolysin
Hlg :	Gamma-hemolysin
HlgA, HlgB, HlgC :	Sub-units A, B and C of gamma-hemolysin
Hld :	Delta-hemolysin
HUVEC :	Human umbilical vein endothelial cells
IFN-γ :	Gamma interferon
IgG :	Immunoglobulin G
iNOS	Inducible nitric oxide synthase
IS element :	Insertion sequence element
kD :	kiloDalton
L-NAME :	N_ω-nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
ManLAM	*M. tuberculosis* PIP3 analog glycosylated phophatidylinositiol lipoarabinomannan
MBC :	Minimal bactericidal concentration
MIC :	Minimal inhinitory concentration
MRSA :	Methicillin resistant *S. aureus*
MSCRAMMs :	Microbial surface components recognizing adhesive matrix molecules
NO :	Nitric oxide
O_2^- :	Superoxide anion
$ONOO^-$	peroxynitrite
ORFs :	Open reading frames

PMNs :	Polymorphonuclear leukocytes
PVL :	Panton-Valentine leucocidin
RNA :	Ribonucleic acid
ROS :	Reactive oxygen species
Sae :	Staphylococcal accessory protein effector
SarA :	Staphylococcal accessory gene regulator
SEs :	Staphylococcal enterotoxins
SEA – SEI :	Staphylococcal enterotoxin A - Staphylococcal enterotoxin I
SpA :	Stahylococcal protein A
SrrAB :	Staphylococcal resiratory response
TSA :	Tryptic soy agar
TSST-1 :	Toxic shock syndrome toxin
VISA :	Vancomycin intermediate resistant *S. aureus*
VRSA :	Vancomycin resistant *S. aureus*
vWF :	Von Willebrand factor

INTRODUCTION

1. *Staphylococcus aureus*

Staphylococci are gram-positive cocci, catalase-positive and facultative anaerobes. The *Staphylococcus* genus comprises more than thirty species which are able to colonize many environments and are part of the cutaneous or mucous flora of human and various animal species. At least ten species of staphylococci are regularly isolated from human and, in rare cases, some atypical species can be recovered from clinical samples like *S. gallinarum* and *S. delphini* which were originally associated with poultry and dolphins respectively **[39]**. However, among all staphylococcal species, only a few of them are pathogenic. *S. epidermidis* is responsible for device-related infections, and *S. saprophyticus* may induce urinary tract infections. But the most pathogenic *Staphylococcus* is undeniably the yellow-pigmented *Staphylococcus aureus*. *S. aureus* is part of the normal flora of humans and several animals and can reside without causing any damage to his host. Nonetheless, this microorganism can also induce a large range of pathologies going from minor skin and soft tissues infections to fatal endocarditis, osteomyelitis or necrotizing pneumonia **[53]**.

2. Niche

In humans, the main ecological niche of *Staphylococcus aureus* are the anterior nares. The second more frequent sites of colonization are the skin, perineum and pharynx. Occasionally, *S. aureus* is found in the gastrointestinal tract, vagina

and axillae **[Fig. 1] [91]**. Three different patterns of *S. aureus* nasal carriage have been described: persistent carriage, intermittent carriage and non-carriage. Results of multiple studies indicate that about 20% of individuals are persistent *S. aureus* nasal carriers, 30% are intermittent carriers and 50% are non-carriers **[91]**. Nasal carriage represents a higher risk of development of post-chirurgical infection, lower respiratory tract infection and blood stream infection. These infections are associated with significant morbidity and mortality rates. Various treatment strategies have been tested to eliminate nasal carriage and by this, try to decrease the frequency of such infections. The most frequent strategies are the use of locally applied antibiotics or disinfectants and systemic antibiotics **[67]**.

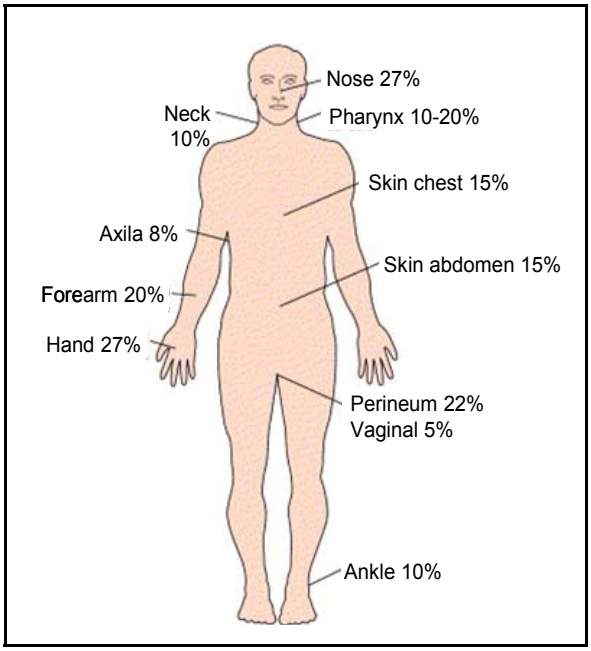

Figure 1 :. [91]. *S aureus* carriage rates per body site in adults.

3. Genome variability

The genome of *S. aureus* is composed of approximately 2.8 10^6 bp and appears to be very flexible. *S. aureus* shares a genus-specific core set of genes with the other species of staphylococci (*S. epidermidis*, *S. haemolyticus, S. carnosus*, and *S. saprophyticus*) that accounts for about 50% of its proteins **[7]**. Inside the species *S. aureus*, the genome presents frequent variations between strains due to the presence of mobile elements such as prophages, genome islands, transposons, IS elements and integrated plasmids. These mobile elements can confer antibiotics resistant or specific virulence factors. In addition, gene deletions and inversions contribute also to the genetic diversity of the species. Consequently, each strain of *S. aureus* may contain different combinations of antibiotic resistance, surface proteins and excreted toxins. Relating the genetic composition with the pathogenic behavior is one major area of staphylococcal research.

4. Antibiotics resistance

Over the past fifty years, most bacterial pathogens developed antibiotic resistance mechanisms, continuously narrowing the scope of effective treatments. Nowadays, antibiotic resistance became a major public health concern. Many staphylococcal infections are becoming more and more difficult

to eradicate due to the acquisition of multiple antibiotic resistance determinants and therapeutic failures are frequent.

The first penicillin resistant strains of *S. aureus* were identified in the 1940s shortly after the introduction of this antibiotic in therapy. Today, more than 90% of *S. aureus* strains are resistant to penicillin due to the production of a β-lactamase. Methicillin was the first semi-synthetic penicillin resistant to β-lactamase degradation. But, as observed for penicillin, the introduction of methicillin in the early 60s was rapidly followed by the emergence of methicillin resistant *S. aureus* (MRSA). This resistance to methicillin is conferred by the gene *mecA* encoding the alternative penicillin binding protein PBP2a. PBP2a is intrinsically insensitive to all β-lactams including cephalosporins and carbapenems **[49]**. At present, resistance mechanisms to virtually all antibiotic classes have been identified among *S. aureus* strains. They include inhibitors of cell-wall synthesis like β-lactams, and glycopeptides, ribosomal inhibitors such as macrolide-lincosamide-streptogramin B (MLS_B), aminoglycosides, tetracyclines, fusidic acid and oxazolidinones, the RNA polymerase inhibitor rifampicin, the DNA gyrase blocking quinolones and the antimetabolite trimethroprim-sulfamethoxazole **[53] [Tabl. 1]** Most hospital-acquired MRSA clones (HA-MRSA) carry multiple resistance mechanisms **[49,53,78]**. In contrast, the community-acquired MRSA (CA-MRSA) are generally susceptible to non β-lactams drugs but they often produce the Panton-Valentine leucocidin and induce severe infections like necrotizing pneumonia. Until the middle of the1990's MRSA were still susceptible to glycopeptides and vancomycin

became the antibiotic of choice to treat MRSA infections. But the first vancomycin intermediate resistant *S. aureus* (VISA) was identified in Japan in 1997 **[33]** and were soon followed by the emergence of fully vancomycin resistant strains (VRSA) **[17,18]**. Therefore it appears that there is a pressing need for optimization and careful use of current active molecules and development of new active drugs.

Antibiotic	Resistance gene(s)	Gene product(s)	Mechanism(s)
Aminoglycosides (e.g., gentamicin)	1) *aac(6')/aph(2'')* 2) *aph(3')-IIIa* 3) *ant(4')-Ia*	1) bifunctional acetyltransferase / phosphotransferase 2) phosphotransférase 3) nucleotidyltransferase	Drug inactivation : aminoglycosides modifying enzymes
β-lactams	1) *blaZ*	1) β-lactamase	1) Drug inactivation : Enzymatic hydrolysis of β-lactam nucleus
	2) *mecA*	2) PBP2a	2) Target modification : Reduced affinity for PBP
Chloramphenicol	*cat*	acetyltransferase	Drug inactivation
Fusidic acid	1) *fusA*	1) elongation factor G (EF-G)	1) Target modification : Mutations in *fusA*
	2) *fusB*	2) EF-G-binding protein	2) Target modification : protection of Ef-G
Glycopeptides	1) Unknown (VISA)	1) altered peptidoglycan	1) Target modification : Trapping of vancomycin in the cell wall
	2) *vanA*	2) D-Ala-D-Lac	2) Target modification : Synthesis of dipeptide with reduced affinity for vancomycin

Antibiotic	Resistance gene(s)	Gene product(s)	Mechanism(s)
Macrolide-lincosamide-streptogramine B			
- Macrolides (e.g., erythromycin)	1) *ermA, ermC*	1) ribosomal methylases	1) Target modification : Reduce binding to 23S
	2) *msrA*	2) efflux protein	2) Efflux of antibiotic
- Lincosamide	1) *ermA, ermC*	1) ribosomal methylases	1) Target modification : Reduce binding to 23S
	2) *LinA'*	2) nucleotidyltransferase	2) Drug inactivation
- Streptogramin B	1) *ermA, ermC*	1) ribosomal methylases	1) Target modification : Reduce binding to 23S
	2) *msrA*	2) efflux protein	2) Efflux of antibiotic
	3) *vgb*	3) viriginiamycin B lyase	3) Drug inactivation
- Streptogramin A	*vat, vatA*	1) acetyltransferase	1) Drug inactivation
	vga, vgaB	2) efflux protein	2) Efflux of antibiotic
- Quinupristin-Dalfopristin (Q-D)	1) Q: *ermA*, *ermB*, *ermC*	1) ribosomal methylases	1) Target modification : Reduce binding to 23S
	2) D: *vat*, *vatB*	2) acetyltransférases	2) Drug inactivation
Oxazolidinones (e.g. Linezolid)	*rrn*	23S rRNA	Target modification : Mutations in the 23S rRNA
Quinolones	1) *parC*	1) ParC (or GrlA) component of topoisomerase IV	1), 2) Target modification : Mutations in the QRDR region, reducing the affinity of enzyme-DNA complexe for quinolones
	2) *gyrA or gyrB*	2) GyrA or GyrB components of gyrase	
	3) *norA*	3) efflux protein	3) Efflux of antibiotic
Rifampicin	*rpoB*	beta-subunit of RNA polymerase	Target modification : mutation in the RNA pol.
Tetracyclines	1) *tetM, tetO*	1) ribosomal protection protein	1) Target modification
	2) *tetK, tetL*	2) efflux protein	2) Efflux of antibiotic
Trimethroprim-sulfamethoxazole (TMP-SMZ)	1) TMP: *dfrA*, *dfrB*	1) dihydrofolate reductase (DHFR)	1) Target modification : Lower affinity for DHFR
	2) SMZ: *dpsA*	2) dihydropteroate synthase	2) Target modification : Overproduction of *p*-aminobenzoïc acid

Table 1 : Adapted from **[3, 40, 49, 53, 59]**. *S. aureus* resistance mechanisms.

5. Role of *S. aureus* virulence factors in infections

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Role of *Staphylococcus aureus* virulence factors in infections.

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ABSTRACT

Staphylococcus aureus is a major human pathogen responsible for a wide variety of infections acquired both in the community and hospital settings. Staphylococcal diseases extend from minor skin infections and food poisoning to fatal pathologies like toxic shock syndrome, endocarditis, osteomyelitis, bacteremia or necrotizing pneumonia. This large diversity of infections can be related to the numerous virulence factors and exotoxins produced by *S. aureus*. This review addresses the *in vivo* significance of the adhesion and invasion-related virulence factors and give an overview of the different staphylococcal exotoxins and their related diseases, in particular the Panton-Valentine leucocidin associated in the past decade with epidemic necrotizing pneumonia and severe skin and soft tissue infection.

INTRODUCTION

The gram-positive cocci *Staphylococcus aureus* are amazingly versatile bacteria able to survive in a wide variety of environments. It colonizes the skin and mucosa of humans and several animal species. Although *S. aureus* may belong to the normal flora of human and reside without causing any damage to its host, it is also frequently responsible for severe pathologies such as invasive endocarditis, osteomyelitis, septic arthritis, septicemia or skin and soft tissues infections. *S. aureus* ranks among the most frequent sources of bacterial infections in humans and is one major nosocomial and community-acquired pathogen.

DISEASE-RELATED TOXINS

Some strains of *S. aureus* generate one or more specific exoproteins for which the correlation with a particular disease has been well established. Among other, the toxic shock syndrome toxin (TSST-1) induces the toxic shock syndrome and staphylococcal scarlet fever, staphylococcal enterotoxins (SEs) are responsible for food poising, the exfoliative toxins (ETs) triggers the scalded skin syndrome or, more recently, the Panton-Valentine leucocidin (PVL) was associated with severe skin and soft tissue infections and necrotizing pneumonia [7,14] **[Fig. 2]**.

The TSST-1 and staphylococcal enterotoxins are pyrogenic toxin superantigens (PTSAgs) that induce a disproportionate response of host immune system [14]. So far, eighteen serologically distinct staphylococcal enterotoxins or enterotoxin-like toxins have been identified. They all have superantigenic properties but only eight of them (SEA, SEB, SECn, SED, SEE, SEG, SEH and SEI) are known to cause emesis when ingested[32].

The exfoliative toxins are responsible for two contagious, blistering skin diseases; the scald skin syndrome characterized by extended epidermal desquamation and the bulbous impetigo characterized by localized lesions with purulent exudates. These two pathologies affect essentially infant and young children or immunocompromised adults[47]. So far four exfoliative toxins have been identified (ETA, ETB, ETC, ETD) but only ETA and ETB have been linked to human pathologies [65].

The general interest toward staphylococcal exotoxins has been recently increasing due to their frequent association with methicillin-resistant *S. aureus* (MRSA) clones. Two categories of MRSA are conventionally accepted, the hospital-acquired MRSA (HA-MRSA) that are the leading cause of nosocomial infections worldwide and the community acquired MRSA (CA-MRSA) [8, 62]. CA-MRSA strains are responsible for contagious and fatal diseases like necrotizing pneumonia, severe sepsis and necrotizing fasciitis that regularly affect previously healthy young patients [8,33]. Although CA-MRSA are more frequently susceptible to non-beta-lactam antibiotics than HA-MRSA, CA-MRSA also tend

to be more virulent. Multiple evidences indicate that HA-MRSA and CA-MRSA evolved from different *S. aureus* lineages [8]. HA-MRSA clones frequently express one or multiple enterotoxins and occasionally the TSST-1 while in contrast the CA-MRSA clones are associated with the Panton-Valentine leucocidin [62]. In rare cases, CA-MRSA can also produce the TSST-1 or exfoliating toxin [62].

Epidemiologic and clinical data provide compelling evidence that links the Panton-Valentine leucocidin with the high virulence potential of CA-MRSA [8,33]. Experimental data also confirm the implication of PVL in the virulence of acute pneumonia in a mouse model [31]. Nonetheless the exact mode of action of PVL during infection is still unclear. PVL is a bicomponent pore-forming leucotoxin encoded by two co-transcribed genes *lukS-PV* and *lukF-PV* that are borne by different integrative phages [62]. PVL is active on human polymorphonuclear leukocytes (PMNs), but unlike other *S. aureus* pore-forming toxin, PVL is not hemolytic [62]. The PMNs cytolytic activity of PVL could represent the initial step of infection allowing the bacteria to evade the first line of host defenses. This is consistent with clinical findings demonstrating that neutropenia is frequent in patient suffering from necrotizing pneumonia due to pvl-positive *S. aureus* [8]. In a number of pathologies, PVL is associated with severe tissue necrosis that may result in poor antibiotic diffusion and suboptimal concentrations at site of infection. It was established that subinhibitory concentrations of antibiotic modify the expression of PVL in several CA-MRSA strains. Subinhibitory concentrations of oxacillin increase PVL production as previously observed for

alpha-hemolysin [16,43]. In contrast, subinhibitory concentrations of clindamycin, linezolid, and fusidic acid significantly reduce PVL synthesis [16]. This demonstrates the importance of good diagnostic and fast identification of CA-MRSA to prevent the use of antibiotic that could increase the severity of infection.

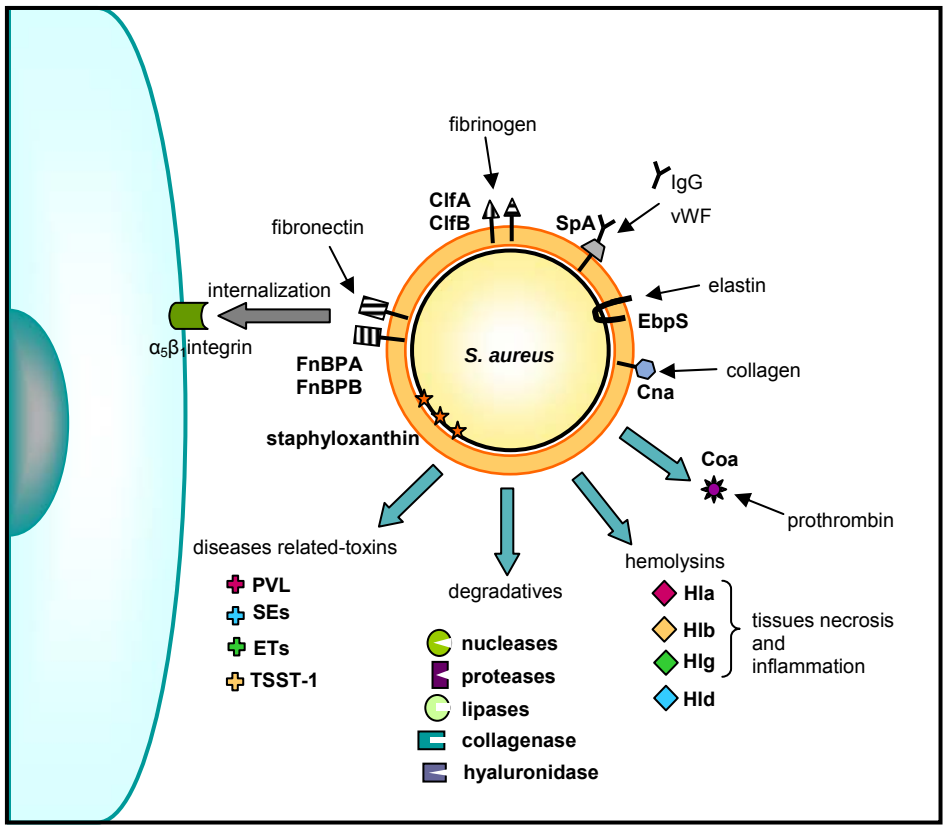

Figure 2 : Schematic representation of *S. aureus* virulence factors. Disease-related toxins: Panton-Valentine leucocidin (PVL), exfoliative toxins (SEs), enterotoxins (ETs) and toxic shock syndrome toxin (TSST-1). Adhesion and invasion-related virulence factors: Fibronectin-binding proteins (FnBPA and FnBPB), Clumping factors (ClfA and ClfB), protein A (SpA), elastin-binding protein (EbpS) collagen-binding protein (Cna), staphyloxanthin, coagulase (Coa), alpha-hemolysin (hla), beta-hemolysin (Hlb), gamma-hemolysin (Hlg), delta-hemolysin (Hld), and degradatives enzymes like nuleases, proteases, lipases, collagenase and hyaluronidase.

ADHESION, COLONIZATION AND PERISTENCE

Yet, severe infections such as chronic endocarditis or osteomyelitis are frequently caused by *S. aureus* strains that do not produce any of these specific staphylococcal exotoxins (TSST-1, enterotoxins, exfoliative toxins or PVL). This clearly indicates that *S. aureus* possesses other virulence factors that promote life-threatening infections in particular condition and/or in certain population. These potential virulence factors include surface adhesins and secreted enzymes and toxins that belong to the core set of staphylococcal proteins and are expressed by virtually all strains of *S. aureus* **[Fig. 2]**. Several studies were undertaken to get a better understanding of their implications *in vivo*.

S. aureus can bind to host extracellular matrix components such as fibrinogen, fibronectin, and collagen thanks to its surface protein adhesins that are collectively termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). So far, at least seven surface proteins have been characterized. The fibronectin-binding proteins A and B, the fibrinogen-binding proteins or clumping factors A and B, the collagen-binding protein (Cna), the elastin-binding protein (EbpS) and the staphylococcal protein A (SpA) that binds to the von Willebrand Factor [22].

S. aureus produces two cell wall-anchored fibronectin-binding proteins (FnBPs), FnBPA and FnBPB. Fibronectin-binding proteins are known to mediate

S. aureus internalization into several non-phagocytic cell types *in vitro*. They include alveolar epithelial cells [36], mammary gland epithelial cells [6,10], fibroblasts [18], keratinocytes [29,37] and endothelial cells [39]. The host extracellular fibronectin attaches to the FnBPs on the surface of the bacterium. The fibronectin-coated bacteria bind to the $\alpha_5\beta_1$–integrin on the surface of the host cell, which elicits the integrin-mediated internalization of the pathogen [1,18]. Most clinical strains seem to contain at least one FnBP gene [1,2] but their precise role in staphylococcal infections remains uncertain. *In vivo,* several studies indicate that FnBPs may contribute to the virulence of certain pathologies and have no implication or even may even decrease the extent of virulence in other infections. On one hand, it was demonstrated that FnBPs take part to the development of murine mastitis [10] and that FnBPA promotes *S. aureus* persistence and propagation to adjacent endothelium during endocaditis [54]. On the other hand, FnBPs are not involved in the early development of murine septic arthritis but could play an important role in the induction of systemic inflammation [44]. Using a rat model of pneumonia, it was suggested that expression of FnBPs promote *S. aureus* elimination from the lungs [36] **[Tabl. 2]**.

S. aureus produces two clumping factors named ClfA and ClfB. ClfA is a major fibrinogen-binding protein that contributes to the platelets binding by *S. aureus* [59]. ClfA is known to be involved in the early setting of infection and probably cooperates with other factors. In a rat model of endocarditis, it was demonstrated *in vitro* and *in vivo* that clumping factor-defective mutants produce statistically less endocarditis than the parental strain [40]. ClfA promotes adhesion to damaged

heart valves but is not sufficient to induce persistence while the fibronectin-binding protein A (FnBPA) seems to allow persistent colonization and propagation to adjacent endothelium [54]. This can be related to the capacity of FnBPA to induce *S. aureus* internalization in endothelial cells. Once inside the cell, the pathogen is protected from host defense mechanism and is able to multiply and invade adjacent cells. In prosthetic device-related infection, it was showed that ClfA, FnBPA and FnBPB are independently sufficient to establish early infections [3]. Clumping factor B (ClfB) appears to have different specificities. ClfB seems to play a minor role in adherence in the prosthetic device-related infection [3]. But ClfB promotes nasal colonization while ClfA, FnBPA and FnBPB were apparently not involved in nasal colonization [58]. In addition, ClfA and ClfB also contribute to the development of murine septic arthritis [44,46].

The staphylococcal collagen-binding protein (Cna) is a cell wall-attached protein that carries a collagen-binding site on its N-terminal domain. *In vivo*, it was demonstrated that Cna facilitates early colonization of the joints in septic arthritis [49,64] and contributes to the pathogenesis of osteomyelitis [17], infective endocarditis [25] and keratitis [55].

Elastin is a hydrophobic protein conferring flexibility and elasticity to tissues like aorta, lung, heart valve, and skin. Staphylococcal infections frequently affect elastin-rich tissues and it was suggested that the membrane-associated elastin-binding protein of *S. aureus* (EbpS) might take part to the colonization of

these tissues [56]. EbpS possesses two transmembrane domains and its N -terminus and C-terminus are both located on the outer face of the cytoplasmic membrane. *In vitro* EbpS seems to promote binding of soluble elastin and its precursor tropoelastin but the significance *in vivo* of this adhesin remains uncertain [15,56].

The staphylococcal protein A (SpA) is a cell wall-anchored protein that has the ability to bind to the Fc region of immunoglobulins. SpA possesses five extracellular domains; and each of them can bind one IgG molecule through its Fcγ binding sites. This Fc-binding function impedes phagocytosis and is believed to contribute to bacterial virulence during staphylococcal infections [22]. Besides, SpA also activates complement and acts as a superantigen for B cells [22,47]. Using *in vivo* animal models, it was demonstrated that protein A takes part to the virulence during septic arthritis and in subcutaneous abscesses [45,48]. In addition, it was shown that SpA is the von Willebrand factor binding protein of *S. aureus*. Von Willebrand factor (vWF) is a large glycoprotein that mediates platelet adhesion at sites of endothelial damage. This suggests that SpA could be involved in the development of endovascular infections such as endocarditis and vascular or heart valve prosthetic infections which are frequent and severe complications of invasive staphylococcal diseases [22].

S. aureus produces a yellow-orange pigment called staphyloxanthin. This triterpenoid carotenoid is located in the cell membrane but is not an adhesin and does not participate to the early step of adhesion, like the cell wall-attached proteins. Yet recent findings indicate that this pigment may take part to the

virulence of *S. aureus*. Like other carotenoids, staphyloxanthin has antioxidant properties that confer resistance to oxidative stress and neutrophil killing. *In vivo*, mutants lacking this pigment are rapidly killed by neutrophils and are unable to induce skin abscess or systemic infection [11,34]. This suggest that staphyloxanthin may facilitate intracellular survival of the pathogen and subsequent relapse or spreading of the infection.

In addition to the membrane protein staphyloxanthin and all the cell wall-attached adhesins, *S. aureus* also secretes several enzyme and toxins like hemolysins (alpha, beta, gamma and delta), coagulase, nucleases, proteases, lipases, hyaluronidase and collagenase that could contribute to the virulence of infections. Nucleases, proteases, lipases, hyaluronidase and collagenase probably participate to the spreading of the pathogen within the host and the degradation of cells and tissues, for both nutrition and protection.

Staphylocoagulase (Coa) is an extracellular protein that has traditionally been used to differentiate *S. aureus* from the less-virulent staphylococci (coagulase-negative staphylococci). The secreted coagulase binds with the host prothrombin leading to the formation of a complex called staphylothrombin. This complex stimulates plasma clotting by converting fibrinogen to fibrin [57]. In a mouse model of blood-borne pneumonia, *S. aureus* coagulase proved to be an important virulence factor in the later stage of infection [57]. It also seems to contribute to the virulence of mastitis [27] but is not involved in endocarditis [4,40].

Among all the secreted proteins produced by *S. aureus* the hemolysins seem to play important roles in the virulence of this pathogen. The four hemolysins (alpha, beta, gamma and delta) are produced by nearly all strains of *S. aureus*. They attack target cells by disrupting their permeability barrier, either through pore formation, by detergent action or via sphingomyelinase activity. Alpha-hemolysin (Hla) is a pore-forming toxin active on a wide range of mammalian cells including erythrocytes, platelets, monocytes, T lymphocytes and fibroblasts [26,38]. Alpha-hemolysin is secreted as monomeric subunits. The binding of monomers to the membrane of the target cell, followed by the oligomerization of these monomers, leads to the formation of a hexa- or heptameric pore [38]. *In vivo*, alpha-toxin appears to be a key virulence factor in animal models of subcutaneous abscess [48,61], intraperitoneal infection [48], mastitis [27], brain abscess and corneal infections [13,28,42]. Besides, it was demonstrated that the concerted action of alpha- and gamma-hemolysins contribute to higher virulence during septic arthritis [41].

Beta-hemolysin (Hlb) is a magnesium-dependent sphingomyelinase C that induces lysis of sheep erythrocytes and human monocytes [63]. The lytic action of beta-hemolysin depends on the sphingomyelin content of cell membrane. A small number of *in vivo* studies brought information concerning the role of beta-hemolysin in infections. Beta-hemolysin induces subcutaneous lesions in mice [61], contribute to tissue necrosis during experimental mastitis [9] and participate, in conjunction with alpha-hemolysin, to severe tissue damage in corneal infection [13,28,42].

The gamma-hemolysin (Hlg) is a pore-forming toxin that belongs to the group of bi-component leucotoxins of *S. aureus* like the Panton-valentine leucocidin. These bi-component leucotoxins are formed by the association of two distinct protein elements called S and F (for slow- and fast-eluting protein). The gamma-hemolysin locus encodes three proteins HlgA (S protein), HlgB (F protein) and HlgC (S protein) that generate two toxins: HlgA + HlgB and HlgC + HlgB [38]. The primary targets are the polymorphonuclear cells, monocytes and macrophages but gamma-hemolysin can also lyses erythrocytes [38]. The contribution of gamma-hemolysin during infection is not very clear. Nonetheless, it seems to be involved, in concert with alpha-hemolysin, in the development of septic arthritis in mice and it could contribute to the severity of infection in endophtalmitis and corneal infections [13,41,60].

The delta-hemolysin (Hld) is a small protein that disturbs cell membranes due to its surfactant properties [52]. This toxin is capable of lysing erythrocytes and other mammalian cells, as well as subcellular structures such as membrane-bound organelles [14]. Moreover, it has been suggested that delta-hemolysin may contribute to the detachment of cell from both *S. aureus* and *S. epidermis* biofilms [66]. It is known that Staphylococci frequently form biofilm in infections such as osteomyelitis or endocarditis. Biofilm-associated infections are often difficult to treat and the cells released from the biofilm may spread and colonize new sites, which could contribute to the recurrent character of these infections.

Virulence factors	Implication *in vivo*	Animal model	Ref.
Fibronecting-binding proteins (without distinction between A or B)	- Promotes internalization in mammary epithelial cells in mastitis	mouse	10
	- Promote *S. aureus* elimination from the lungs in pneumonia	rat	36
	- Contribute to the induction of systemic inflammation in septic arthritis	mouse	44
Fibronecting-binding protein A (FnBPA)	- Involved in persistent adhesion and propagation in endocarditis	rat	54
	- Promotes adhesion in prosthetic device-related infection	mouse	3
Fibronecting-binding protein B (FnBPB)	- Promotes adhesion in prosthetic device-related infection	mouse	3
Clumping factor A (ClfA)	- Promotes early adhesion in endocarditis	rat	40,54
	- Promotes adhesion in prosthetic device-related infection	mouse	3
	- Contributes to the virulence of septic arthritis	mouse	44,46
Clumping factor B (ClfB)	- Promotes nasal colonization	mouse	58
	- Contributes to the virulence of septic arthritis	mouse	44
Collagen-binding protein (Cna)	- Facilitates early colonization of the joints in septic arthritis	mouse	49,64
	- Contributes to the virulence of :		
	osteomyelitis	mouse	17
	infective endocarditis	rat	25
	keratitis	rabbit	55
Elastin-binding protein (EbpS)	- Unknown function *in vivo*		
Protein A (SpA)	- Contributes to the virulence of subcutaneous lesions but to a lesser extend than alpha-hemolysin	rabbit	48
	- Contributes to the virulence of septic arthritis	mouse	45

Staphyloxanthin	- Contributes to the development of systemic infection - Contributes to the virulence of skin abscess - Not involved in nasal colonisation	mouse	11,34
Coagulase (Coa)	- Involved in the later stages of blood-borne pneumonia	mouse	57
	- Contributes to the virulence in mastitis	mouse	27
	- Seems not involved in the virulence in endocarditis	rat	4,40
Alpha-hemolysin (Hla)	- Major virulence factor of subcutaneous lesions	mouse	48,61
	- Contributes to the severity and high morbidity in intraperitoneal infection	mouse	48
	- Involved in severe tissue damage during corneal infection	rabbit	13,42
	- Concerted action with gamma-hemolysin contributes to virulence in septic arthritis	mouse	41
	- Contributes to the virulence of infection in mastitis	mouse	9,27
	- Major virulence determinant in brain abscess	mouse	28
Beta-hemolysin (Hlb)	- Involved in tissue damage during corneal infection but less than Hla	rabbit	13,42
	- Contributes to the virulence of subcutaneous lesions	mouse	61
	- Contributes to the virulence in mastitis	mouse	9
Gamma-hemolysin (Hlg)	- Concerted action with alpha-hemolysin contributes to virulence during septic arthritis	mouse	41
	- May contribute to the virulence in endophtalmitis and corneal infection	rabbit	9,13,60
Delta-hemolysin (Hld)	- Could be involved in the detachment of cell from *S. aureus* and *S. epidermis* biofilms	/	66

Table 2 : Implication of *S. aureus* adhesion and invasion-related virulence factors *in vivo*.

INTRACELLULAR *S. AUREUS*

Staphylococcal infections are often difficult to eradicate and present a high frequency of relapses. Several elements can contribute to this recurrent character such as biofilms, nasal carriage and intracellular persistence. The production of bioflim, frequently observed during device-related infections, protects the bacterial colonies from the host immune system and antibiotics action. Persistent or intermittent nasal carriage of *S. aureus* also constitutes a reservoir for chronic re-infection of patients. Finally it is believed that the ability of *S. aureus* to survive inside cells also contributes to the recurrent character of several staphylococcal infections. The following paragraphs will focus on this intracellular residency and its association with some chronic pathologies.

It has been demonstrated that intracellular *S. aureus* are frequently associated with recurrent rhinosinusitis, and intracellular bacteria were recovered up to twelve months after the first identification [12,51]. And it is believed that this intracellular survival also participates to the relapses frequently observed in osteomyelitis and endocarditis [19]. These infections are often difficult to eradicate even after prolonged and adapted treatment, suggesting that the intracellular residency protects *S. aureus*, at least partially, from host defense mechanisms and antibiotic action.

It is now recognized that *S. aureus* can enter and survive in diverse non-professional phagocytic cell types including keratinocytes [29] , epithelial cells

[6,10,53], endothelial cells [35,39], fibroblasts [18] and enterocytes [24]. But *S. aureus* can also survive inside professional phagocytes [21,23]. It was showed that mouse polymorphonuclears (PMNs) isolated from the site of infection contain viable intracellular *S. aureus* and that these infected PMNs are sufficient to establish infection in a naïve animal [21]. Besides, a recent study demonstrated that *S. aureus* persisted up to five days inside human macrophages [30]. During infection of human lung epithelial cells *S. aureus* could persist intracellularly for up to two weeks [19]. To penetrate inside a cell, *S. aureus* seems to rely on its MSCRAMMs, in particular the fibronectin-binding proteins as reported earlier in this paper. Following internalization in epithelial cells, it was observed that *S. aureus* rapidly modulates its gene expression to promote adaptation and survival in this new environment. Genes involved in major metabolic pathways including cell division were significantly down-regulated whereas genes encoding transporters were up-regulated to allow maintenance of vital functions but limiting the pathogen multiplication [19]. This fine regulation of bacterial growth and limited expression of virulence factors ensures prolonged bacterial persistence inside cells. Upon internalization, the bacteria not only adapt their expression profile but they also induce modifications in the host cell gene expression. In endothelial cells, it was shown that *S. aureus* internalization triggers the up-regulation of many proteins involved in cell signaling and metabolism but also adhesion proteins, cytokines, and proteins contributing to antigen presentation [35].

Finally, the intracellular persistence may lead to the emergence of antibiotic resistant clones. It was shown that the intracellular activity of many antibiotics is much lower than their extracellular activity [5]. This reduced activity in the intracellular environment can result from multiple factors (poor cellular accumulation, protein binding, lower affinity in the intracellular medium, …). Yet, as a consequence, intracellular bacteria may be exposed to subhinibitory concentrations of drug and could be more prone to develop resistance mechanisms.

CONCLUSION

This paper is an attempt to summarize the information available on the role of specific *S. aureus* virulence factors in the development of infections. *S. aureus* is a remarkably versatile pathogen that produces a large amount of well-established and potential virulence factors that can be classified in two categories; the specific diseases-related exotoxins comprising TSST-1, enterotoxins, exfoliative toxins and PVL and the adhesion and invasion-related virulence factors including multiple surface proteins and secreted proteins. To investigate the potential role of virulence factors during infection, most authors compared the virulence of a mutant deleted for a specific virulence factor to its wild type parental strain. The cell wall-attached proteins contribute to the first steps of invasion *in vivo*. Among them, the two fibronectin-binding proteins and the clumping factor A appears to be the major adhesins in various infection models

and present overlapping functions. Even though these three MSCRAMMs seem to be independently sufficient to promote bacterial adhesion [3], they most probably cooperate in concert with the protein A, coagulase, collagen-binding protein and elastin-binding protein to initiate infection *in vivo*. This clearly indicates that *S. aureus* has developed multiple strategies to colonize the various environments it can encounter. In contrast, the secreted proteins most likely participate later in the infection and probably contribute to the spreading of the pathogen within the host and the degradation of cells and tissues, for both nutrition and protection.

In the overall, all these virulence factors enable *S. aureus* to survive inside the host and launch the infection. Therefore, any substances that could inhibit or reduce their production would be of great interest for the treatment of staphylococcal infections. In addition, it has been demonstrated that some antibiotics that are currently used in the treatment of staphylococcal infections modulate the expression of virulence factors. Linezolid impairs the expression of coagulase, alpha- and delta-hemolysin [20]. Subinhibitory concentrations of aminoglycosides and macrolides reduce the expression of alpha-hemolysin while β-lactams and fluoroquinolones enhance this expression [43]. Subinhibitory concentrations of oxacillin increase PVL synthesis while clindamycin, linezolid, and fusidic acid significantly reduce its production [16]. These findings suggest that some antibiotics may in fact amplify the virulence of some strains of *S. aureus* and that the choice of treatment must be carefully considered.

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6. Regulation of virulence

The pathogenesis of *S. aureus* infections is an intricate mechanism that requires the synchronized expression of multiple genes. *S. aureus* possesses numerous regulatory systems that interact in a complex and highly regulated manner to control the expression of virulence factors in response to cell density, energy supplies and environmental signals. There are two major types of regulators in *S. aureus*, the two-component systems, represented by agr and sae, that are composed of a membrane sensor and an intracellular signal transducer and the DNA binding-proteins illustrated by sarA and sigmaB **[96]**.

Agr

The accessory gene regulator (agr) is a quorum-sensing system that responds to cell density and regulates the expression of many exoproteins genes, among which hemolysins, toxic shock syndrome toxin, lipase or protease but also membrane proteins. During post-exponential growth phase, agr increases the production of toxins like hemolysins whereas the production of cell wall-associated proteins such as fibronectin-binding proteins and protein A is decreased **[96]**.

The agr system is an approximately 3kb locus made of two divergent transcription units under the control of promoters P2 and P3. The P2 operon is

composed of four genes (*agrBDCA*) and acts as a sensor of population density. The P3 promoter controls the expression of RNAIII, the intracellular effector of gene regulation. The *agrD* gene encodes the precursor of the autoinducing peptide (AIP). This pro-AIP is processed and secreted by the membrane protein AgrB. The mature AIP can bind the N-terminal transmembrane domain of the membrane sensor AgrC that in turn activates the intracellular response regulator AgrA. Activated AgrA upregulates the transcription from promoters P2 and P3 that, consequently, intensify the response and initiate the production of the RNAIII effector **[Fig. 3] [57]**. Therefore, the direct outcome of agr autoinduction is the increase of RNAIII quantity, which is the intracellular effector of the agr regulon. RNAIII has a long half-life, and a complex secondary structure, which is well conserved among a number of Staphylococcal species **[57]**. RNAIII activates the transcription of several extracellular protein genes and represses that of many surface protein genes.

Considering this cell-density dependent activation of agr, it has generally been proposed that agr play a major role in infections. Initially, the bacteria, present in small numbers, express their cell wall-associated proteins (FnBPs, Clfs, SpA, …) allowing the adhesion and colonization of the host tissue. The pathogens start to multiply and once they reach a sufficient cell density, agr activation induces the repression of cell wall-associated proteins and activates the expression of secreted toxins and enzymes. It has been confirmed that agr is activated during certain infections. In a mouse arthritis model, agr mutants are less virulent than a wild type *S. aureus* **[1]**. Nevertheless, not all *S. aureus* toxins

are under the control of agr. It has been recognized that among the enterotoxins, two of them are not regulated by agr (enterotoxins A and K) while other (enterotoxins B, C, and D) are partially regulated by agr and are also controlled by one or more additional regulatory system **[96]**.

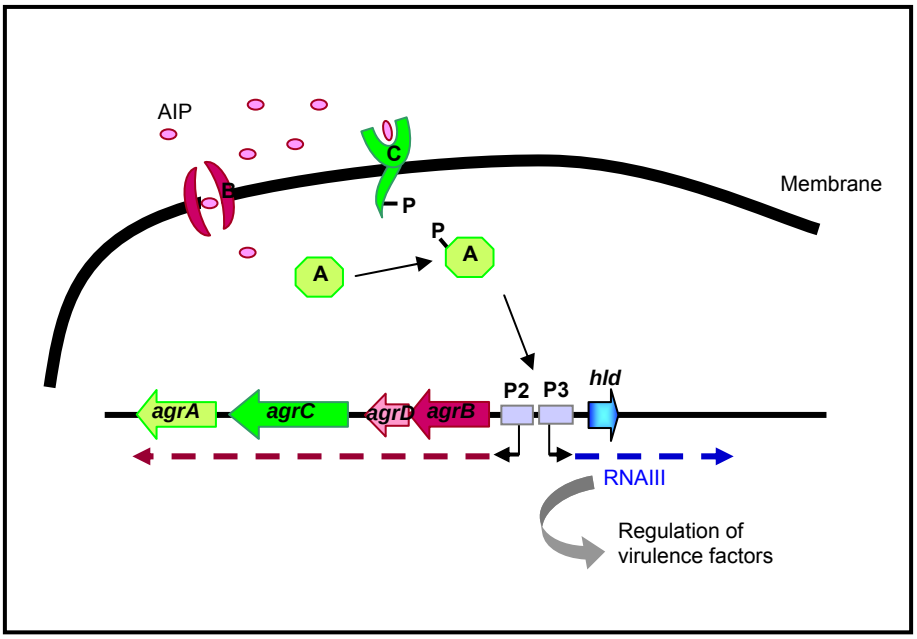

Figure 3 : Schematic illustration of quorum sensing system agr. When bacterial density is low, the AIP peptide, encoded by the gene *agrD*, is produced in small amount. AIP is processed and secreted by AgrB. The multiplication of bacteria increases the extracellular concentration of AIP and enhances the probability that AIP attached to the AgrC receptor. The binding of AIP to AgrC induces the autophosporylation (or dephosphorylation) of this receptor that will in turn provoke the phosphorylation of AgrA. AgrA activates the transcription from promoters P2 and P3 and induces the synthesis of RNAIII that regulates the transcription of numerous virulence factors.

A few studies have addressed the role of agr in the development of intracellular infections. Upon internalization, bacteria are surrounded by an endosomal membrane, the presence of which may allow AIP to rapidly accumulate and trigger the expression of RNAIII. Agr activation will then up-regulate the production of exoproteins, such as hemolysins and degradative enzymes, which could facilitate bacterial escape from the endosome. In epithelial cells, it was established that RNAIII expression increases rapidly and reaches a peak at 2h after internalization. This rise of RNAIII concentration correlated with the escape of *S. aureus* from the endosomes **[69,77]**. This indicates that some virulence factors controlled by agr play a role in virulence *in vivo* and are involved in intracellular survival of *S. aureus*.

Sae

The staphylococcal accessory protein effector (sae) is a two-component system known to activate the expression of nuclease, coagulase, alpha-, beta- and gamma-hemolysin but also cell wall-associated proteins like fibronectin-binding proteins and protein A **[13,95]**. Sae probably responds to environmental factors such as high salt concentration, low pH and glucose **[57,96]**. Sae seems to be the principal activator of alpha-hemolysin expression during experimental endocarditis **[93]**. In addition, *in vitro* experiments demonstrated that sae induces *S. aureus* hemolytic activity in presence of subinhibitory concentrations of β-lactam **[44]**.

SarA

The staphylococcal accessory gene regulator (sarA) and its homologues belong to the second type of *S. aureus* regulatory systems, the DNA-binding proteins. SarA regulates the expression of numerous virulence factors, and affects the expression of the agr system. *In vitro*, sarA increases the expression of hemolysins and fibronectin-binding proteins but represses the expression of protein A **[13,27,57]**.

The *sarA* locus is made of three overlapping transcripts initiated from three promoters, P1, P2 and P3. Cheung *et al.* **[20]** demonstrated that these three promoters are expressed differentially *in vivo* and *in vitro*. *In vitro*, P1 is stronger than P2 and P3. Besides, activation of these promoters varies with the growth cycle stage. The promoters P1 and P2 are expressed during the exponential phase while P3 is mainly expressed during the postexponential phase **[20]**. *In vivo*, using a rabbit endocarditis model, it was shown that P1 is activated both in the center and on the surface of the vegetations. P2 promoter became highly expressed on the surface of the vegetation but not in the center of the lesion and the P3 promoter appeared to be silent. This study demonstrated that the activation of *sarA* promoters differs *in vitro* and *in vivo* **[20]**. Furthermore, the three promoters seem to be differentially expressed in function of the pathogen location in the infected areas. This indicates that *S. aureus* has the ability to modulate its response to adapt to distinct host microenvironments.

SigmaB

SigmaB is an alternative transcription factor homologous of *Bacillus subtilis* sigmaB. In *S. aureus*, sigmaB is activated by energy depletion and environmental stimuli such as ethanol, salicylic acid, heat shock and salt stress **[57,73]**. Its activity is regulated by a multi-factorial post-translational process involving rsbU, rsbV and rsbW **[Fig. 4]**. Based on the known functions of the rsbU, rsbV, and rsbW homologues from *B. subtilis*, it was proposed that rsbW acts as an anti-sigma factor and binds to sigmaB through protein-protein interactions. RsbW from *S. aureus* can form mutually exclusive complexes with either sigmaB or its antagonist, rsbV. In normal conditions, rsbV is phosphorylated. This inactive form (rsbV-P) is unable to complex with rsbW, leaving the latter free to interact with sigmaB. When bound to rsbW, sigmaB is unable to aggregate with the RNA polymerase core enzyme to form an active holoenzyme. The phosphatase rsbU is the positive activator of sigmaB. Upon stress, rsbU removes a phosphate group from rsbV-P and thus reactivates rsbV. Unphosphorylated rsbV forms a highly specific complex with rsbW, thereby releasing sigmaB which is free to form an active complexe with the RNA polymerase holoenzyme **[9,73]**.

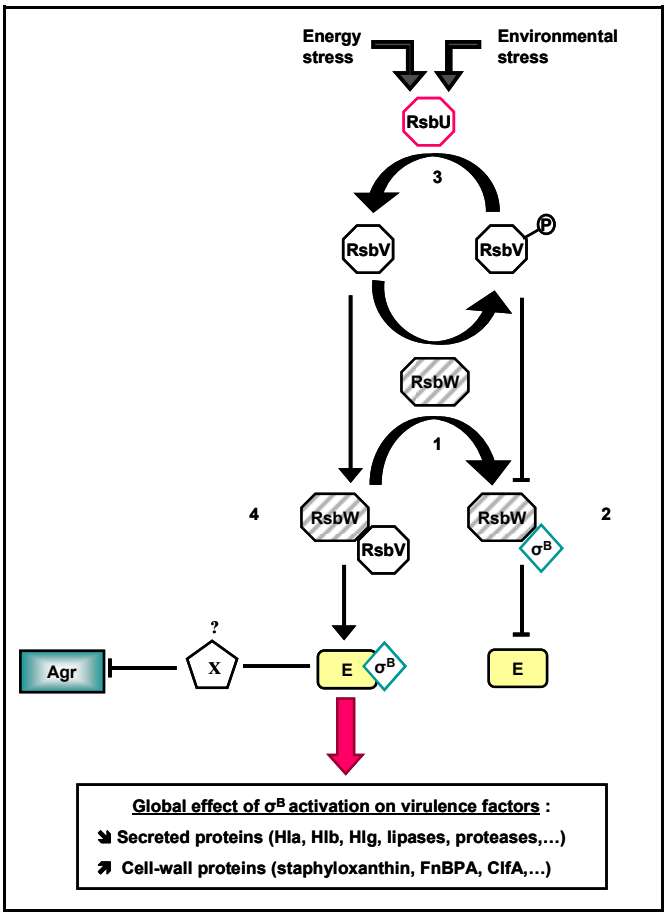

Figure 4 : Adapted from **[9,76]** Proposed model for the regulation of sigmaB in *S. aureus*. RsbW can form mutually exclusive complexes with either sigmaB or its antagonist, rsbV (step 1). RsbV is normally inactive (rsbV-P) due to phosphorylation by rsbW and is thus unable to complex with rsbW, leaving the latter free to interact with sigmaB (step 2). When bound to rsbW, sigmaB is unable to aggregate with the RNA polymerase core enzyme (E) to form an active holoenzyme (E-σ^B). Upon stress, the rsbV-P-specific phosphatase activity of rsbU, a positive activator of sigmaB, becomes activated and thus reactivates rsbV (step 3). Unphosphorylated rsbV forms a complex with rsbW (step 4), thereby releasing sigmaB. RsbW, if complexed with rsbV, is unable to bind to sigmaB, leaving the latter free to form an active sigmaB-holoenzyme (E-σ^B). SigmaB activation leads to the down-regulation of several secreted proteins and up-regulation of cell-wall proteins either directly or indirectly by the repression of agr. This repression of agr is believed to rely on an unidentified mediator.

A microarray based study demonstrated that 251 open reading frames (ORFs) were influenced by sigmaB activity **[10]**. Most of the genes upregulated by sigmaB were preceded by a nucleotide sequence sharing homologies with the sigmaB consensus promoter sequence of *B. subtilis*. The controlled genes are presumably involved in general metabolic processes, cell wall synthesis, or signaling pathways , but also in virulence expression. In this respect, sigmaB appears to act conversely to agr, by up regulating the expression of many adhesins and repressing the transcription of exoproteins and toxins **[Fig. 4, Tabl. 3]**. Among other, SigmaB modulates the expression of hemolysins, clumping factors, elastin-binding protein, coagulase, fibronectin-binding proteins, protein A, lipases and proteases but also global regulators of virulence including SarA, arl and agr **[10]**. Besides, SigmaB was shown to affect pigmentation and biofilm production.

Among the genes involved in metabolic processes and regulated by sigmaB some of them could contribute to the response to oxidative stress like the genes *katA* and *sodM* encoding respectively a catalase and a superoxide dismutase, or *crtN* and *crtM* encoding two enzymes necessary for the synthesis of staphyloxanthin pigment **[10,64]**.

Virulence determinant	Gene	Result for:	
		agr	**σ^B**
Aureolysin	***aur***	+	−
Capsular polysaccharide synthesis enzyme 5J	*cap5J*	+	+
Clumping factor B	*clfB*	+	⊘
Coagulase	***coa***	−	+
Cystein protease	***sspC***	+	−
Enterotoxin A	*sea*	+	Unknown
Enterotoxin B	***seb***	+	−
Exotoxin 2	*set8*	+	Unknown
Factor effecting methicillin resistance B	*femB*	+	⊘
Fibronectin-binding protein A	***fnbA***	−	+
Fibronectin-binding protein B	*fnbB*	−	⊘
Glycerol ester hydrolyase	***geh***	+	−
α-Hemolysin	***hla***	+	−
β-Hemolysin	***hlb***	+	−
γ-Hemolysin	***hlgBC***	+	−
δ-Hemolysin	*hld*	+	⊘
Hyaluronate lyase	*hysA*	+	⊘
Lipase	***lip***	+	−
LrgAB (holin-like proteins)	***lrgAB***	+	−
Myosin-cross-reactive antigen	**N315-SA0102**	−	+
Phosphatidylinositol-specific phospolipase C	***plc***	+	−
Protein A	*spa*	−	⊘
Secretory antigen A	***ssaA***	−	+
Serine protease A, B, D, and F	***splA***,***B***,***D***,***F***	+	−
Staphylokinase	***spc***	+	−
Toxic shock syndrome toxin 1	*tst*	+	Unknown
V8 protease	***sspA***	+	−

Table 3 : [10] Influence of sigmaB on virulence determinants regulated by the agr locus. Genes that are regulated conversely by *agr* and sigmaB are shown in boldface type. Influence of *agr* and sigmaB on transcription of the respective gene. ⊘, not influenced; +, increased; −, decreased. Based on transcript levels detected in strains COL and IK183.

Other regulatory systems

Agr, sae, sarA and sigmaB are four important regulatory systems of *S. aureus* but numerous other regulators have been identified. The whole genome sequence of *S. aureus* strains N315 and Mu50 reveals the presence of sixteen pairs of putative two-component systems **[44].** The autolysis-related locus (arl) and the staphylococcal respiratory response (srrAB) belong to the two-component systems. Arl represses the expression of hemolysins and exoenzymes and appears to regulate autolytic activity and control the multidrug efflux pump NorA. **[27,96].** SrrAB is activated in conditions of oxygen depletion. SrrAB regulates the expression of genes involved in energy metabolism and interacts directly with the agr system since it inhibits RNAIII expression. So srrAB probably participates to the connection between the bacterial energy metabolism and the quorum-sensing response **[57,96]**. Other transcription regulators include the rot repressor that seems to counteract agr activity **[96]**.

These numerous regulatory systems appear to contribute to the global regulatory network of *S. aureus*. They can not only affect directly the expression of virulence factors but also modulate the expression of the other regulators. Sae and agr up-regulate each other, srrAB and agr down-regulate each other and arl and agr have opposite effect on each other, with agr activating arl and arl repressing agr **[57]**. Rot is believed to be repressed by agr **[13]**. And SarA is reported to activate agr, while in contrast sigmaB represses it **[11] [Fig. 5]**. This large number of regulatory systems, their complex interactions and numerous

feedbacks ensure an optimized expression of all the accessory genes and contribute to the ability of *S. aureus* to survive in a wide range of environments and to induce infection. They act in a time- and population density-dependent manner and integrate various environmental signals (pH, O_2, heat shock, nutrient availability, …) to control internal metabolic processes and the production of particular subsets of accessory/virulence factors at the time and in quantities that are appropriate to the needs of the organism in any given situation **[57]**.

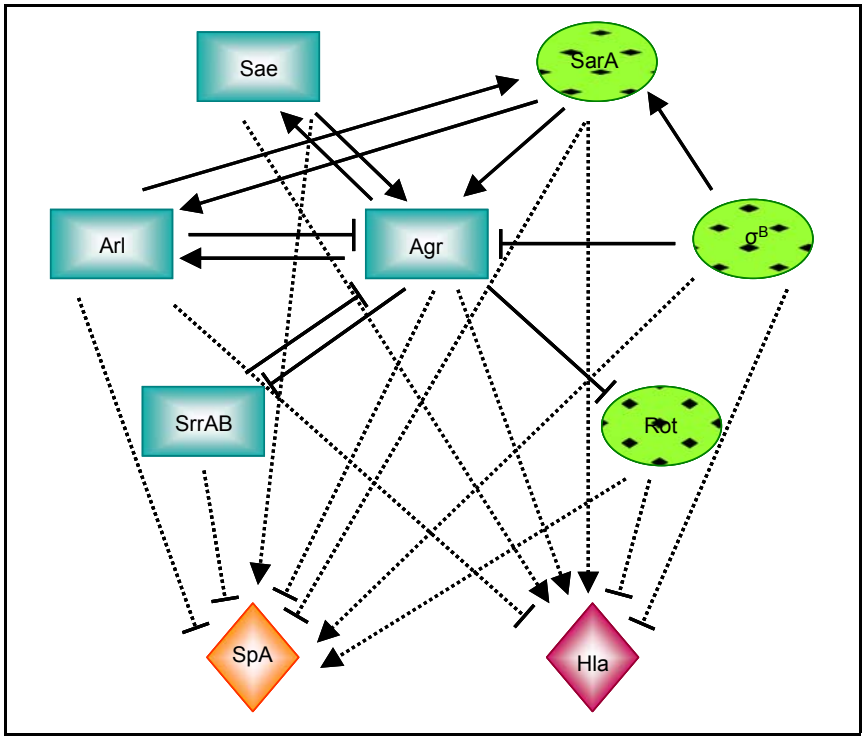

Figure 5 : Adapted from **[11,13,27,57]**. Illustration of the interactions between global regulators and effect on the expression of two major virulence factors : the alpha hemolysin (Hla) and cell-wall protein A (SpA). The Two-component systems are represented by blue rectangles and the DNA-binding proteins with green ellipses. Plain lines describe the interactions between the different regulatory systems and dotted lines show the effect on Hla or SpA. Arrows and perpendicular bars indicate positive and negative regulation respectively.

Step by step, we are getting a clearer picture of *S. aureus* regulation of gene expression. Yet, the general knowledge on the intricate interactions between all these regulators remains fragmentary. In addition, the best part of the data

obtained on virulence factor expression and regulation are the result of *in vitro* studies, usually in broth culture. However, these observations can hardly be extrapolated to *in vivo* conditions. This type of studies does not take into account the most probable influence of the interactions with host tissues and host defense mechanism on the expression profile of the pathogen.

7. Mechanisms for intracellular survival

S. aureus is one among many other bacteria able to penetrate and survive inside eukaryotic cells. If, the intracellular fate of *S. aureus* remains largely unknown, some of the strategies developed by other intracellular pathogens have been well described. Three types of intracellular bacteria can be considered; the obligate, facultative and opportunistic intracellular organisms. Obligate intracellular pathogens such as *Coxiella brunetii*, *Chlamydia* spp. and *Rickettsia* spp. need to be inside a cell to replicate. The facultative intracellular pathogens are able to survive in the extracellular environment as well as inside the cells like *Brucella* spp., *Legionella pneumophila*, *Francisella tularensis*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Salmonella* spp. and *Shigella flexineri*. Finally, the opportunistic intracellular pathogens are bacteria that demonstrated an ability to survive inside host cells under certain circumstances but this intracellular survival is not an essential determinant in the life cycle of the bacteria. They include *Staphylococcus aureus*, streptococci, *Helicobacter pylori* and *Escherichia coli* **[84]**. In each case, pathogens proliferating or maintaining

themselves inside cells or simply taking transient refuge therein are shielded from humoral defenses and probably also from antibiotics action. This may therefore contribute to the chronic or recurrent character of many intracellular infections.

7.1. Internalization

The process of bacteria internalization by professional phagocytes such as neutrophils, macrophages, monocytes or dendritic cells is termed phagocytosis **[86]**. This mechanism relies on the interaction of bacterial surface elements with the phagocytes membrane receptors. The binding of bacteria to one or more receptor(s) induces a signaling cascade in the cytoplasm of phagocytes and lead to a massive polymerization of actin, rearrangement of cytoskeleton and formation of large pseudopodes that surround the bacteria and engulf them **[81]**. Receptors involved in the recognition of pathogens include complement receptors (CRs), the mannose receptor, Fc receptors and scavenger receptors **[54]**. Among other, it was established that *Mycobacterium tuberculosis* and *Salmonella typhi* are internalized by macrophages through recognition of complement receptor CR3 **[54]**.

In non-professional phagocytic cells, the invasive bacteria induce their own uptake and can be classified in two different groups on the basis of their entry mechanisms, the “zipper” and the “trigger” mechanisms **[86]**. The zipper

mechanism relies on the continuous interactions of bacterial surface proteins and cellular receptors. These interactions induce a signaling cascade leading to the activation of actin cytoskeleton and the production of small membrane extensions that zip around the bacterium and surround it. The closely apposed membrane extensions can stretches around the bacteria only so far as its surface has ligands to engage those receptors. The contacts between the cell receptors and the bacterial ligands guide the membrane extensions to form a thigh vacuole around the bacteria **[81,86] [Fig. 6]**. Pathogens inducing the zipper mechanism include *Listeria monocytogenes, Staphylococcus aureus*, or *Streptococcus pyogenes*. The internalization of *L. monocytogenes* is mediated through its interaction with the typrosine kinase Met receptor and the adhesion protein E-cadherin **[12,86]**. The fibronectin-binding proteins (FnBPs) of *S. aureus* bind to the host extracellular fibronectin which act as a bridge and recognize the $\alpha_5\beta_1$–integrin on the surface of the host cell **[2]**. A very similar process contributes to the internalization of *S. pyogenes* **[42]**.

In contrast, *Salmonella* and *Shigella* illustrate the second mechanism of entry, the trigger mechanism **[Fig. 6]**. These pathogens actively inject specific bacterial products into the cytoplasm of the cell using a type III secretory system. The injected products induce polymerization of actin, modification of cytoskeleton and formation of large pseudopodes very similar to the phagocytosis in professional phagocytes **[86]**. In contrast to the zipper mechanism that requires the constant receptor-ligand interactions to proceed, the trigger mechanism is an all-or-none response. The initial attachment of the bacteria to the cell is

sufficient to stimulate its internalization **[81,86,86]**. For exemple, *Shigella* proteins IpaC and VirA were shown to trigger actin polymerization **[86]**.

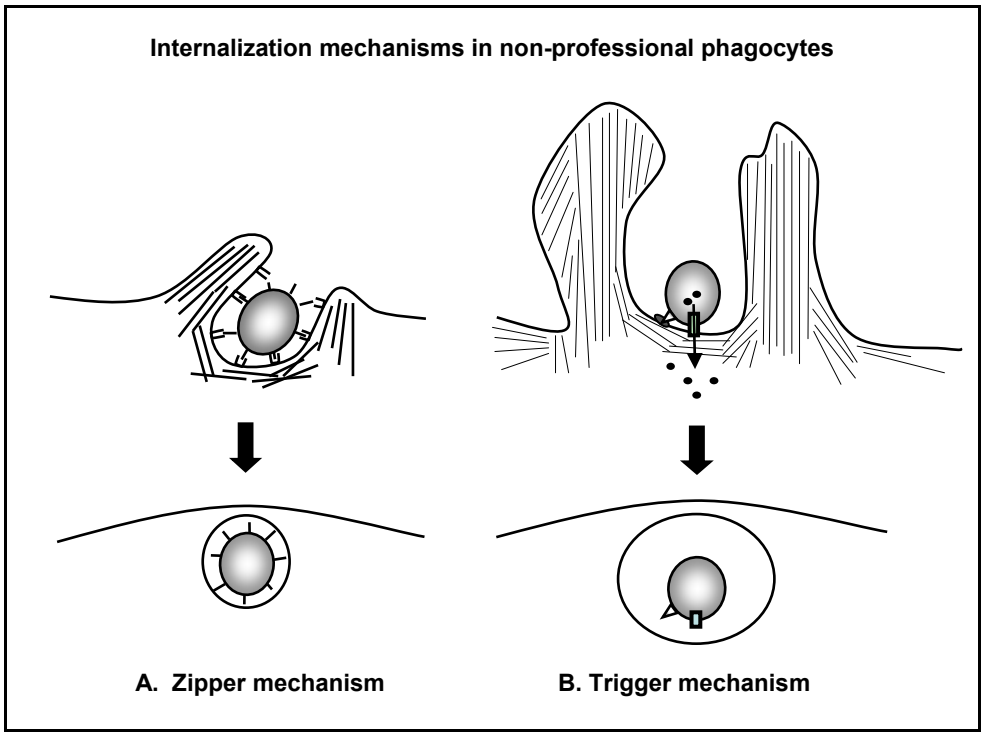

Figure 6 : Illustration of the internalization mechanisms in non-professional phagocytes. **A.** The zipper mechanism used, for example, by *Listeria*, *Staphylococcus* and *Streptococcus*. **B.** The trigger mechanism exploited by *Shigella* and *Salmonella*.

7.2. Intracellular location and protection against cellular defenses

Classically, when extracellular elements, such as bacteria, are internalized by a cell, they are transferred to the endocytic pathway to be degraded. The vacuole containing the internalized bacteria fuses with a lysosome containing hydrolytic enzymes. Inside this newly formed phagolysosomes, bacteria are exposed to acid pH and various proteases, nucleases and lipases contributing to bacterial degradation.

The mechanisms of intracellular survival are highly variable according to the pathogen considered but they all share a common purpose : the protection of bacteria against the cellular defenses and prevention of bacterial degradation by the host cells. Upon internalization, some bacteria such as *Listeria monocytogenes*, *Shigella flexineri* or *Rickettsia spp.* rapidly escape from the internalization vacuoles and replicate into the cytoplasm therefore avoiding the exposure to lysosomal enzymes and acid pH. *Listeria* escapes from its vacuole by secreting the pore forming toxin listeriolysin O and take advantage of the cellular actin to move within the cytoplasm **[12]**. *Shigella* requires at least three proteins to reach the cytoplasm; IpaB and IpaC probably create pores while IpaD is needed for regulation and efficient insertion of IpaB and IpaC into the membrane **[63,92]**. *Rickettsia* seems to rely on *tld* and *tlyC* genes encoding respectively a putative phospolipase D and a protein displaying hemolytic activity **[92]**.

Other bacteria including *Brucella* spp., *Salmonella* spp., *Francisella tularensis* and *Mycobacterium* spp. remain inside phagosomes but prevent the fusion with lysosomes **[15,22,54]**. This process probably involves several cellular and bacterial components that remain largely unidentified. In the case of *Mycobacterium tuberculosis,* one component of the bacterial cell wall, the ManLAM (*M. tuberculosis* PIP3 analog glycosylated phophatidylinositiol lipoarabinomannan) was reported to block phagosomes maturation **[54]**. The gram-negative *Legionella pneumophila* prevents the fusion with lysosome and alters the phagosome transport to induce the fusion of phagosome with the endoplasmic reticuculm where it replicates **[70]**. *Chlamydia* spp. replicate into specific modified-vacuoles that will not enter the common endocytic pathway **[31]**. Finally some bacteria such as *Coxiella brunetii* **[88],** *Staphylococcus aureus* and to some extend *Legionnella* pneumophila may simply resist the fusion with lysosomes and multiply within this acidic compartment **[15] [Fig. 7]**.

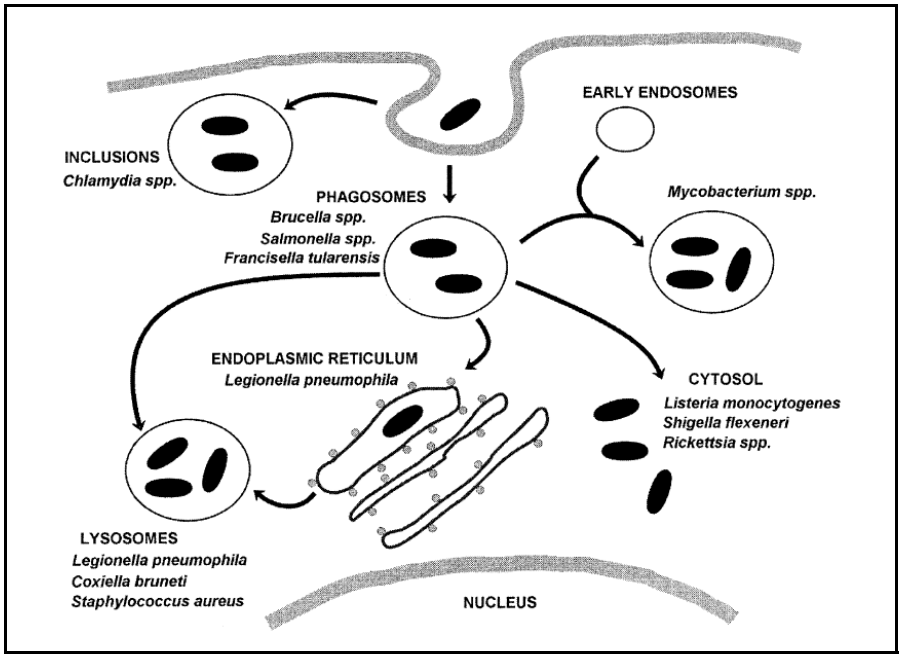

Figure 7 : [15]. Illustration of various strategies exploited by intracellular pathogens to evade cellular defense mechanisms and prevent bacterial destruction. Some bacteria (e.g. *Listeria*, *Shigella*, *Rickettsia*) escape from phagosomes rapidly after internalization and proliferate into the cytoplasm. Others remain in phagosomes that continue to fuse with newly formed endosomes but not with lysosomes (*Mycobacterium*); in phagosomes that are unable to fuse with other vacuoles (e.g. *Brucella*, *Salmonella*, *Francisella*); or in specialized vacuoles (*Chlamydia*). In some cases, the phagosomes pathway is modified and the phagosomes containing living bacteria fuse with the endoplasmic reticulum (*Legionella*). Finally, certain bacteria simply resist to the fusion with lysosomes and multiply within phagolysosomal vacuoles (*Coxiella*, *S. aureus* and to some extend *Legionella*).

Along with the production of hydrolytic enzymes and reduction of local pH, the secretion of various reactive oxygen species (ROS) constitutes a third cell

defense mechanism against intracellular pathogens. ROS include hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite ($ONOO^-$) or superoxide anion (O_2^-) **[26,19].** They provoke severe damages to bacterial DNA, proteins and lipids and contribute to the destruction of the internalized microorganisms. Yet many intracellular pathogens have developed protection mechanisms to counter the deleterious effects of ROS.

A first strategy is to reduce the exposure to ROS. In macrophages, an inducible nitric oxide synthase (iNOS) catalyzes the formation of nitric oxide from L-arginine. *Francisella tularensis* can modify its lipopolysaccharide (LPS) in a manner that this altered LPS is no longer a stimulus for the inducible nitric oxide synthase (iNOS) and therefore minimize the production of NO **[19]**. *Helicobacter pylori* produces an arginase that degrades the iNOS substrate, L-arginine. In absence of its substrate the iNOS is not able to generate sufficient NO to reach bactericidal concentrations. Other bacteria produce detoxification enzymes that degrade ROS like the *S. aureus* catalase KatA, the *Salmonella* superoxide distmutases sodCI and sodCII or peroxiredoxins frequently found in a variety of species that catalyse the formation of nitrite from peroxynitrite **[19]**.

The second mechanism implies the restoration of ROS-induced damages to bacterial macromolecules. They include DNA repair mechanisms such as the RecBC system of *Salmonella* and heat shock proteases such as Clp of *Salmonella* and *Listeria* that degrade the damaged proteins **[19]**. All theses examples illustrate the multiple strategies that invasive bacteria have developed

to promote internalization and counter cellular defenses to finally allow their multiplication inside the host cells.

8. Intracellular infection models

In the present work, we attempted to get a better understanding of the intracellular fate of *S. aureus* and tried to identify some underlying mechanisms contributing to its intracellular survival. We have compared the development of intracellular infection in professional phagocytic cells and non-professional phagocytes, and have explored the implication of some bacterial virulence factors and one global regulator of virulence.

8.1. Selected cell types : THP-1 and HUVEC

To investigate the fate of *S. aureus* in phagocytic cells versus non phagocytic cells, we selected two human cell types, the macrophages THP-1 and endothelial cells HUVEC. THP-1 is a human myelomonocytic cell line displaying macrophage-like activity; originally isolated from the blood of a one-year-old boy with acute monoctytic leukemia **[82]**. THP-1 monocytes are rather "naïve" cells with low intrinsic defenses against intracellular pathogens **[16]**. This feature was exploited in our infection model since it permitted the intracellular multiplication of bacteria and allowed us to explore the bacterial mechanisms for

intracellular survival in phagocytic cells. In addition, THP-1 monocytes could be differentiated into active macrophages with increased bactericidal activity by incubation with phorbol myristate acetate (PMA) **[85]**.

HUVEC (human umbilical vein endothelial cells) are the endothelial cells lining the vein of the umbilical cord. These cells displayed the classical large polygonal morphology of endothelial cells with an oval centrally located nucleus **[36]**.

These two cell types present multiple advantages for our project. First, THP-1 cells are professional phagocytes whereas HUVEC are non-professional phagocytes which allowed us to compare the intracellular fate of *S. aureus* in these two different conditions. Then, both cell types are from human origin and therefore maybe more appropriate to study models of human infections as well as the activity of antibiotics classically used to treat these infections in hospitals. At last, both cell types have been widely used in numerous fields of research including for infection models and it would permit to compare our results with previously published data **[50,89]**. In our laboratory, THP-1 cells have been used to explore the cellular pharmacokinetics of drugs and the activity of antibiotics or cytokines against intracellular pathogens **[4,5,16,46]**.

To set up the intracellular infection models we infected both cell types with an initial low inoculum of bacteria to ensure cell viability for the 24h infection. In previous publications, the intracellular bacteria were frequently expressed as the

number of bacteria per cell (initial cell number at the beginning of infection) **[37]** or as the number of bacteria per ml of cell lysate **[14,43]**. But these two methods do not take into account the fraction of cells that died during the infection process. Here, we express the level of internalized bacteria as the number of CFU per mg of proteins (in $\log_{10}$ unit) and the intracellular growth as the difference of CFU/mg of proteins (T24 – T0, with T0 being the number of intracellular bacteria post-internalization). This approach appears much more precise since it takes into account the internalization rate but also the unavoidable death of a certain number of infected cells during the 24h infection. Dead cells are eliminated during the multiple washing and the living infected cells are then collected and lysed for protein quantification. Therefore the protein concentration reflects more accurately the number living infected cells after the 24h of infection.

8.2. Selected virulence factors : hemolysins and sigmaB

A described earlier in this work, *S. aureus* expresses a large range of virulence factors and some of them are most probably involved in the intracellular survival of the pathogen. Among others, *S. aureus* produces at least four membrane damaging toxins called hemolysins that could possibly be involved in the intracellular development of the bacteria like it was described for another intracellular pathogen, *Listeria monocytogenes*. Indeed, *L. monocytogenes* was shown to secrete a pore forming toxin, the listeriolysin O (LLO), which allows the bacteria to escape from the phagosome and proliferate into the cytoplasm.

Therefore, we have chosen to study the implication of three major hemolysins of *S. aureus*, the alpha- and gamma-hemolysins that are pore-forming toxins and the beta-hemolysin which is a sphingomyelinase.

In addition to its numerous virulence factors *S. aureus* possesses a large array of regulatory systems that control the expression of virulence-related genes as well as general metabolic pathways. Among them, the alternative transcription factor sigmaB is involved in stress response and modulates the expression of several virulence factors. In the intracellular environment, the bacteria have to face the cellular defense mechanisms and acidic pH that could be activators of stress response. Hence we have investigated the contribution of sigmaB and its principal positive regulator rsbU during the intracellular infection.

8.3. Selected bacterial strains : the *S. aureus* 8325-4 lineage

S. aureus genome is very flexible and each strain may express a different combination of antibiotic resistance mechanisms, surface proteins and excreted toxins. Consequently, to study the implication of a small number of specific virulence factors, it appears essential to use a set of isogenic staphylococcal strains. We selected a set of strains disrupted for alpha-, beta- and/or gamma-hemolysin which were constructed in the laboratory of Dr. Timothy J. Foster (Trinity College, Dublin, Ireland). These strains had previously been used to investigate the role of hemolysins in animal models of subcutaneous abscess or

corneal infections **[58,65]**. The techniques employed for hemolysins disruption were published and, in addition, all these strains were derived from the reference laboratory strain *S. aureus* 8325-4 which had been fully sequenced and used for more than twenty years for genetic studies **[56]**. Besides, *S. aureus* 8325-4 carries a natural deletion in the gene coding for the phosphatase rsbU, a positive regulator of the alternative transcription factor sigmaB. So we have included in our study the strain SH1000 **[34]**, a *rsbU+* derivative of *S. aureus* 8325-4, which allowed us to explore the significance of the *rsbU* deletion in 8325-4 and the role of rsbU and sigmaB during the development of intracellular infection. The choice of these strains did not seem inappropriate since such mutations in the regulatory network of sigmaB had been described in several human isolates **[38]**.

AIMS

Staphylococcus aureus is the etiological agent of severe infections including invasive endocarditis, osteomyelitis or skin and soft tissues infections. These staphylococcal infections present a high degree of relapses, and it is believed that the ability of *S. aureus* to survive inside the host cells contributes to this recurrent character. However the precise fate of intracellular *S. aureus* is still poorly understood.

The general objective of this project was to study the intracellular development of *S. aureus* in two human cell types; the phagoctyic cells THP-1 and endothelial cells HUVEC. We compared the internalization, intracellular growth and intracellular location in both cell types after 24h of infection as well as the activity of antibiotics against intracellular bacteria.

S. aureus expresses a wide range of virulence factors; some of them are most probably required for intracellular survival. Hence we studied the implication of the phosphatase rsbU and the alternative transcription factor sigmaB involved in stress response and searched for the role of three membrane-damaging toxins, the alpha-, beta- and gamma-hemolysin in the development of intracellular infection.

RESULTS

CHAPTER I:

Role of rsbU in the intracellular fate of *Staphylococcus aureus*. Comparison between human phagocytic (THP-1 macrophages) and non-phagocytic (HUVEC) cells.

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Role of rsbU in the intracellular fate of *Staphylococcus aureus*. Comparison between human phagocytic (THP-1 macrophages) and non-phagocytic (HUVEC) cells.

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ABSTRACT

S. aureus intracellular survival is assumed to contribute to the persistence of infection. RsbU is a phosphatase regulating the alternative transcription factor sigmaB. We compared the intracellular fate of *S. aureus* 8325-4 (*rsbU-*) and SH1000 (*rsbU+*) in human THP-1 macrophages and HUVEC over 24h.

SH1000 showed higher internalization and intracellular growth in both cell types, and an increased resistance to H_2O_2. Inhibition of staphyloxanthin, the golden pigment of *S. aureus*, severely impaired the intracellular growth of SH1000. Both strains had similar intracellular location. Functional rsbU favors *S. aureus* intracellular survival probably by its regulatory effect on sigmaB and subsequent enhanced production of staphyloxanthin.

INTRODUCTION

Staphylococcus aureus ranks among the most frequent causes of nosocomial and community-acquired bacterial infections in humans. This pathogen produces a large collection of virulence factors that induce immediate local and general damages during infections. These factors are under the control of global regulators with antagonistic effects like agr, a well established global regulator of staphylococcus virulence and the transcriptional factor sigmaB, which plays a central role in stress response [11] and in persistence of infection *in vivo* [5]. In this respect, staphylococcal infections like rhinosinusitis, endocarditis or osteomyelitis often present a recurrent character which is thought to be in part related to the existence of an intracellular pool of bacteria [9,10]. Former publications have demonstrated the ability of *S. aureus* to penetrate and survive inside a variety of professional and non-professional phagocytes. Nevertheless, the experimental procedures were usually dissimilar which makes difficult to identify the factors determining the intracellular fate of *S. aureus* in these various cell types.

The relationship between virulence and intracellular survival is never easy to establish. A recent study suggests that, once internalized in epithelial cells, *S. aureus* adjusts its gene expression to cope with intracellular environment, with a noteworthy reduction in the expression of global regulators of virulence factors [3]. The present study therefore explores a potential role for transcription factor sigmaB in intracellular infection. We focused our attention on rsbU, a

phosphatase that positively controls sigmaB, which itself regulates the expression of virulence factors either directly or indirectly by modulating expression of global regulators like agr [7,11] (and references cited therein). To this effect, we have compared the intracellular fate (internalization, intracellular growth and location) of two isogenic strains of *S. aureus*, 8325-4 and SH1000, in two human cell types, namely the Human Umbilical Vein Endothelial Cells (HUVEC) and THP-1 phagocytic cells. *S. aureus* 8325-4 is a common laboratory strain that bears a natural deletion in the *rsbU* gene [4]. Natural deletions in *rsbU* or *sigB* encoding the transcription factor sigmaB have been identified in several human isolates [6]. *S. aureus* SH1000 is a rsbU+ derivative strain from 8325-4 [4], which shows an enhanced pigmentation, but a reduced secretion of exoproteins, and a dowregulation of agr [4]. We show that these strains differ by their internalization and intracellular growth but not by their subcellular location, suggesting a role of rsbU and sigmaB in controlling the expression of genes determinant for intracellular survival. Among the factors regulated by sigmaB we demonstrate that the golden pigment staphyloxanthin plays a major role in the intracellular development of *S. aureus*.

MATERIALS & METHODS

Antibiotics and reagents

Gentamicin was obtained as a commercial product (GEOMYCIN®) registered in Belgium for parenteral administration by Glaxo-SmithKline s.a., Genval,

Belgium. Gelatin, catalase and H_2O_2 were from Sigma-Aldrich (St Louis, MO). The dehydrosqualene synthase inhibitors BPH-652, BPH-751, BPH-752 were obtained from E. Oldfiled (University of Illinois, Urbana, USA)

Bacteria

All experiments were performed with *S. aureus* 8325-4 and SH1000 [4]. Bacteria were grown in Mueller-Hinton broth and plated on TSA.

Cells and culture media

Human THP-1 cells (ATCC TIB-202), a human myelomonocytic cell line displaying macrophage-like activity, were obtained from American Type Culture Collection supplied trough LGC Promochem Ltd, Teddington, UK. THP-1 were cultivated with RMPI 1640 medium supplemented with 10% fetal calf serum (Invitrogen Ltd, Paisley, UK) as described previously [1]. Human umbilical vein endothelial cells (HUVEC) from pooled donors and EGM culture medium were purchased from Lonza Inc., Walkersville, MD. HUVEC were sub-cultured in gelatin-coated flasks with EGM medium according to supplier's instructions. They were used up to passage 8 and plated in 12-well gelatin-coated plates in DMEM-glutamax medium (Invitrogen) supplemented with 10% FCS for experiments.

Intracellular infection

Infections of THP-1 were carried on in 6-well plates (~10^6 cells/well) with RMPI medium supplemented with 10% FCS. For infection, HUVEC were

grown to confluence in 12-well plates (~2.10^5 cells/well) previously coated with gelatin with DMEM-glutamax medium (Invitrogen) supplemented with 10% FCS.

HUVEC or THP-1 were incubated with previously opsonised bacteria with an initial inoculum of 4 bacteria per cell (Multiplicity Of Infection [MOI] = 4) during 1h to allow internalization of bacteria. The cells are washed with PBS and incubated for 45 min with gentamicin (100 x MIC) to eliminate extracellular bacteria. Next the cells are washed with PBS and the infection is carried out for 24h with the cell culture medium in presence of low concentration of gentamicin (1 x MIC) to prevent the development of extracellular bacteria. After 24h, the cells are washed and collected, lysed with water and plated on TSA for CFU counting. Proteins are measured with the method of Lowry as described previously [13]. Intracellular growth was assessed by the number of CFU/mg of protein. Each sample was done in triplicates. Extracellular contamination was assessed in pilot studies by plating a sample of culture medium before harvesting the cells. The bacterial growth in the cell culture media (RMPI + 10% FCS or DMEM-glutamax + 10% FCS) and the MIC of antibiotics were similar as observed in M-H broth.

Resistance to hydrogen peroxide

The effect of H_2O_2 on bacterial growth was tested as previously described [2] but the concentration of catalase used to destroy the remaining H_2O_2 at the end of

incubation period was increased to 100 U/ml and samples were plated on TSA for determination of CFU.

Microscopy

Confocal microscopy was performed using the general methodology described before [13]. Bacteria were stained overnight with 0.25 mg/ml fluorescein-5-isothiocyanate (FITC) (Invitrogen) and lysosomes were stained 1h before the end of the incubation period with 50 µM LysoTracker® Red DND 99 (Invitrogen). Infection was carried out for 3h to limit the dilution of FITC upon intracellular multiplication of the bacteria. Observations were made in a MRC 1024 confocal microscope (Bio-Rad laboratories, Richmond, Ca) and images were analysed using the Confocal Assistant software (version 4.02). For electron microscopy, cells were harvested at 24h post infection, washed with PBS, fixed with glutaraldehyde 2% and osmium tetraoxyde 1%, and stained "en bloc" with uranyle acetate as previously described [13].

Statistical analyses

Statistical analyses (ANOVA) were performed with GraphPad Instat 3.06.

RESULTS

In a first set of experiments, we compared the internalization and intracellular growth of *S. aureus* 8325-4 and its SH1000 derivative. To this effect, HUVEC

and THP-1 macrophages were exposed to a same inoculum (MOI of 4); intracellular bacteria were counted after 1h of incubation with the cells and elimination of extracellular bacteria to calculate the internalization rate, and after 24h of incubation, to evaluate the intracellular growth. For both strains, the internalization was more important in THP-1 than in HUVEC (~ 0.5 log difference). In addition, the internalization of *S. aureus* SH1000 was statistically higher compared to 8325-4 in HUVEC (0.3 log increase) but this difference was much less pronounced in THP-1 (0.1 log) **[Fig. 8A]**. After 24h of infection, it appears that both strains can multiply and survive inside cells. Each strain displays a similar intracellular growth in both HUVEC and THP-1 cells. Yet, the presence of a functional *rsbU* gene seems to confer an advantage to strain SH1000 that shows a higher intracellular growth than 8325-4 in both cell types (about 0.7-0.8 log CFU/mg prot. difference with 8325-4 in 24h) **[Fig. 8B]**.

Resistance to oxidative stress is determinant for intracellular survival of bacteria. We therefore compared the resistance of both strains to H_2O_2. SH1000 appears much more resistant than 8325-4; the concentration of H_2O_2 needed to kill 50 and 90 % of the bacteria being approximately 42 and 100 mM for SH1000 instead of 16 and 22 mM for 8325-4 **[Fig. 8C]**.

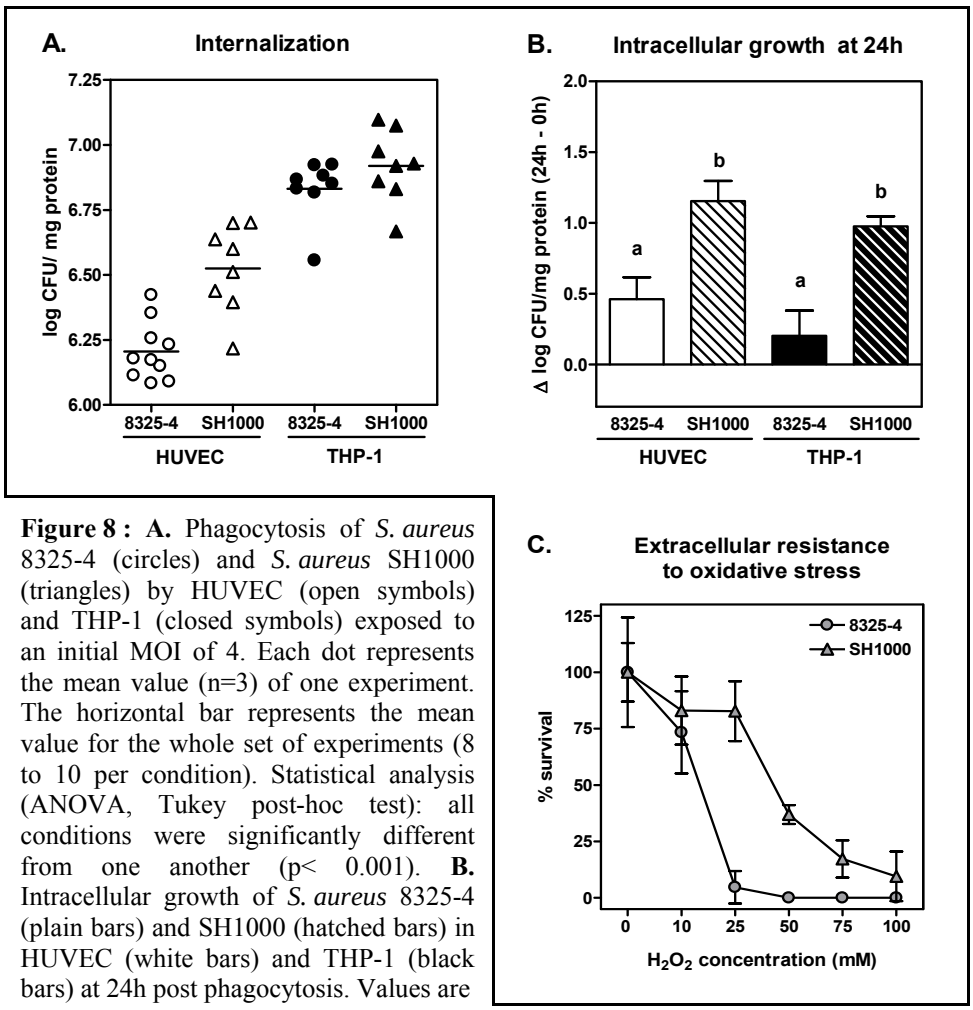

Figure 8 : A. Phagocytosis of *S. aureus* 8325-4 (circles) and *S. aureus* SH1000 (triangles) by HUVEC (open symbols) and THP-1 (closed symbols) exposed to an initial MOI of 4. Each dot represents the mean value (n=3) of one experiment. The horizontal bar represents the mean value for the whole set of experiments (8 to 10 per condition). Statistical analysis (ANOVA, Tukey post-hoc test): all conditions were significantly different from one another ($p < 0.001$). **B.** Intracellular growth of *S. aureus* 8325-4 (plain bars) and SH1000 (hatched bars) in HUVEC (white bars) and THP-1 (black bars) at 24h post phagocytosis. Values are mean ± SD of 3 independent samples. Statistical analysis (ANOVA, Tukey post-hoc test): bars with different letters are significantly different from one another ($p < 0.001$). **C.** Effect of H_2O_2 on the survival of *S. aureus* 8325-4 (circles) and SH1000 (triangles). Bacteria were incubated for 45 min with increasing concentrations of H_2O_2 (from 0 mM to 100 mM).The reaction was stopped by the addition of catalase. Values are expressed as the percentage of CFU as compared to the control sample (no H_2O_2). Values are mean ± SD of 3 independent samples.

Among the factors regulated by sigmaB, the staphyloxanthin golden pigment was shown to have antioxidant properties and confers protection against neutrophil killing [2]. *S. aureus* SH1000 and 8325-4 present an obvious difference in staphyloxanthin synthesis **[Fig. 10B]**. The rsbU-deficient 8325-4 is known to have an impaired production of staphyloxanthin while the rsbU-restored SH1000 produces large amount of pigment [4]. To assess the contribution of staphyloxanthin in intracellular infection, we compared the intracellular growth of both *S. aureus* strains in presence of inhibitors of staphyloxanthin synthesis [8]. BPH-652, BPH-751 and BPH-752 are three inhibitors of the dehydrosqualene synthase, an enzyme involved in the early step of staphyloxanthin synthesis. **[Fig. 9, Fig. 10C]**. After 24h of infection, the inhibition of staphyloxanthin synthesis totally prevents the intracellular growth of SH1000, while having little but significant effect on the growth of 8325-4 **[Fig. 10A]**. This indicates that the positive effect of rsbU restoration on the intracellular growth of SH1000 is largely related to the high production of staphyloxanthin and that this pigment confers a major advantage for intracellular persistence. However the addition of inhibitors did not modify the internalization of SH1000 suggesting that the higher intracellular growth and higher internalization of this strain result from two independent mechanisms (data not shown).

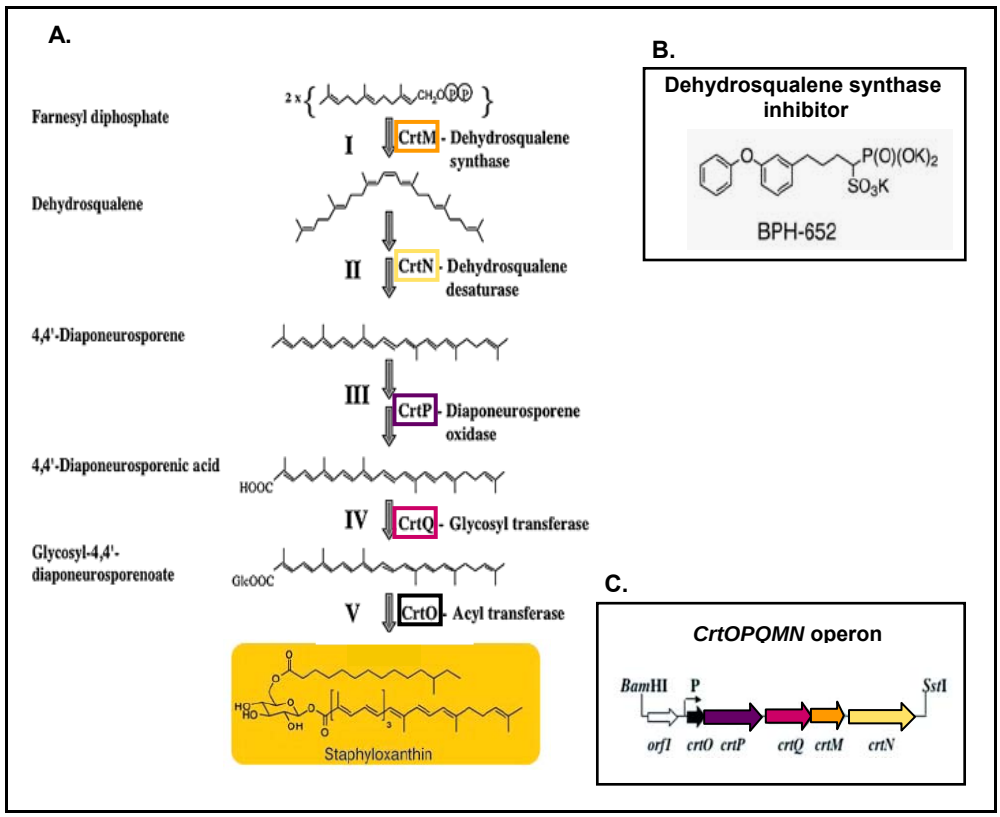

Figure 9 : Adapted from **[48,66]**. **A.** Staphyloxanthin biosynthesis pathway. **B.** Chemical structure of the dehydrosqualene synthase inhibitor BPH-652. BPH-751 is the enantiomer of BHP-652 and BPH-752 is a mixture of BPH-652 and BPH-751. **C.** organization of the *crtOPQMN* operon encoding five enzymes involved in staphyloxanthin synthesis.

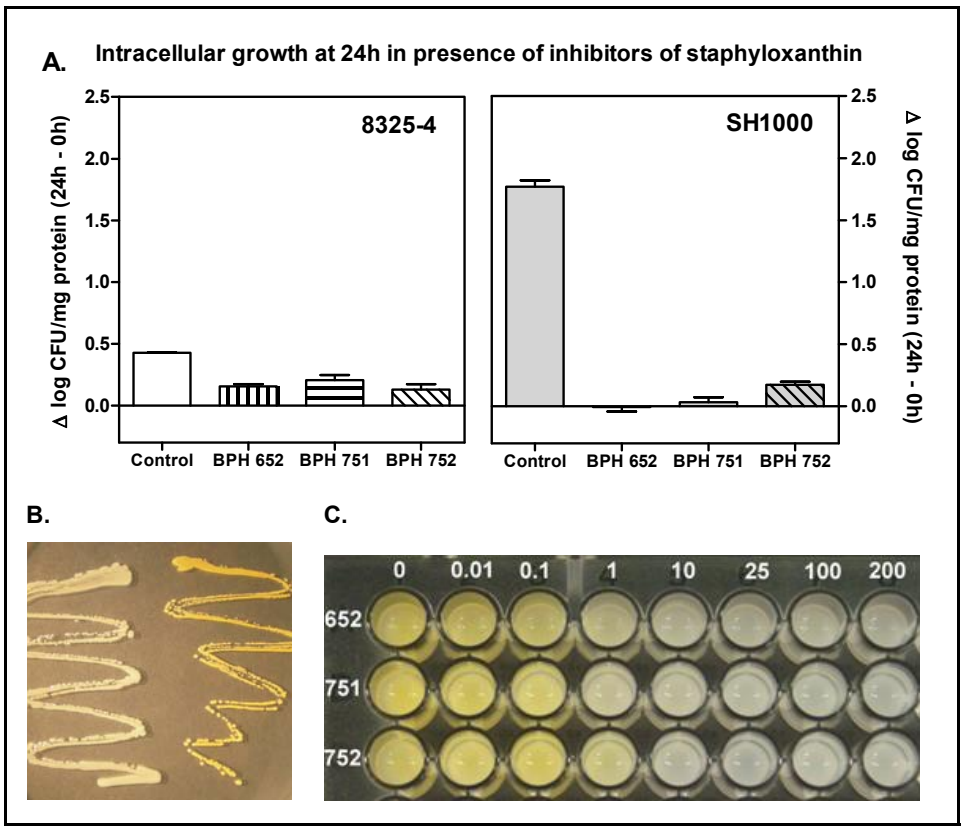

Figure 10 : A. Intracellular growth after 24h of infection in THP-1 for *S. aureus* 8325-4 (left panel) and SH1000 (right panel) without inhibitor (control) or with 100 µM of inhibitors BPH-652 (vertical lines), BPH-751 (horizontal lines), BPH-752 (diagonal lines). Values are mean ± SD of 3 independent samples. **B.** Colonies of *S. aureus* 8325-4 (left) naturally deficient for pigment synthesis and SH1000 (right) that produces staphyloxanthin. **C.** Picture of SH1000 culture in M-H broth incubated with increasing concentration of inhibitors (0 to 200 µM) illustrating the inhibition of pigment synthesis.

In a last set of experiments, we examined the subcellular location of bacteria in both cell types **[Fig. 11]**. Electron microscopy revealed that, after 24h of infection in THP-1 cells, both *S. aureus* strains appear confined in membrane-enclosed vacuoles where they are able to divide. In HUVEC, most bacteria are also surrounded by a membrane but a small number is not enclosed in membrane and appear free in the cytoplasm. To determine whether bacteria were localized in vacuoles from the phagolysosomal apparatus, infected cells were examined in confocal microscopy, with bacteria stained with FITC and acidic compartments with LysoTracker® Red. While both strains colocalized with the acidotropic marker in THP-1 cells, part of the FITC signal was clearly localized in a compartment that is not labeled by LysoTracker® Red in HUVEC (similar observations were made for both strains; data are illustrated for one strain in each cell line).

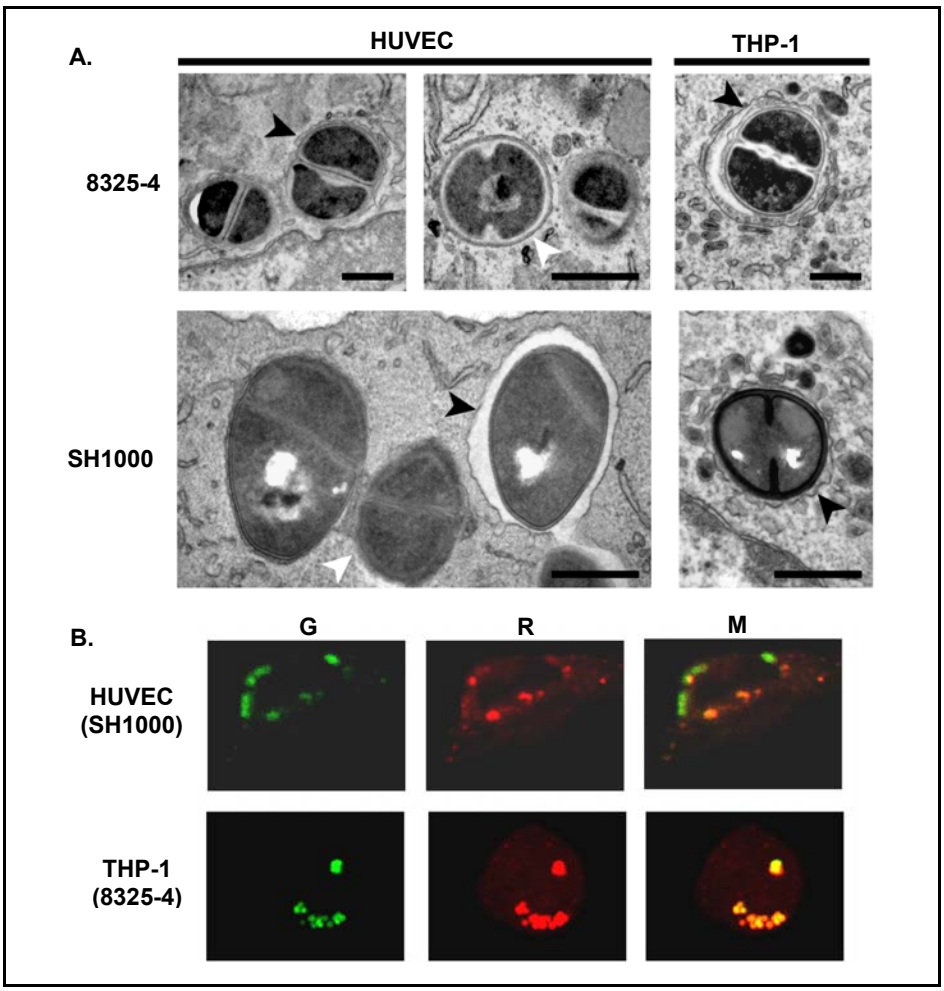

Figure 11 : Intracellular location of *S. aureus*. **A.** Electron microscopy after 24h of infection with 8325-4 (upper row) and SH1000 (lower row) in HUVEC (left) or THP-1 cells (right). Black arrowheads point to membranes limiting infected compartments; white arrowheads, to bacteria free in the cytosol. Bars are 0.5 µm. **B.** confocal microscopy after 3h of infection in HUVEC with SH1000 (upper row) or THP-1 with 8325-4 (lower row). Green channel (G): FITC (labeling bacteria); Red channel (R): LysoTracker® Red (labeling acidic compartments); Merged image (M). Observations were made under oil immersion with a 63x objective.

DISCUSSION

The main observation made in the present study is that restoration of rsbU in *S. aureus* SH1000 markedly increases internalization and intracellular growth as compared to the parental rsbU-deficient strain 8325-4, in both phagocytic and non-phagocytic cells, while at the same time not affecting its subcellular location. Note that in this study, we choose to use a low MOI which guaranteed an equivalent survival of both cell types over 24h, as opposed to other reports in which infecting inoculum was so high that it caused cell death by apoptosis [9,15].

These effects on both invasion and intracellular persistence can be ascribed to restoration of *rsbU* gene and consecutive sigmaB upregulation and possibly to the following downregulation of agr. Previous studies using bovine mammalian cells showed indeed an increased internalization and intracellular survival for an agr-deficient mutant as compared to its parental strain [15]. Invasion of eukaryotic cells implies a binding of the bacterium at the cell surface. In endothelial cells, this interaction with surface integrins occurs via fibronectin-binding proteins, which are upregulated and show improve binding capacity in agr mutants [12]. This may explain the larger internalization for SH1000 evidenced here. In THP-1 cells, the difference of internalization between SH1000 and 8325-4 was much less evident but, for both, strains the phagocytosis was slightly higher than in HUVEC. This may rely on a higher phagocytic capacity of THP-1 cells. It has indeed been proposed that, as opposed to the integrin-mediated zipper mechanism that takes place in endothelial cells, phagocytosis by professional

phagocytes occurs via a trigger mechanism, in which cells produce pseudopodes that engulf the bacterium attached at the cell surface, making the process more efficient [14].

Intracellular survival implies resistance to cell defense mechanisms among which oxidative stress. We show here that SH1000 is much more resistant to H_2O_2 than 8325-4. Among the proteins regulated by the transcription factor sigmaB, the staphyloxanthin pigment responsible for the golden color of *S. aureus* colonies was indeed shown to confer protection against oxidative stress and neutrophil killing [2]. Here, we demonstrated that this pigment is a major factor contributing to the intracellular multiplication of *S. aureus*. And the higher intracellular growth observed after rsbU restoration is largely related to the enhanced production of pigment. However, this pigment does not contribute to the higher internalization of SH1000 since the addition of pigment inhibitors did not modified the internalization rate of SH1000. Hence staphyloxanthin appears to confer a significant advantage for intracellular survival but probably contribute with many other factors in the development of intracellular infection.

Despite these major differences in their capacity to infect both phagocytic and non-phagocytic cells, both strains show a similar subcellular location, in the line of what has been previously observed for 8325-4 in HUVEC cells [9] and for other wild-type strains like ATCC25923 or Newman in macrophages [1,7]. This reinforces therefore our hypothesis that the higher intracellular growth of SH1000 is only mediated by an improved adaptation to the intracellular

environment and not to a different intracellular localization and escape from acidic compartment.

Taken together, our data plead therefore for the search of rsbU, and sigmaB functionality as well as staphyloxanthin production in clinical isolates from persistent infections. As not only SH1000 [7], but also Small Colony Variants, in which many genes are under the positive control of sigmaB [10], show a much higher capacity to survive for prolonged periods of time intracellularly.

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CHAPTER II:
Activity of antibiotics against intracellular *S. aureus*

1. Background

Over the last decades, the dramatic increase of bacterial resistance to antibiotics became a major public health concern. The continuous emergence of new resistance mechanisms and the scarcity of new antibiotic compelled scientist to optimize the use of current active molecules. To successfully eradicate intracellular pathogens, an antibiotic must (i) enter the cell and reach adequate concentration, (ii) be localized in the same subcellular compartment than its target (cytosol, phagosome, phagolysosome, …) and (iii) be active in the intracellular environment (low pH, protein binding, …). In this regard, previous publications from our laboratory demonstrated that the activity of many antibiotics in common use for the treatment of staphylococcal infections was much lower against intracellular *S. aureus* in phagocytic cells than the activity of the same antibiotics against extracellular *S. aureus* in broth culture **[4,5,45]**.

In this chapter we compared the activity of some classical antistaphylococcal drugs in endothelial and phagocytic cells on the two strains of *S. aureus* previously described, 8325-4 and SH1000. Could the antibiotics have different effects in the two cell types? Considering that the bacterial intracellular location is not exactly similar in both cell types, we wonder if this could have some implication on antibiotics activity. The selected drugs belong to different pharmacological classes of antibiotic with oxacillin (β-lactam), gentamicin (aminoglycoside), vancomycin (glycopeptide), and oritavancin, a new glycopeptide that appears successful in the treatment of staphylococcal

infections **[83]**. These antibiotics present different mode of action like inhibition of peptidoglycan synthesis (β-lactams and glycopeptides) or inhibition of protein synthesis (aminoglycosides) and different cellular accumulation or location. Gentamicin is known to enter very slowly in eukaryotic cells by the endocytic pathway and concentrates in the lysosomes. Vancomycin and oritavancin also concentrate in lysosomes but the cellular accumulation of oritavancin can be up to 30 times higher than vancomycin accumulation **[84]**. Here we compared the activity of these three lysosome-located antibiotics with the activity of oxacillin which is known to localize in the cytoplasm.

2. Materials and methods

Antibiotics

Oritavancin was obtained from Targanta Therapeutics Corp., Cambridge, MA. Gentamicin and vancomycin were obtained as GEOMYCIN® and VANCOCIN® distributed in Belgium by Glaxo-SmithKline s.a., Genval, Belgium. Oxacillin was purchased from Sigma-Aldrich, St-Louis, MO.

Extracellular dose-response curve

Extracellular activity were measured by incubating 10^6 CFU/ml in Mueller-Hinton broth with increasing concentration of antibiotics (from 0.001 x MIC to 1000 x MIC) during 24h. Samples were plated on TSA for CFU counting. Each sample was done in triplicates.

Intracellular dose-response curve

Cells were infected just as described in chapter I but the gentamicin used to prevent the development of extracellular bacteria was replaced by increasing concentrations of selected antibiotics (from 0.001 x MIC to 1000 x MIC).

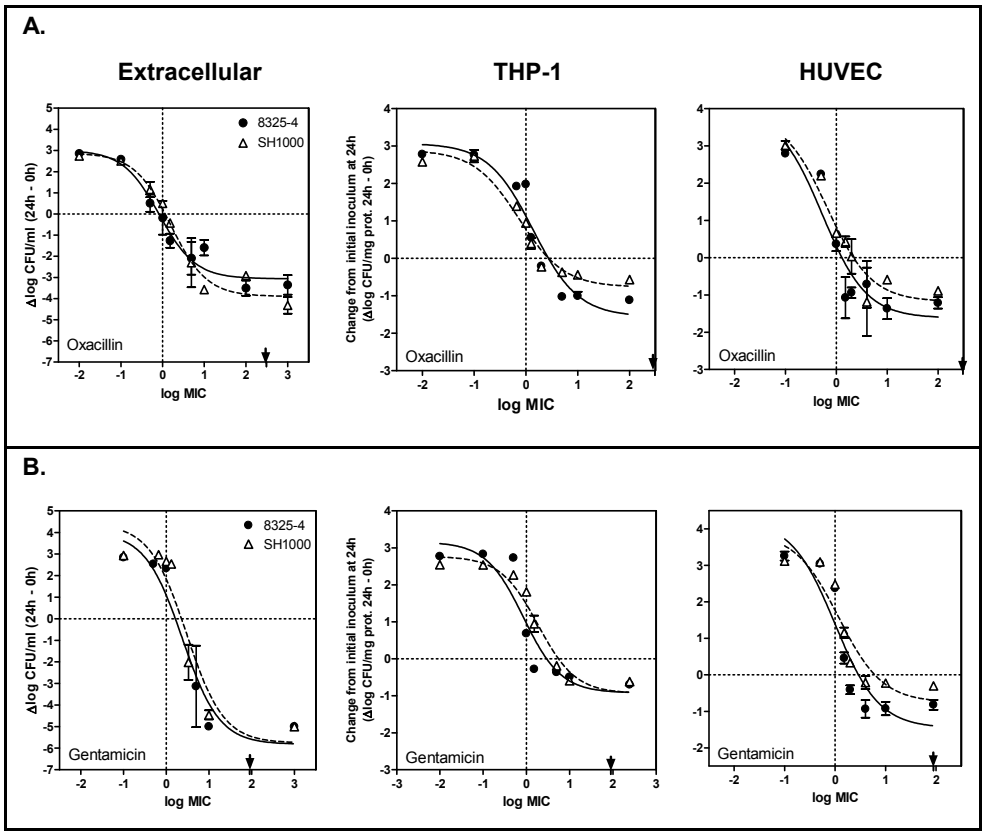
Statistical analysis

Curve fitting analyses were performed using GraphPad Prism 4.02 for Windows (GraphPad Prism Software, San Diego, CA). Statistical analyses were performed with GraphPad Instat 3.06.

3. Activity of antibiotics

The four selected drugs show a high activity in broth culture against both strains of *S. aureus.* Their maximal effect (E_{max}) as extrapolated from the dose-response curve reached - 3.5 log decrease from the initial inoculum for oxacillin, < - 5.0 log for gentamicin, - 5.0 log for vancomycin and oritavancin **[Fig. 12]**. All samples yielding less than three colonies per plates were considered to have a - 5.0 log decrease from the original inoculum which was set as the limit of detection **[4]**. Their activity against intracellular *S. aureus* is, in contrast, much weaker. Oxacillin, gentamicin and vancomycin reduced the intracellular inoculum for about -1.0 log after 24h of infection. Only oritavancin presented a suitable intracellular activity close to -3.0 log decrease **[Fig. 12]**. We examined

two classically employed pharmacological parameters, the maximal effect (E_{max}), being the maximal CFU decrease (in log 10 units) reached after 24h of infection and the EC_{50}, defined as the drug concentration causing a reduction of the inoculum half-way between the effect in absence of drug (E_0) and the maximal effect (E_{max}) **[Tab. 4].** Each antibiotic had a similar effect against the two strains of S. *aureus* and no difference of activity was noticed between THP-1 and HUVEC.



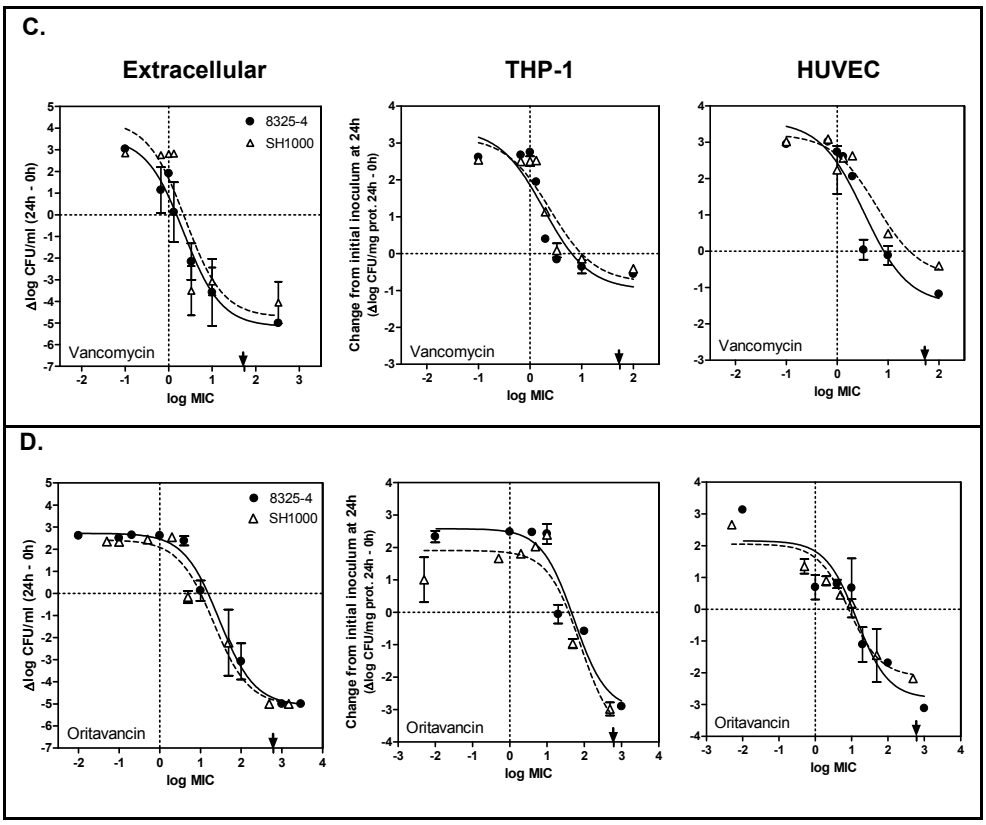


Figure 12 : Dose-response curves of antibiotics against *S. aureus* 8325-4 (black circles – continuous line), and SH1000 (white triangles – broken line) in Mueller-Hinton broth (left panel), THP-1 (middle panel) and HUVEC (right panel). **A.** oxacillin; **B.** gentamicin; **C.** vancomycin and **D.** oritavancin. The graphs show the variation in the number of CFU per ml of culture medium (left panels) or the variation in the number of CFU per mg of protein (middle and right panels) (Δlog CFU 24h-0h; means ±SD; n = 3 ; most SD bars are smaller than the symbols). The horizontal dotted line represents a bacteriostatic effect. The vertical dotted line represents the concentration corresponding to MIC. Sigmoidal dose-response curves were obtained by non-linear regression. See table 3 for regression parameters, statistical analysis, pharmacological and microbiological descriptors.

	Extracellular		Intracellular - THP-1		Intracellular - HUVEC	
Antibiotics	**8325-4**	**SH1000**	**8325-4**	**SH1000**	**8325-4**	**SH1000**
Oxacillin						
MIC (µg/ml)	**0.2**	**0.2**				
E_{max} (95% CI) (Δlog CFU/mg)	-3.06 [a,b] (-3.81 to -2.31)	-3.89 [a] (-4.66 to -3.13)	-1.56 [b,c] (-2.61 to -0.49)	-0.77 [c] (-1.28 to -0.25)	-1.62 [b,c] (-2.89 to -0.36)	-1.18 [c] (-2.04 to -0.32)
EC_{50} (95% CI) (µg/ml)	0.17 [A] (0.08 to 0.35)	0.31 [A] (0.17 to 0.58)	0.29 [A] (0.10 to 0.79)	0.15 [A] (0.07 to 0.33)	0.11 [A] (0.02 to 0.56)	0.13 [A] (0.04 to 0.40)
C_{static} (µg/ml)	0.16	0.22	0.55	0.55	0.26	0.43
R^2	0.963	0.975	0.923	0.962	0.876	0.935
Gentamicin						
MIC (µg/ml)	**0.2**	**0.2**				
E_{max} (95% CI) (Δlog CFU/mg)	-5.82 [a] (-9.29 to -2.35)	-5.76 [a] (-8.89 to -2.64)	-0.92 [b] (-2.16 to 0.32)	-0.93 [b] (-1.62 to -0.25)	-1.46 [b] (-3.13 to 0.22)	-0.75 [b] (-2.07 to 0.56)
EC_{50} (95% CI) (µg/ml)	0.48 [A,C] (0.07 to 3.38)	0.73 [A] (0.17 to 3.09)	0.17 [B] (0.04 to 0.81)	0.37 [A,B] (0.16 to 0.86)	0.20 [B,C] (0.04 to 1.03)	0.24 [B,C] (0.054 to 1.05)
C_{static} (µg/ml)	0.32	0.51	0.25	0.64	0.59	1.23
R^2	0.944	0.931	0.883	0.965	0.863	0.882
Vancomycin						
MIC (µg/ml)	**0.9**	**0.9**				
E_{max} (95% CI) (Δlog CFU/mg)	-5.17 [a] (-6.76 to -3.57)	-4.73 [a,b] (-9.29 to -0.17)	-0.98 [c] (-2.68 to 0.72)	-0.79 [c] (-2.29 to 0.72)	-1.46 [b,c] (-3.07 to 0.14)	-0.71 [c] (-1.88 to 0.46)
EC_{50} (95% CI) (µg/ml)	1.99 [A] (0.79 to 4.96)	2.16 [A] (0.18 to 25.72)	1.67 [A] (0.26 to 10.54)	2.21 [A] (0.39 to 12.45)	3.04 [A] (0.76 to 12.12)	5.20 [A] (1.36 to 19.93)
C_{static} (µg/ml)	1.38	1.99	5.88	8.91	7.41	23.4
R^2	0.970	0.817	0.825	0.851	0.906	0.955

Oritavancin						
MIC (µg/ml)	**0.05**	**0.1**				
E_{max} (95% CI) (Δlog CFU/mg)	-5.08 [a] (-5.75 to -4.40)	-5.07 [a] (-6.46 to -3.68)	-3.02 [a] (-5.38 to -0.66)	-3.89 [a] (-7.71 to -0.07)	-2.81 [a] (-4.94 to -0.67)	-2.12 [a] (-3.31 to -0.94)
EC_{50} (95% CI) (µg/ml)	1.44 [A] (0.82 to 2.52)	2.16 [A] (0.80 to 5.85)	2.65 [A] (0.45 to 15.42)	7.85 [A] (0.67 to 91.30)	0.70 [A] (0.096 to 5.13)	0.86 [A] (0.24 to 3.11)
C_{static} (µg/ml)	0.78	1.0	2.24	3.80	0.52	0.83
R^2	0.991	0.991	0.919	0.880	0.874	0.945

Table 4 : Microbiological and pharmacological parameters of dose-response curves. MIC = minimal inhibitory concentration (µg/ml), E_{max} = maximal effect expressed as the reduction in CFU at 24h from the original inoculum (Δlog CFU/mg of protein 24h-0h), EC_{50} = concentration causing a reduction of the inoculum halfway between the minimal (E_0) and the maximal (E_{max}) values as obtained from the Hill equation (slope factor of 1); C_{static}= concentration resulting in no apparent bacterial growth after 24h as determined by graphical interpolation. For statistical analysis (one-way ANOVA with Tukey's post test for multiple comparisons) values with different letters are significantly different ($p < 0.05$) from each other within the pertinent comparison group. For each antibiotic we compared the two strains (8325-4 and SH1000) and the three conditions (Extracellular, THP-1 and HUVEC). Lowers case letter, comparison of E_{max}. Upper case letter, comparison of $EC_{50.}$

4. Conclusion

It was previously demonstrated that many antibiotics are less active against intracellular *S. aureus* in phagocytic cells and that their intracellular activity can not be predicted by their activity in broth culture **[4,45]**. Here we demonstrate that this activity is similar in macrophages and endothelial cells. The restoration of RsbU in *S. aureus* SH1000 which was associated with a higher intracellular growth and resistance to oxidative stress compared to 8325-4 (see Chap. I) did not confer any advantage when considering the antibiotics activity. The four selected antibiotics had similar effect against both strains of *S. aureus*. Although very active against extracellular *S. aureus,* oxacillin, gentamicin, and vancomycin displayed a weak activity against intracellular *S. aureus* in THP-1 as well as in HUVEC. Only oritavancin shows a more pronounced intracellular activity approaching a – 3.0 log decrease. The low intracellular activity of antibiotics can have different causes like a reduced bioavailability due to the binding to cytoplasmic components; a poor cellular accumulation, an alteration of the drug in the intracellular environment, or a modification of bacterial metabolism reducing its susceptibility to antibiotics. Oxacillin is known to have a weak cellular accumulation and is localized essentially in the cytoplasm thus providing some explanation for the poor activity on intracellular *S. aureus*. Concerning its activity in our two infection models, the presence of a small number of cytoplasmic bacteria in HUVEC didn't perceptibly modify the general effect of oxacillin. Gentamicin concentrates in phagolysosomes but its accumulation is very slow and it was demonstrated that the acid pH severely

reduces its activity **[84]**. The discrepancy in the activity of the two selected glycopeptides should probably be related to their intrinsic activity and level of accumulation. Vancomycin is bacteriostatic and has a low level of cellular accumulation after 24h with an accumulation factor of 8 (ratio between the cellular concentration and the extracellular concentration). In contrast, the high activity of oritavancin can be linked to its bactericidal effect, its very high cellular accumulation with accumulation factor of 300, and possibly to an additional mechanism of action due to its anchorage in bacterial membrane **[83,84]**.

CHAPTER III:

Role of hemolysins in the development of intracellular infection

1. Background

Intracellular bacteria have developed multiple strategies to survive inside the host cells. Following internalization, some pathogens like *Listeria monocytogenes* rapidly escape from the phagosome and replicate into the cytoplasm. Other bacteria including *Mycobacterium spp.* and *Brucella spp.* remain inside phagosomes but prevent subsequent fusion with lysosomes. Finally, some bacteria like *Coxiella brunetii* resist to the fusion with lysosomes and are able to multiply within acidic phagolysosomes **[15]**. To escape from the phagosome, *Listeria monocytogenes* produces a phagosome-specific pore-forming toxin called Lysteriolysin O **[72]**. *S. aureus* possesses at least four membrane-damaging toxins named alpha-, beta-, gamma- and delta-hemolysins, which could possibly have similar function than the listeriolysin O.

Alpha-hemolysin is a 33kD protein encoded by the gene *hla*. It is a pore-forming toxin active on many mammalian cells including human erythrocytes, platelets, monocytes, T lymphocytes and fibroblasts **[32,51]**. Alpha-hemolysin is secreted as monomers that bind to the target cells and generate a hexa- or heptameric pore **[51]**.

The gene *hlb* encodes a 35 kD enzyme know as staphylococcal beta-hemolysin. This magnesium-dependent sphingomyelinase C is very active on sheep erythrocytes and human monocytes **[90]**. Beta-hemolysin specifically cleaves

sphingomyelin and its specificity seems to correlate with the sphingomyelin content of cell membrane.

The gamma-hemolysin belongs to the group of staphylococcal bi-component leucotoxins formed by the association of two distinct sub-units named S and F. The gamma-hemolysin locus *hlg* encode three proteins, HlgA (S component), HlgB (F component) and HlgC (S component) with molecular weight of 32kD, 34kD and 32.5kD respectively. This three proteins associate to generate two active toxins: HlgA + HlgB and HlgC + HlgB **[51]**. Like alpha-hemolysin, gamma-hemolysin is a pore forming toxin. Its principal targets are the polymorphonuclear cells (PMNs), monocytes and macrophages but gamma-hemolysin can also lyses erythrocytes **[51]**. *In vivo*, alpha-, beta- and gamma-hemolysins seem to contribute to tissue necrosis and local inflammation in various models of infections.

The fourth hemolysin encoded by the gene *hld* is a small peptide of 3 kD that has surfactant properties. Delta-hemolysin cytolytic activity seems not very specific since it degrades erythrocytes and other mammalian cells, as well as subcellular structures such as membrane-bound organelles **[23]**. A mechanism of action involving the formation of transitory pores was proposed. In this model, delta-hemolysin binds to the membrane and associates in trimers that sink into the membrane leading to the formation of transient pores and efflux of vesicle content **[68]**. The implication *in vivo* of delta-hemolysin is still unclear.

In this chapter we addressed the role of staphylococcal hemolysins in the development of intracellular infection. We used various *S. aureus* strains disrupted for alpha-, beta- or gamma-hemolysin and compared their intracellular growth and localization in macrophages and endothelial cells.

2. Materials and methods

Bacteria

All *S. aureus* strains used in this study are listed in **table 5**. Most strains were obtained from Dr. Timothy J. Foster (Trinity College, Dublin, Ireland). Bacteria were grown in Mueller-Hinton broth and plated on TSA. Hemolysins disruptions were verified by PCR with primers listed in **table 6**. The complemented mutant of the gamma-hemolysin disrupted strain was constructed by electroporating a plasmid carrying the full *hlg* locus. Plasmid pCU1-*hlg*+ was extracted from *S. aureus* Newman N65+ **[80]** and electroporated in *S. aureus* DU5942 following a method described by Schenk and Laddaga **[71]**.

Intracellular infections and microscopy

Intracellular infections of THP-1 cells and HUVEC, electron and confocal microscopy were carried out as described in chapter I.

***S. aureus* strains**	**Genotype**	**Characteristics**	**Reference**
8325-4	*rsbU-*	Wild type strain cured of all prophage; carries a natural deletion in *rsbU*	Novick, 1967 **[56]**
DU1090	*hla::*Em^R *rsbU-*	Derived from 8325-4. Disrupted for alpha-hemolysin	O'Reilly, 1986 **[60]**
DU5719	*hlb::*Φ42E *rsbU-*	Derived from 8325-4. Disrupted for beta-hemolysin	Patel, 1987 **[65]**
DU5942	*hlg::*Tet^R *rsbU-*	Derived from 8325-4. Disrupted for gamma-hemolysin	Nilsson, 1999 **[55]**
DU5942-M1	*hlg::*Tet^R *rsbU-* pCU1-hlg+	Derived from 8325-4. Disrupted for gamma-hemolysin and complemented with plasmid pCU1-hlg+ that carries a functional gamma-hemolysin locus	This study
DU5938	*hla::*Em^R *hlb::*Φ42E *hlg::*Tet^R *rsbU-*	Derived from 8325-4. Disrupted for alpha-, beta- and gamma-hemolysin	Nilsson, 1999 **[55]**

Table 5 : *S. aureus* strains. *rsbU* = gene coding for phosphatase rsbU, *hla* = alpha-hemolysin gene ; *hlb* = beta-hemolysin gene; *hlg* = gamma-hemolysin locus; Em^R = erythromycin resistance gene; Tet^R = tetracycline resistance gene ; Φ42E = phage 42E.

Primers	**Sequences**	**Size of PCR product**
hlaF	GAAAACACGTATAGTCAGCTCAGTAAC	951 pb
hlaR	GTCATTTCTTCTTTTTCCCAATCG	
hlbF	GGTGAAAAAAACAAAATCCAATTCAC	985 pb
hlbR	ACTATAGGCTTTGATTGGGTAATGATC	
hlb4-rt-F	CGTTTATATGTTATCGACCGTTT	191 pb
hlb4-rt-R	GGCCGAGTACAGGTGTTT	
hlgCF	AACTTTATCTGTGAGCTTACTTGC	} 1146 pb } 1950 pb
hlgR2	CTTTATCATAACTTTTATCTTTGATG	
hlgF3	CAACATTGTCACACGAAAGAGGT	

Table 6 : Primers used to check hemolysins disruptions. hlaF/hlaR amplify alpha-hemolysin gene (*hla*); hlbF/hlbR amplify beta-hemolysin gene (*hlb*) and hlb4-rt-F/hlb4-rt-R were used check beta-hemolysin disruption by Real-Time PCR; hlgCF/hlgR2 and hlgF3/hlgR2 amplify two fragment of gamma-hemolysin locus *hlg*.

3. Characterization of hemolysins disruptants

Alpha-, beta-, and gamma-hemolysins were inactivated by disruption of their respective genes. The alpha-hemolysin gene was disrupted by insertion of an erythromycin resistance marker **[60]**, beta-hemolysin was inactivated by lysogenization with a converting bacteriophage (42E) which integrates in the *hlb* gene **[65]** and the gamma-hemolysin locus was excised and replaced by a tetracycline resistance gene **[55,80]**. All strains were checked by PCR as illustrated for alpha-hemolysin disruption **[Fig. 13]**. All these mutants derived from the common laboratory strain *S. aureus* 8325-4 **[56]**. *S. aureus* 8325-4 bears a small natural deletion in the gene *rsbU*, coding for a regulator of transcriptional factor sigmaB. The implication of this mutation was addressed in the first chapter of this work by comparing the intracellular fate of *S. aureus* 8325-4 with strain SH1000 which carries a functional *rsbU* gene **[34]**. The complemented gamma-hemolysin mutant DU5942-M1 was obtained by electroporating plasmid pCU1-*hlg*+ **[80]** carrying the complete *hlg* locus into the strain DU5942. And the expression of gamma-hemolysin in DU5942-M1 was subsequently verified by real-time quantitative PCR **[Fig. 14]**.

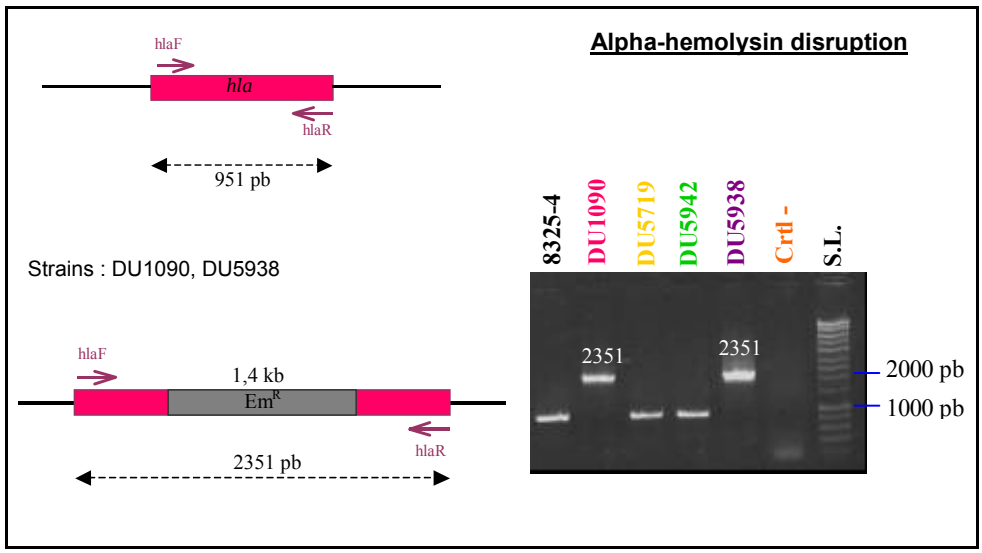


Figure 13 : PCR amplification of *hla* gene. Strains DU1090 disrupted for alpha-hemolysin and strain DU5938 disrupted for alpha-, beta- and gamma-hemolysins carry a 1.4 kb erythromycin resistance maker inside the *hla* gene. *hla* is intact in strains 8325-4 as well as in strain DU5719 disrupted for beta-hemolysin and strain DU5942 disrupted for gamma-hemolysin. Ctrl- = negative control; S.L. = Smart Ladder (Eurogentec).

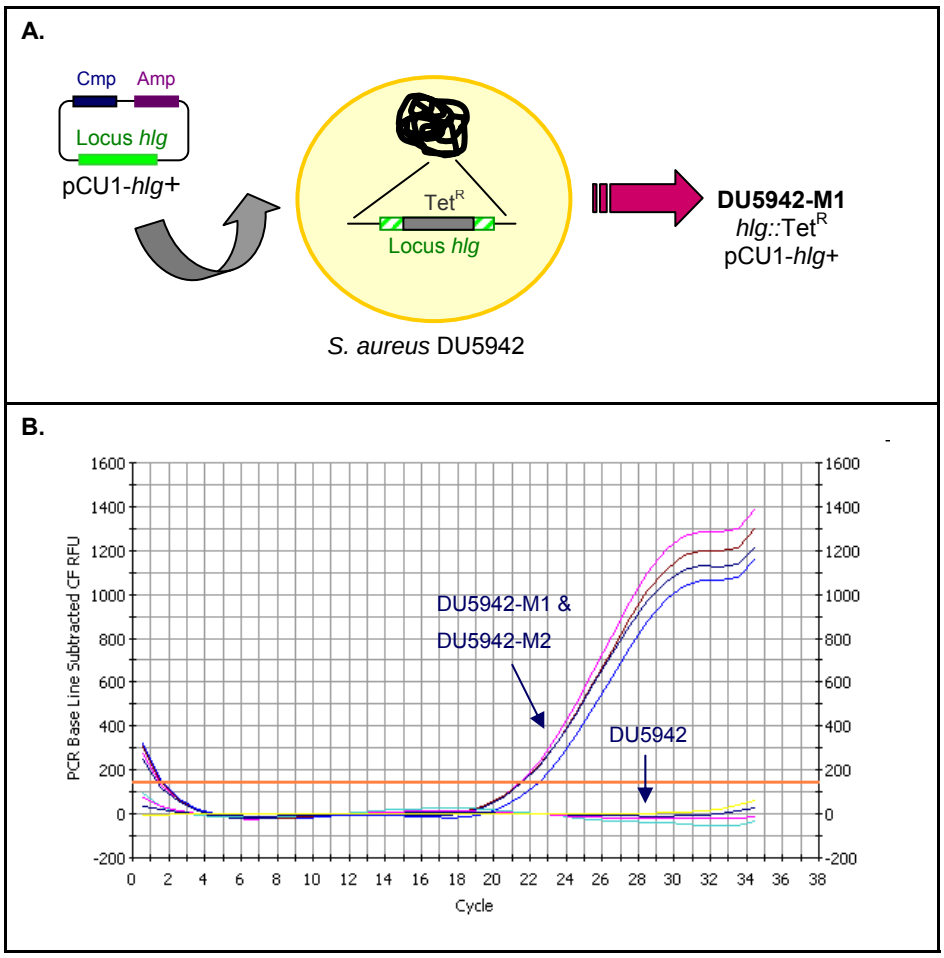


Figure. 14 : A. Construction of complemented mutant *S. aureus* DU5942-M1. Cmp = chloramphenicol resistance gene; Amp = ampicillin resistance gene. Ten clones were selected on NYE agar with 10 µg/ml chloramphenicol, and four of them were tested for plasmid stability after intracellular infection. **B.** The expression of gamma-hemolysin was checked by real-time PCR. The four clones tested had the same profile and clone DU5942-M1 was selected for further experiments.

4. Intracellular growth of hemolysin disruptants

The effect of hemolysin disruption on the development of intracellular infection was studied in THP-1 cells and HUVEC. The different profiles of hemolysin production of all the strains did not seem to affect the cellular viability before the cell uptake since the amount of cellular proteins immediately after internalization was similar for all the strains. Just before internalization, the freshly opsonised bacteria were centrifuged and resuspended in fresh culture media thereby removing the hemolysins already produced. Next, the short time allowed for internalization (1h) and the small volume of bacterial culture added to the cells also limited the amount of hemolysin present in the cell culture medium.

After 5h of infection in THP-1 macrophages, all strains tested seem in an apparent latency phase since there is no perceptible modification of intracellular inoculum compared to the initial intracellular inoculum (post-internalization). After 24h of infection, the disruption of alpha- and beta-hemolysin does not seem to affect the intracellular fate of *S. aureus* as strains DU1090 (*hla*-) and DU5719 (*hlb*-) show an intracellular growth of 0.2 log and 0.4 log respectively, similar to the growth of parental strain 8325-4 (0.2 log) **[Fig. 15A]**. Unexpectedly, the gamma-hemolysin disrupted strain DU5942 presented a very high intracellular growth of 2.0 log. Nonetheless it rapidly appeared that this important intracellular growth was not due to *hlg* disruption. A first indication came from strain DU5938, disrupted for the three hemolysins, which has an

intracellular growth of 0.3 log, similar to the parental strain. The second evidence came from the complemented mutant DU5942-M1. If the disruption of gamma-hemolysin was responsible for the important intracellular growth of DU5942, it could be expected that the complementation with a functional *hlg* locus would reduce the intracellular growth to the same level than the parental strain 8325-4. In contrast, the complemented mutant DU5942-M1 reached the same elevated intracellular growth than DU5942 **[Fig. 15B]**. Similar results were obtained in THP-1 cells and HUVEC with all the strains **[Fig. 16]**. After 24h of infection in THP-1, the cellular toxicity of *S. aureus* 8325-4 and DU5942 was examined by trypan blue staining. For each strain approximately 10 to 15% of cells had incorporated the dye indicating cell damages.

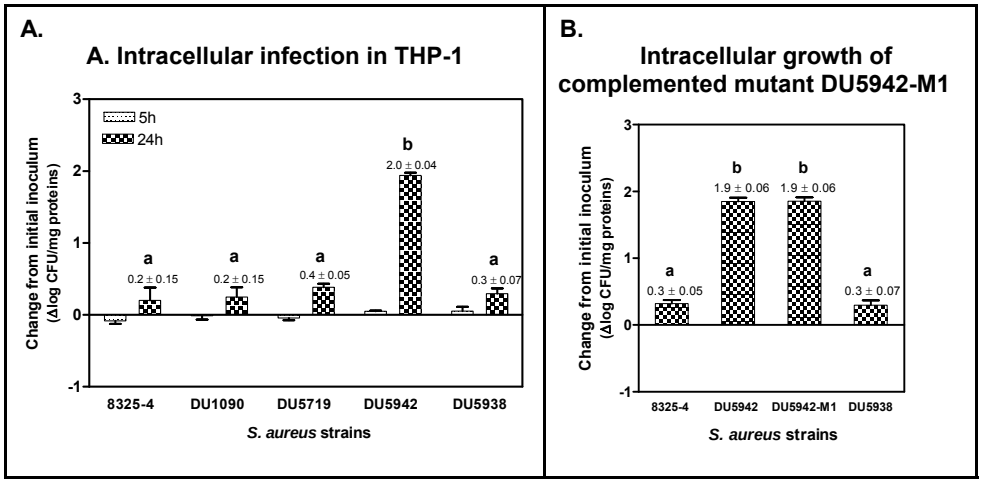


Figure 15 : A. Intracellular growth in THP-1 cells after 5h and 24h for strains 8325-4 (parental strain), DU1090 (*hla*-), DU5719 (*hlb*-), DU5942 (*hlg*-) and DU5938 (*hla*-, *hlb*-, *hlg*-). The intracellular growth was measured by the delta log CFU/mg protein at 5h or 24h post infection. **B.** Intracellular growth in THP-1 at 24h for strains 8325-4, DU5942, complemented mutant DU5942-M1 and DU5938. Plotted values are the mean ± standard deviation of three independent samples. Each experiment was reproduced at least twice with similar results. For statistical analysis (ANOVA), bars with different letters are significantly different from each others. (n=3; $p < 0.001$).

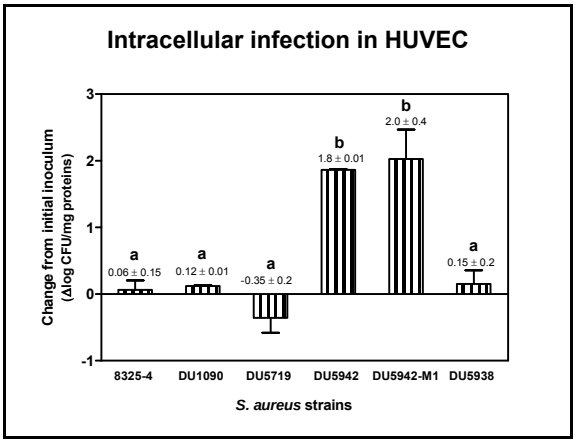


Figure 16 :
Intracellular growth at 24h in HUVEC. For statistical analysis (ANOVA), bars with different letters are significantly different from each others (n=3; $p < 0.001$).

5. Intracellular location

The intracellular location of the triple disruptant DU5938 was investigated using confocal microscopy. In THP-1, DU5938 colocalize almost exclusively with the LysoTracker dye labeling the acidic compartments. In HUVEC, the largest part of bacteria colocalize with acidic compartments but a small part of the FITC signal labeling the bacteria was clearly localized in a compartment that is not labeled by LysoTracker, exactly as described in the first chapter for the parental strain 8325-4 **[Fig. 17]**.

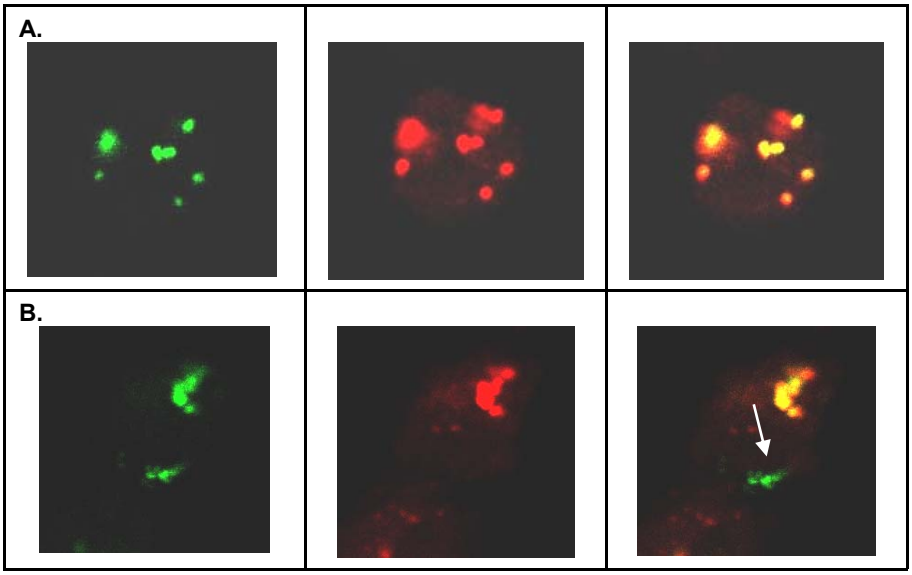


Figure 17 : Intracellular location of *S. aureus* DU5938. Confocal microscopy after 3h of infection. **A.** THP-1; **B.** HUVEC. Bacteria stained with FITC (left panels), acidic compartment stained with LysoTracker® Red (middle panels), merged images (right panels). The white arrow point to bacteria free in the cytoplasm. Observations were made under oil immersion with a 63x objective.

6. Conclusion

The results presented in this chapter indicate that alpha-, beta- and gamma-hemolysin are not required for the development of intracellular infection in macrophages and endothelial cells. The intracellular growth of all hemolysin-disrupted strains and parental strain 8325-4 was similar in HUVEC and THP-1 cells. *S. aureus* is able to cope with aggressive phagolysosomal environment and replicates in these acidic vacuoles in contrast to *Listeria monocytogenes* that need to escape from the phagosome for intracellular multiplication.

One could argue that, since bacteria are still enclosed in vacuoles in THP-1 after 24h, the action of hemolysins would occur after that time point, when the bacteria will finally leave these vacuoles. But we demonstrated here that, in HUVEC, a small number of bacteria were not localized in the acidic compartments and are believed to reside freely into the cytoplasm. Yet, no difference of intracellular location was noticed between the triple disruptant DU5938 and parental strain 8325-4. The mechanism allowing *S. aureus* to leave the phagolysosomes in HUVEC remains undefined but our data indicate that this mechanism, whatever it is, is highly regulated and influenced by the infected cell type. The fourth staphylococcal hemolysin, named delta-hemolysin, could be a candidate but some of its characteristics tend to indicate that this toxin is not the principal contributor of phagolysosomal escape. First, its mode of action that creates transitory pores and destabilizes membrane may not be sufficient to trigger the total destruction of phagosomal membrane **[68]**. Then its poor cell

specificity **[23]** and the evidence that its gene is expressed shortly after internalization **[77]**, while the bacteria remain confined in vacuoles for up to 24h in our models reduce the possibilities for a specific role of this toxin during phagolysosomal escape.

Other intracellular pathogens known to escape from the phagolysosomes include *Shigella flexineri*, *Listeria monocytogenes* or *Rickettsia* spp. Phagosomal escape by *L. monocytogenes* has been the most widely studied of that set of bacteria and is known to be mediated by the secretion of the pore-forming toxin listeriolysin O **[72]**. The escape mechanisms of other pathogens are less evident but appear to be associated with several proteins including phophoplipases. *Shigella* requires at least three proteins to escape from the phagosome. IpaB and IpaC probably create pores in phagosomal membrane while IpaD is needed for regulation and efficient insertion of IpaB and IpaC into the membrane **[63,92]**. *Rickettsia* seems to rely on *tld* and *tlyC* genes encoding respectively a putative phospolipase D and a protein displaying hemolytic activity **[92]**. Therefore we cannot exclude that *S. aureus* phagolysosomal escape depends on the secretion of several proteins, possibly including phospholipase(s).

CHAPTER IV:

S. aureus DU5942

1. Background

In the previous chapter we identified a strain of *S. aureus* with a very high intracellular growth (~2.0 log) in THP-1 cells and HUVEC. We demonstrated that this important intracellular growth was not due the disruption of gamma-hemolysin gene. We constructed a complemented mutant DU5942-M1 that presented the same intracellular growth than the gamma-hemolysin deficient strain DU5942. The expression of gamma-hemolysin in the complemented mutant DU5942-M1 was verified as well as plasmid stability after 24h of infection.

Here we attempted to further characterize strain DU5942. At first, we searched for a potential interference of the tetracycline resistance marker used to disrupt the gamma-hemolysin locus. Next we studied the intracellular location of DU5942, the growth at acidic pH and the kinetic of intracellular multiplication. Finally we tested the effect of antibiotics and oxidative stress on the intracellular development of DU5942.

2. Materials and methods

Antibiotics and reagents

Oritavancin was obtained from Targanta Therapeutics Corp., Cambridge, MA. Gentamicin and vancomycin were obtained as GEOMYCIN® and

VANCOCIN® distributed in Belgium by Glaxo-SmithKline s.a., Genval, Belgium. Oxacillin was purchased from Sigma-Aldrich, St-Louis, MO. Others reagents (reserpine, H_2O_2, catalase, L-NAME) were from Sigma-Aldrich.

Susceptibility testing

MICs and MBCs (minimal bactericidal concentration) were measured by microdilutions as described earlier **[75]**.

Intracellular infections, microscopy techniques, antibiotics dose-effect studies and resistance to hydrogen peroxide were performed exactly as described in previous chapters.

3. Disruption of hlg – role of TetK?

The locus *hlg* is composed of three genes encoding the three sub-units of gamma-hemolysin, HlgA, HlgB and HlgC. Disruption of *hlg* in strain DU5942 was obtained by excising a ~1.8 kb DNA fragment in *hlg* locus and inserting a ~2 kb tetracycline resistance gene **[55,80] [Fig.18]**. The *tetK* tetracycline resistance gene encodes an efflux pump **[30]**. In order to seek out a possible interaction of this efflux pump in our infection model we added a specific inhibitor of efflux pump, reserpine, during the 24h infection of THP-1 cells. The inhibitory effect of reserpine on the tetK efflux pump was initially checked with conventional microdilution MIC technique **[Tabl. 7]**. And it was demonstrated

that resperpine was active in the intracellular environment by Lismond *et al.* **[47]**.

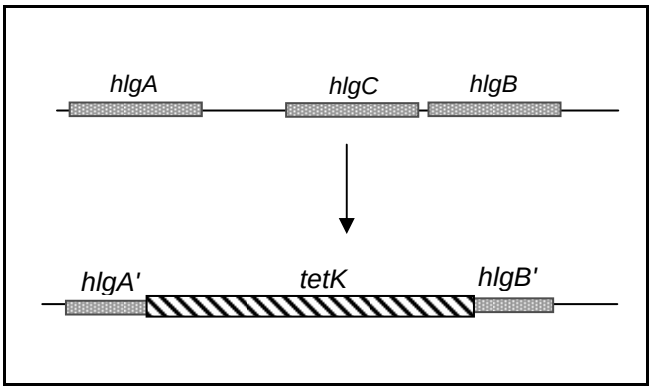


Figure 18 : Illustration of the disruption of gamma-hemolysin locus *hlg* with *tetK* gene.

S.aureus	**genotype**	**MIC Tet (µg/ml)**	**MIC Tet + resrpine (µg/ml)**
8325-4	*rsbU-*	< 0.25	< 0.25
DU5942	*hlg::* Tet^R *rsbU-*	8	0.25
DU5942-M1	*hlg::*Tet^R *rsbU-* pCU1-hlg+	8	0.5
DU5938	*hla::*Em^R *hlb::*Φ42E *hlg::*Tet^R *rsbU-*	8	< 0.25

Table 7 : Tetracycline MIC for the various *S. aureus* strains in absence or in presence of reserpine (20 µg/ml).

During the 24h intracellular infection, reserpine did not modify the intracellular growth of DU5942 and DU5942-M1 excluding a possible interference of *tetK* resistance marker in our infection model **[Fig. 19]**.

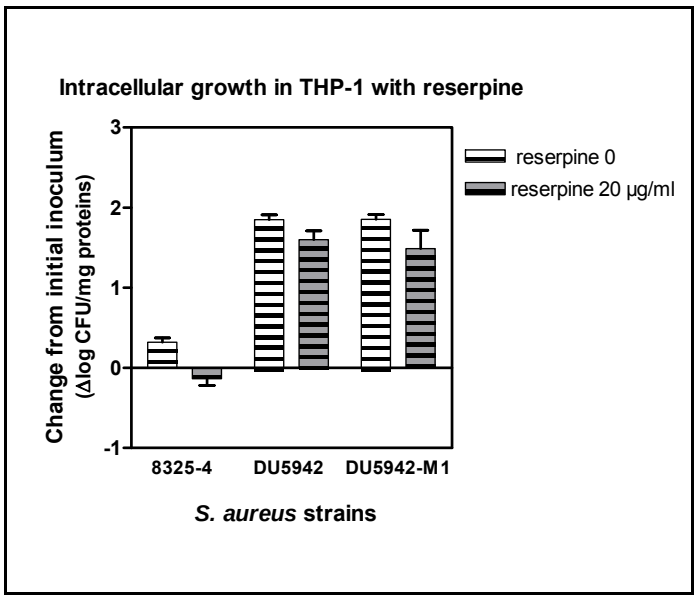


Figure 19 : Intracellular growth of strains 8325-4, DU5942 and DU5942-M1 with or without reserpine after 24h of infection in THP-1 cells

4. Intracellular location

Since DU5942 presents a very high intracellular growth, we first hypothesized that this could be related to a different intracellular location. But confocal and electron microscopy indicate that the intracellular position of DU5942 does not noticeably differ from that of strains 8325-4 or DU5938 as illustrated in previous chapters. In THP-1 cells, DU5942 is confined in acidic compartment whereas in HUVEC it appears to localize both in acidic compartment and in the cytoplasm **[Fig. 20]**. In some of the confocal microscopy pictures of infected HUVEC, a shadow of the cytosolic bacteria can be detected in the red channel.

This is due to the strong fluorescent signal of the FITC green dye that leaks into the red channel.

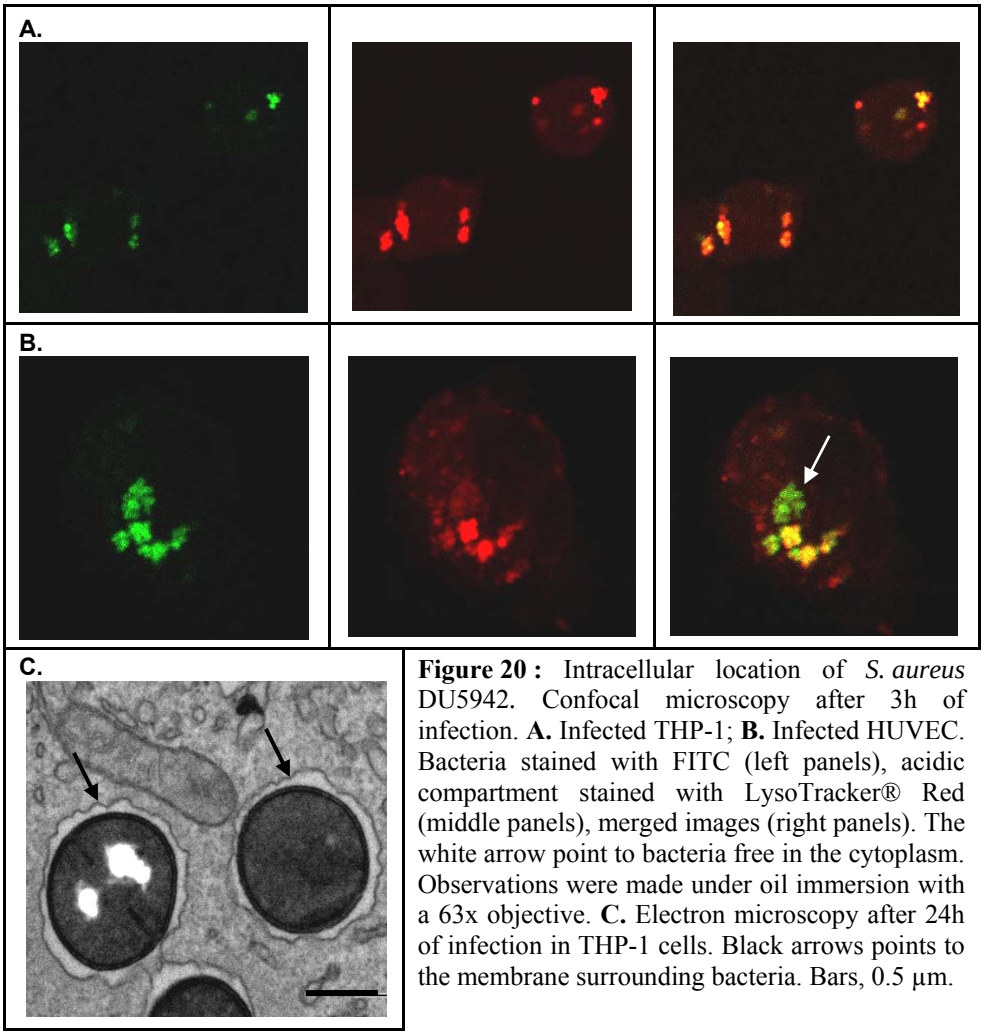


Figure 20 : Intracellular location of *S. aureus* DU5942. Confocal microscopy after 3h of infection. **A.** Infected THP-1; **B.** Infected HUVEC. Bacteria stained with FITC (left panels), acidic compartment stained with LysoTracker® Red (middle panels), merged images (right panels). The white arrow point to bacteria free in the cytoplasm. Observations were made under oil immersion with a 63x objective. **C.** Electron microscopy after 24h of infection in THP-1 cells. Black arrows points to the membrane surrounding bacteria. Bars, 0.5 µm.

5. Growth at pH 5.5 and 7.2

As DU5942 appears to multiply in acidic compartment during intracellular infection, we look for a better tolerance or a higher growth of this strain at acidic pH that could explain the higher intracellular growth. We compared the growth in broth at pH 7.2 and 5.5, the latest corresponding to the lysosomal pH **[61]**. However all the strains tested had identical growth at both pH **[Fig. 21]** indicating that the important intracellular growth of DU5942 could not be attributed to a better tolerance of acidic environment.

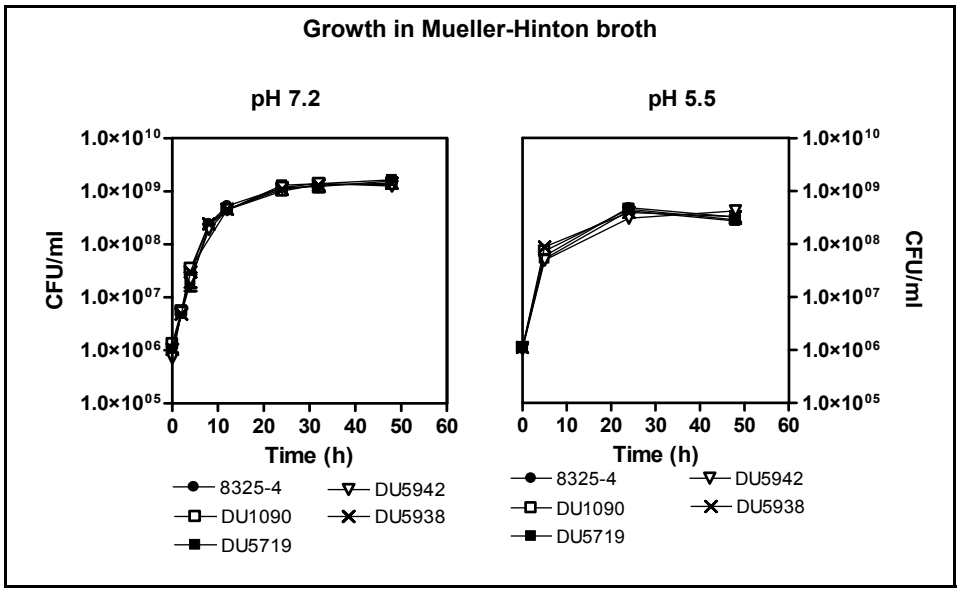


Figure 21 : Bacterial growth in Mueller-Hinton broth at pH 7.2 and 5.5.

6. Kinetic of intracellular growth

In the previous chapter we demonstrated that the intracellular inoculum of all hemolysin-disrupted mutants and parental strain was unmodified after 5h of infection indicating that growth set off between 5h and 24h **[see Fig. 15A]**. We wondered if the important intracellular growth of DU5942 may be correlated to a shorter latency phase and an earlier start of multiplication. Therefore we studied the intracellular growth at different times post infection. For all the strains the growth seems to start between 12h and 16h but reaches a "stationary phase" at 16h post infection for *S. aureus* 8325-4 and DU5938 while DU5942 and DU5942-M1 keep on multiplying. Thus DU5942 does not start earlier its intracellular multiplication but instead appears able to reach a higher equilibrium status in the ratio of intracellular bacteria per cell **[Fig. 22]**.

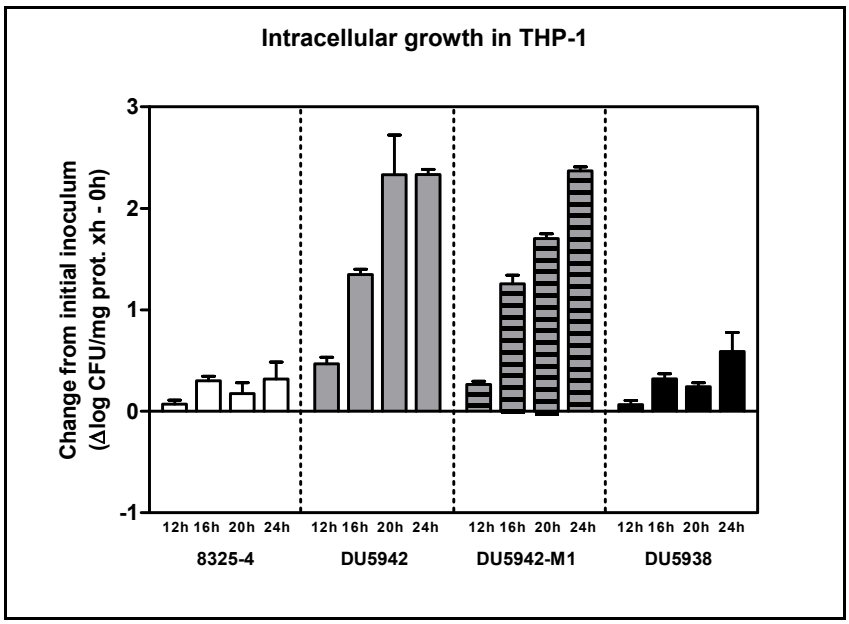


Figure 22 : Intracellular growth in THP-1 cells after 12h, 16h, 20h and 24h for strains 8325-4 (parental strain), DU5942 (*hlg*-), DU5942-M1 (*hlg*-, pCU1-*hlg*+) and DU5938 (*hla*-, *hlb*-, *hlg*-).

7. Effect of antibiotics

In the following set of experiments, we tested the effect of antibiotics on the intracellular growth of *S. aureus* DU5942. In the protocol of infection of both THP-1 cells and HUVEC, cells are incubated with gentamicin (1 x MIC) to prevent the development of extracellular bacteria. We therefore examined in more details the effect of gentamicin against extracellular and intracellular bacteria. All strains had similar minimal inhibitory concentration (MIC) and

minimal bactericidal concentration (MBC) in Mueller-Hinton broth and these were higher at acidic pH **[Tabl. 8]**. When exposed to increasing concentrations of gentamicin in Muller-Hinton broth, *S. aureus* 8325-4, DU5942 and DU5942-M1 present identical responses **[Fig. 23]**. In the intracellular environment the maximal effect (E_{max}) of gentamicin was similar for the three strains however, DU5942 and its complemented mutant appeared more tolerant to gentamicin since a higher concentration of this antibiotic was necessary to reach a static effect after 24h of infection (C_{static}) **[Fig. 23, Tabl. 9]**. No difference was seen between theses strains with other antibiotics like oxacillin, vancomycin or oritavancin and their intracellular activity was similar as what was observed in chapter II for strain 8325-4 and SH1000. (Data not shown)

	pH 7.2		pH 5.5	
Strains	**MIC (µg/ml)**	**MBC (µg/ml)**	**MIC (µg/ml)**	**MBC (µg/ml)**
8325-4	0.125	0.25	2	2
DU5942	0.25	0.5	2	4
DU5942-M1	0.25	0.25	2	8
DU5938	0.125	0.25	2	4

Table 8 : Gentamicin minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) in Mueller-Hinton broth.

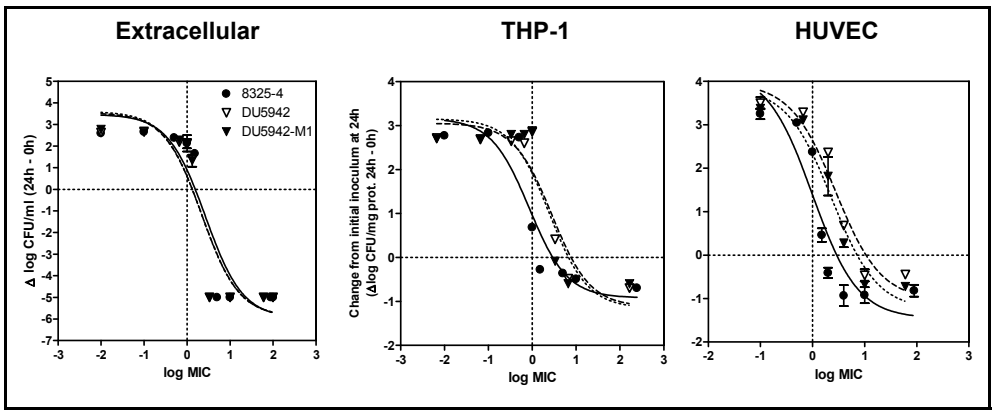


Figure 23: Dose-response curves of gentamicin against *S. aureus* 8325-4 (black circles – continuous line), DU5942 (white triangles – broken line) and DU5942-M1 (black triangles – dotted line) in Mueller-Hinton broth (left panel), THP-1 (middle panel) and HUVEC (right panel). The graphs show the variation in the number of CFU per ml of culture medium (left panel) or the variation in the number of CFU per mg of protein (middle and right panels) (Δlog CFU 24h-0h; means ±SD; n=3 ; most SD bars are smaller than the symbols). The horizontal dotted line represents a bacteriostatic effect. The vertical dotted line represents the concentration corresponding to MIC. Sigmoidal dose-reponse curves were obtained by non-linear regression. See table 9 for regression parameters, statistical analysis, pharmacological and microbiological descriptors.

	Extracellular			Intracellular _ THP-1			Intracellular _ HUVEC		
Antibiotics	**8325-4**	**DU5942**	**DU5942-M1**	**8325-4**	**DU5942**	**DU5942-M1**	**8325-4**	**DU5942**	**DU5942-M1**
Gentamicin									
MIC (µg/ml)	0.2	0.3	0.3						
E_{max} (95% CI) (Δlog CFU/mg)	-5.96 [a] (-8.55 to -3.36)	-5.89 [a] (-8.75 to -3.04)	-5.89 [a] (-8.72 to -3.07)	-0.92 [b] (-2.16 to 0.32)	-1.12 [b] (-2.66 to 0.42)	-1.16 [b] (-3.05 to 0.74)	-1.46 [b] (-3.13 to 0.22)	-1.04 [b] (-2.93 to 0.85)	-1.26 [b] (-2.85 to 0.34)
EC_{50} (95% CI) (µg/ml)	0.51 [A] (0.12 to 2.07)	0.65 [A] (0.14 to 2.93)	0.65 [A] (0.15 to 2.84)	0.17 [A] (0.04 to 0.81)	0.85 [A] (0.19 to 3.69)	0.75 [A] (0.12 to 4.53)	0.20 [A] (0.04 to 1.03)	0.84 [A] (0.15 to 4.73)	0.71[A] (0.16 to 3.16)
C_{static} (µg/ml)	0.31	0.39	0.39	0.60	2.34	2.19	0.59	3.16	2.18
R^2	0.894	0.866	0.869	0.883	0.898	0.856	0.863	0.943	0.958

Table 9 : Microbiological and pharmacological parameters of dose-response curves. MIC = minimal inhibitory concentration (µg/ml), E_{max} = maximal effect expressed as the reduction in CFU at 24h from the original inoculum (Δlog CFU/mg of protein 24h-0h), EC_{50} = concentration causing a reduction of the inoculum halfway between the minimal (E_0) and the maximal (E_{max}) values as obtained from the Hill equation (slope factor of 1); C_{static}= concentration resulting in no apparent bacterial growth after 24h as determined by graphical interpolation. For statistical analysis, values with different letters are significantly different ($p < 0.05$) from each other within the pertinent comparison group. Lowers case letter, comparison of E_{max} for the three strains (8325-4, DU5942, DU5942-M1) and the three conditions (Extracellular, THP-1 and HUVEC). Upper case letter, comparison of EC_{50}.

8. Oxidative stress

Eucaryotic cells possess diverse protection mechanisms against intracellular pathogens including the secretion of hydrolytic enzymes in phagolysosomes and production of oxygen reactive species (ROS) like hydrogen peroxide (H_2O_2), nitric oxide (NO) or superoxide anion (O_2^-) **[19,26]**.

In the first chapter, we showed that the higher intracellular growth of *S. aureus* SH1000 was associated with a higher resistance to hydrogen peroxide. Therefore we tested the resistance of strain DU5942 to oxidative stress. Extracellularly, the resistance to H_2O_2 was not significantly different for DU5942 and parental strain 8325-4 or triple disruptant DU5938 **[Fig. 24A].** Nonetheless to check a possible effect inside cells, we assessed the intracellular growth in presence of L-NAME (N_ω-nitro-L-arginine methyl ester), an inhibitor of NO synthase, or catalase, an enzyme converting H_2O_2 to water and oxygen. After 24h of infection in THP-1 cells, L-NAME had no effect on the intracellular growth of DU5942, 8325-4 or DU5938. In contrast, the addition of catalase severely reduces the intracellular growth of DU5942 (- 1.4 log) while having no apparent effect on the development of 8325-4 or DU5938 **[Fig. 24B]**.

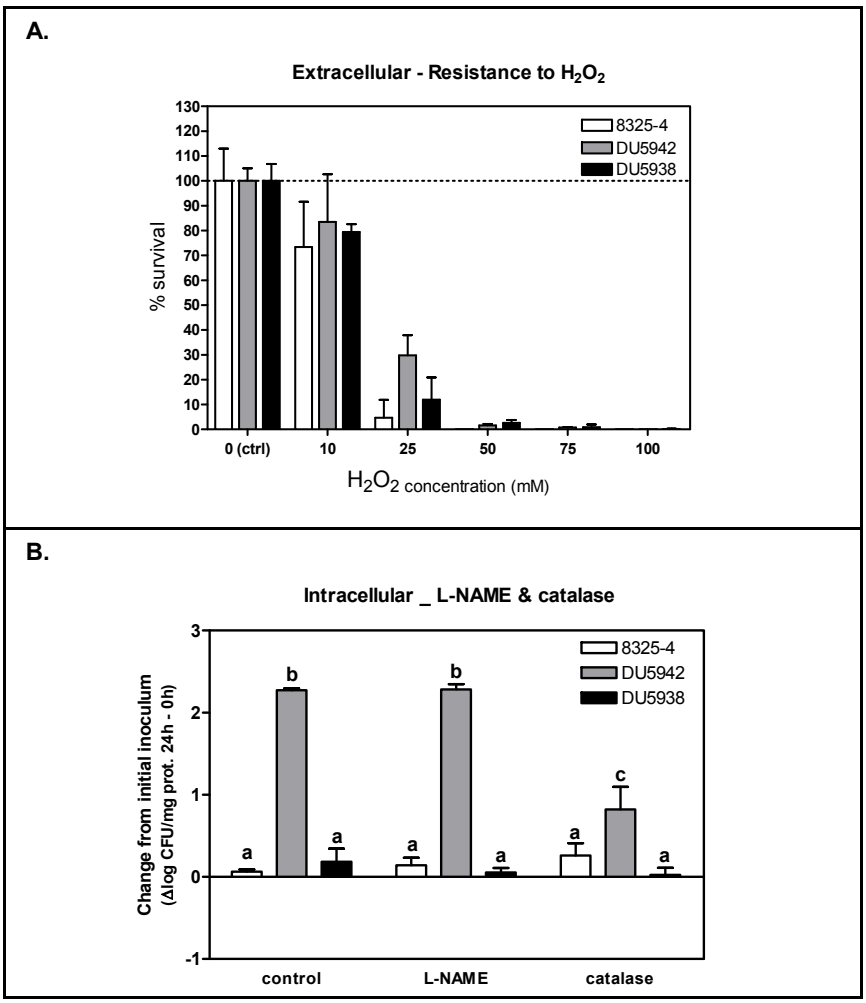


Figure 24 : Resistance of strains 8325-4, DU5942 and DU5938 to oxidative stress. **A.** Effect of H_2O_2 in extracellular culture. For each concentrations of H_2O_2, resistance of 8325-4, DU5942 and DU5938 were not statistically different (ANOVA; n=3; $p>0.05$) **B.** Intracellular infection in THP-1 with control samples, or in presence of 400 µmole/L of L-NAME or 1500 U/ml of catalase. For statistical analysis (ANOVA), bars with different letters are significantly different from each others (n=3; $p < 0.001$).

9. Conclusion

In this chapter we attempted to characterize *S. aureus* DU5942 and try to get indications on the origin of its important intracellular growth. We could not identify the probable mutation(s) allowing a higher intracellular growth to this strain however we eliminated some potential explanations. First we could exclude an interaction of the tetracycline resistance mechanism used to disrupt the *hlg* locus. Then we demonstrated that the intracellular location, the growth at acidic pH and the intracellular latency phase of strain DU5942 were not different from that of parental strain 8325-4 or the triple disruptant DU5938.

In a second time we tested the activity of antibiotics. In broth, *S. aureus* 8325-4, DU5938, DU5942 and its complemented mutant DU5942-M1 have similar gentamicin MIC and MBC which are higher at acidic pH. This reduced efficacy of gentamicin at pH 5.5 is due to an impaired transport into bacteria at acidic pH **[24]**. All the strains presented the same response in extracellular (broth) dose-effect studies. In contrast, DU5942 and DU5942-M1 showed a tolerance to gentamicin that seems to be expressed only in the intracellular environment. This appears as a shift of the dose-effect curve toward the right indicating that a higher concentration of gentamicin is required to reach a static effect. This tolerance to gentamicin could be part of the explanation to the important intracellular growth of DU5942. In both infection models (THP-1 and HUVEC), gentamicin at low concentration (1x MIC) is added to the cell culture media to prevent the development of extracellular bacteria. Gentamicin penetrates very

slowly inside eukaryotic cells and has reduced activity at acidic pH **[84]** and we showed that the bacteria co-localised essentially in acidic compartments. Therefore, gentamicin appeared well appropriate since it can prevent extracellular contamination with a minimal effect on intracellular bacteria. Other described methods to prevent extracellular contamination include the addition of lysostaphin, an endopeptidase that specifically cleaves the cell wall of staphylococci **[14,37]**. But when tested in our infection models, lysostaphin led to highly irreproducible results. But, although a low concentration of gentamicin in culture media is expected to have a minimal effect on intracellular bacteria, a difference in the intracellular response to this antibiotic could lead to a more efficient intracellular multiplication of *S. aureus* DU5942. No difference was observed extracellularly or intracellularly with oxacillin, vancomycin and oritavancin. And the activity of all these antibiotics was similar to the activity observed for *S. aureus* 8325-4 and SH1000 in chapter II.

Finally we tested the effect of oxidative stress. When exposed to increasing concentrations of H_2O_2 in extracellular culture, DU5942 shows the same survival rates than 8325-4 and DU5938. During intracellular infection, the addition of L-NAME and subsequent inhibition of NO production does not seem to affect the intracellular development of strains 8325-4, DU5942 or DU5938. In contrast the addition of catalase reduced the intracellular growth of DU5942 but had no noticeable effect on 8325-4 and DU5938. Thus the presence of H_2O_2 appears to contribute to the important intracellular growth of DU5942. We could not link this particular effect of gentamicin and H_2O_2 to a specific gene, or a metabolic or

regulatory path of *S. aureus* but in both case, this characteristic appears to be expressed only when the bacteria are intracellular.

GENERAL DISCUSSION

The research fields of our laboratory explore the interactions between antibiotics and eucaryotic cells, the activity of antibiotics against sensible and resistant microorganisms, and the intracellular survival of pathogens. In two studies published in 2005, it was shown that, for the most part, the intracellular activity of antibiotics in macrophages differed from their extracellular activity and that this intracellular activity was also different against two major human pathogens, namely *L. monocytogenes* and *S. aureus* **[46,74]**. The fate of intracellular *L. monocytogenes* has been widely investigated and its intracellular cycle was well described. In contrast little was known about the fate of *S. aureus* once it has been internalized. It was reported that *S. aureus* could penetrate inside different cell types but the mechanisms allowing its intracellular survival were poorly understood. Moreover, it was thought that the intracellular residency possibly contributes to the relapses frequently observed in chronic staphylococcal infections such as osteomyelitis and endocarditis which are often difficult to eradicate even after prolonged and adapted treatment. This work was therefore designed to get further in our knowledge of *S. aureus* intracellular fate; explore the role of some virulence factors that could potentially be involved in the intracellular survival, and compare the intracellular fate of this pathogen as well as the activity of antibiotics in different cell types.

1. Main findings of this work

In this work, we showed that *S. aureus* were efficiently internalized in phagocytic cells THP-1 but also in non-professional phagocytes endothelial cells. *S. aureus* proliferated and reached similar intracellular growth in both cells types. After 24h of infection in THP-1, *S. aureus* was confined in acidic compartments and was able to replicate in these vacuoles. Our observations correlate with a recent publication showing that *S. aureus* could persist up to four days inside macrophages vacuoles before moving to the cytoplasm **[43]**. In HUVEC the intracellular location was slightly different. Most bacteria were trapped in acidic compartments but a small number did not colocalize with the acidotropic marker and appeared to be free in the cytoplasm. In contrast, in epithelial cells, the largest part of bacteria were reported to escape from the vacuoles and were free in the cytoplasm as soon as 3h post-infection **[6,77]**. This discrepancy of intracellular location between the various cell types tends to indicate that cell-specific factor(s) influence the intracellular development of *S. aureus*. There is a sort of gradation in the persistence inside phagolysosomal compartment which looks very short in epithelial cells, intermediate in endothelial cells, and longer in macrophages. Long persistence of a large number of bacteria in the cytoplasm has not been described so far, and the escape from phagolysosomes generally results in subsequent lysis of the host cell **[6,43,77]**. Thus the phagolysosomal persistence is most probably correlated with the general intracellular persistence.

The factors influencing the phagolysosomal escape of *S. aureus* are still unidentified but this divergence between epithelial cells, endothelial cells and macrophages may somewhat explain the success of staphylococcal infections. Epithelial cells are, in many situations, the primary border the bacteria have to overcome to settle the infection. The ability to enter epithelial cells conjugated with a short time of intracellular persistence allows *S. aureus* to spread more rapidly to adjacent cells and deeper tissues. *S. aureus* is frequently responsible for skin infections with important tissue necrosis and extensive spreading **[94]**. In contrast, a longer persistence inside endothelial cells shields the bacteria from the extracellular host defense mechanism and might take part to the persistence and recurrence regularly observed in infections like endocarditis **[94]**. Finally the long persistence in mobile macrophages may contribute to the ability of the pathogen to invade the vascular system from a localized infection site and disseminate in other organs and tissues.

A second objective of this work was to compare the activity of antibiotics against intracellular *S. aureus* in both phagocytic and non phagocytic cells. Most antibiotics had a poor activity against intracellular bacteria with the exception of oritavancin that reduced the intracellular inoculum up to 3 log. No difference of activity was detected between phagocytic and non phagocytic cells. The small number of cytosolic bacteria observed in endothelial cells is probably too scarce to notice a difference of activity with antibiotics like oxacillin which is localized in the cytoplasm **[84]**. In addition, we cannot exclude that the cytosolic bacteria

in HUVEC may have an altered metabolism that could reduce their susceptibility to antibiotics **[29]**.

A third objective was to identify bacterial virulence factors that may influence the intracellular survival of *S. aureus*. We showed that a deficiency in phosphatase rsbU and the ensuing down regulation of transcriptional factor sigmaB affects the intracellular survival of *S. aureus*. A strain of *S. aureus* expressing a functional rsbU had a greater level of internalization in endothelial cells, a much higher intracellular growth after 24h of infection and was more resistant to oxidative stress compared to the rsbU-deficient strain. Among the factors influenced by sigmaB, we proposed that the carotenoid pigment staphyloxanthin might contribute to intracellular survival of *S. aureus*. The strain with functional rsbU produces noticeably more staphyloxanthin and it was demonstrated that this membrane-bound pigment confers protection against oxidative stress and neutrophil killing **[21]**. Inhibition of staphyloxanthin synthesis attenuates the virulence of infection in animal model **[48]**. Our results indicate that the higher intracellular growth and higher resistance to hydrogen peroxide observed after rsbU-restoration are largely related to the production of staphyloxanthin. However the addition of staphyloxanthin inhibitors did not modify the internalization of SH1000 suggesting that the higher intracellular growth and higher internalization of this strain result from two independent mechanisms. We suggested that the enhanced internalization of SH1000 may be related to the down-regulation of agr by sigmaB resulting in an up-regulation of several adhesion proteins such as the fibronectin-binding proteins. Hence

staphyloxanthin appears to confer a significant advantage for intracellular survival but probably contribute with many other factors in the global development of intracellular infection.

We also investigated the role of hemolysins in the development of intracellular infection. After 24h of infection, some bacteria appeared free in the cytoplasm of endothelial cells indicating that they had to escape somehow from the phagolysosomes. Yet, the disruption of alpha-, beta- and gamma-hemoysin seemed to have no influence on the intracellular location of *S. aureus* in endothelial cells. We hypothesized that the escape from the phagolysosome relies on at least one other unidentified factor possibly a phospholipase as it was described for others intracellular pathogens **[63,92]**.

Our results may seem contradictory with some publications suggesting that alpha-hemolysin is important for intracellular development and is involved in phagolysosomal escape in epithelial cells **[69,77]**. This apparent discrepancy could be related to the different infection models used. Indeed from these publications it appears that *S. aureus* escapes from the vacuoles of epithelial cells much earlier than what we observed in endothelial cells and macrophages. It is therefore possible that *S. aureus* virulence factors are differentially regulated in function of the infected cell type. Moreover, these publications suggested a role for alpha-hemolysin but so far it was never demonstrated that alpha-hemolysin was sufficient to mediate phagolysosomal escape alone or in combination with another virulence factor. Shompole *et al.* **[77]**, showed that

agr expression was induced before the phagolysosomal escape in epithelial cells and that an *agr* mutant could not escape from vacuoles. These authors proposed that this escape was mediated by alpha-hemolysin since they observed an increase of *hla* expression following agr activation. Nonetheless, as described earlier in this work, agr regulates the expression of many virulence factors among which the four hemolysins but also various enzymes such as lipases, proteases or nucleases. Many of these virulence factors are most probably also up-regulated upon agr activation and could as well be responsible for phagolysosomal escape.

In macrophages, it was demonstrated that alpha-hemolysin was important for long term persistence since a *hla*-deficient mutant was eradicated by two days while the wild-type strain persisted intracellularly up to five days. However, in this study, no significant difference in the intracellular location of the WT and *hla* mutant was noticed thus correlating with our observations **[43]**.

2. Interests and limits of our intracellular infection models

2.1. Interest of the models

Our infection models allowed us to compare the intracellular behavior of *S. aureus* in professional phagocytes and non-professional phagocytes. We selected two human cell types, the macrophages THP-1 and endothelial cells

HUVEC. These two cell types presented multiple advantages for our project. First, these cells are from human origin and therefore may be more appropriate to study models of human infections. Then, both cell types have been largely used in diverse fields of research and it would permit to compare our results with previously published data **[50,89]**.

We performed this study with a set of *S. aureus* strains that were all derived from a common parental strain. This allowed us to compare the effect of specific virulence factors while avoiding interferences due to the large variation in the level and nature of synthesized proteins frequently observed between strains from different lineages.

In our two intracellular infection models, we used similar and carefully defined experimental procedures to assess the internalization, the intracellular growth and the bacteria subcellular location. We could also explore the contribution of specific virulence factors, and put in light the importance of the transcription factor sigmaB and the golden pigment staphyloxanthin in the intracellular development of *S. aureus*. More generally these models may be helpful to evaluate the role of any specific bacterial virulence factors suspected to take part to the internalization or early step of intracellular development, but also to study the activity of new and old antibiotics or combination of drugs, and any substance that would modify the expression and regulation of bacterial virulence factors, like described in this work with the inhibitors of staphyloxanthin synthesis.

2.2. Limits of models

Although our two infection models gave us valuable information on the intracellular fate of *S. aureus*, they have some of limitations. First, the selected phagocytic cells THP-1 are rather "naïve" cells and this characteristic was exploited in our model since it permitted the intracellular multiplication. However, our results can not be directly extrapolated to other types of phagocytic cells that display more active cellular defense mechanisms.

Next, the *S. aureus* strains selected for this work were all isogenic derivative of strain 8325-4. *S. aureus* 8325-4 is a laboratory strain cured of all prophage **[56]** and does not possess any of the specific disease-related toxins (SEs, ETs, TSST-1 and PVL) or antibiotic resistance mechanisms. This strain may be very useful for genetic studies, to investigate the specific mode of action virulence factors or their role in infections. Yet, the observations made with this strain can not simply be extrapolated to the various clinical isolates that generally possess a varying collection of antibiotic resistance and disease-related toxins.

Finally, these *in vitro* models can bring valuable information on the complex interactions between bacteria and host cells nonetheless they do not take into account many parameters that are present during infection *in vivo* including host immune defense mechanisms, bacterial propagation or antibiotics distribution and accessibility to the site of infection. In addition our experiments were limited to 24h and could not evaluate the persistence for several days. So in

order to get a more complete view of the infection process, these infection models will need to be complemented with *in vivo* animal studies.

3. Perspectives

3.1. Short term perspectives

What's happening next ?

Our results established that, after 24h of infection, bacteria were confined in the acidic phagolysosomes in THP-1 cells while in HUVEC the intracellular location was slightly different. Most bacteria were also located within the acidic compartments but a small number did not colocalize with the acidotropic marker and were apparently free into the cytoplasm.

A recent publication demonstrated that *S. aureus* could persist several days inside macrophages **[43]**. This raises a first major question from our observations; what is the fate of intracellular bacteria after the first 24h of infection?

A first approach would be to check the evolution of intracellular inoculum. Did the intracellular bacteria reach a plateau? Will they keep on multiplying or finally be killed by the cells? Is the overall intracellular persistence equal in both

cell types? To get some answers, our two infection models should be optimized in order to extend the time of infection. This requires some adaptation of our standard protocols like changing culture media once or twice a day, checking the absence of extracellular contamination, and possibly adapting the initial amount of cells to ensure a sufficient number of living cells at the end of the infection process.

In a second time it would be interesting to study deeper the phagolysosomal persistence and difference of location between THP-1 and HUVEC. From the cellular point of view; what are the cellular factors influencing the bacterial intracellular location? And, more generally, what are the factors involved in the cell response to bacterial invasion? Is there any difference in the response of THP-1 and HUVEC? A first approach would be to compare the gene expression profile of uninfected and infected cells by RNA microarray. A second technique, complementary to microarray, would be to extract the total proteins of cells and compare the proteomic profiles in 2-D gels electrophoresis.

From the bacterial point of view; what is/are the factor(s) involved in phagolysosomal escape in HUVEC? Are these factors differentially regulated in HUVEC and THP-1? A clear way to answer this would be to clone the virulence factor(s) suspected to be involved in phagolysosomal escape in another bacterial species that would, in normal conditions, be trapped in phagolysosomes. This simple and clever technique was used by Bielecki *et al.* that inserted the gene of listeriolysin O from *Listeria monocytogenes* into the gram positive *Bacillus*

subtilis and demonstrated that this protein was sufficient to induce the phagosomal escape of *B. subtilis* **[8]**. Nonetheless, this procedure necessitates a strong presumption of the factor(s) involved in the lysis of phagosomes. A more complete approach was used to identify the factors involved in phagosomal escape of *Rickettsia prowazekii* **[92]**. This second method had the advantage to explore the implication of multiple factors. At first, these authors identified potential virulence factors responsible for phagosomal escape by searching which membrane-damaging toxins were specifically expressed before the bacteria got out of phagosomes. Among the four potential toxins they had selected, only two were expressed before the escape into the cytoplasm. Next they inserted theses two factors named *tlyC* and *pld* in *Salmonella enterica*, an intracellular pathogen that naturally replicates in the vacuoles, and demonstrated that *tlyC* and *pld* were both involved in phagosomal escape. The identification of *S. aureus* virulence factors that allow the bacteria to escape into the cytoplasm, will probably required a similar two steps process. The first step necessitates the detection of the toxins and enzymes that are up-regulated prior bacterial escape and, among them, select those that are more susceptible to contribute to the lysis of phagosomal membrane. In a second step, the selected factors should be expressed alone or in combination in a bacterial species that normally does not reside in the cytoplasm.

Is Staphyloxanthin a major virulence factor involved in intracellular persistence?

In chapter I, we demonstrated that *S. aureus* SH1000 presented a higher intracellular growth and resistance to hydrogen peroxide and suggested that this was related to a higher production of staphyloxanthin. Additional information on the capacity of the sole staphyloxanthin pigment to induce intracellular survival could be obtained by the cloning process described in the previous paragraph. Expressing the operon *crtOPQMN* that encodes the five enzymes required for staphyloxanthin production **[66]** in a non-pigmented and non-pathogenic bacterial species could bring more information on the general properties of this pigment. Is the pigment sufficient to induce the intracellular persistence of another bacterial species? Could the pigmented bacteria proliferate more inside the cells compared to the non-pigmented control?

Another aspect that should be investigated is the long term persistence (over 24h) of 8325-4 and SH1000. Does *S. aureus* SH1000 persist longer inside cells than *S. aureus* 8325-4? Is the persistence similar in HUVEC and THP-1? *In vivo*, could *S. aureus* SH1000 induce more recurrent or long lasting infections than 8325-4?

What is the origin of the high intracellular growth of *S. aureus* DU5942?

In the last chapter of this work we described a strain of *S. aureus* with remarkable intracellular growth. In addition this strain demonstrated an intracellular tolerance to gentamicin and resistance to oxidative stress. A first approach to try to identify the mutation(s) responsible for this particular intracellular behavior would be to compare the expression profile of DU5942 and parental strain 8325-4 by RNA microarray. This technique could put in light a modification in the expression of one or multiple genes. Nonetheless this method has some limits. First, our results tend to indicate that the specific properties of DU5942 are expressed only in the intracellular environment. Therefore we would have to compare the expression profile of DU5942 and 8325-4 in broth culture and during intracellular infection. Then, we cannot exclude that the new properties of DU5942 are the consequence of a point mutation, or a small deletion that does no't induce any modification in the expression of the altered gene. In this case, the mutated gene could not be identified by microarray.

How can we get a global view of virulence expression during infection?

To investigate the potential role of a specific virulence factors during infection, many scientists compared the virulence of a mutant deleted for this specific factor to its wild type parental strain. In this work we have used the same

method to explore the implication of three hemolysins and one global regulator on the development of intracellular infection. This technique appears very helpful to put in light the contribution of a particular factor but does not give a general view of the overall set of proteins, enzymes and metabolic pathways involved in infection.

Global *in toto* studies can bring a more complete view of the effective virulence of a strain or on the overall modifications of gene expression during infection. The study of the entire transcriptome or proteome of a strain during an infection gives a global view of the expression of the virulence-related genes, as well as general metabolic processes that all together contribute to the development of infection. Therefore *in toto* studies are a useful complement to the more classical studies that concentrate on one or a few virulence factors or regulators. For example, a recent study of *S. aureus* whole genome expression demonstrate that, upon internalization in endothelial cells, genes involved in metabolic pathways such as cell division or nutrient transport were down-regulated whereas numerous genes involved in iron scavenging and virulence were up-regulated **[29]**. In the same way it could be interesting to compare the transcriptome or proteome of our two strains 8325-4 and SH1000 to get a larger view of the key elements involved in intracellular infection.

3.2. Long term perspectives

in vivo models

After the acquisition of significant information with *in vitro* models it appears natural to study the implication of intracellular persistence *in vivo*. What is the contribution of intracellular persistence in the global severity of infection in animal models? What is the impact of intracellular persistence in the different type of staphylococcal infections? To achieve this, we could search for intracellular persistence and compare the virulence potential of strain 8325-4 and SH1000 in endocarditis **[52]** or osteomyelitis infection models **[25]** and cutaneous abscess model **[41]**.

Characterization of clinical strains / prevision of infective potential

Next, our infection models could be employed to compare the invasive potential or cytotoxicity of clinical strains of *S. aureus* in order to correlate their behavior in *in vitro* models with the severity of clinical symptoms or the persistence of infection. Could some strains be more predisposed to induce intracellular persistence? Can we identify specific factors in strains isolated from recurrent pathologies that could be linked to the intracellular persistence?

Effect of cytokines on the intracellular development of *S. aureus*

The innate immune system represents the first line of defense against bacterial invasion.

It consists of professional phagocytes of the granulocytes and monocytes lineage, including polymorphonuclear leucocytes (PMNs), circulating monocytes and tissue-based macrophages **[35]**. Genetic disorders affecting constituents of the innate immune system are generally associated with a higher susceptibility to bacterial infection. One of these pathologies, the chronic granulomatous disease (CGD) is a primary immune deficiency (affecting 1/200.000 newborn infants) caused by a defect in phagocyte bactericidal activity that results in recurrent and severe infections **[79]**. Phagocytes are unable to kill ingested microorganisms which lead to the formation of granulomas and abscesses. At the molecular level, the disease is caused by mutations in the gene encoding the NADPH oxydase, an enzyme involved in the production of reactive oxygen species for the destruction of intracellular pathogens. *S. aureus* and *Aspergillus* spp. are among the most frequently implicated microorganisms in CGD infections **[79]**.

The therapeutic use of cytokines such as interferon-γ (IFN-γ) or granulocyte-macrophage colony-stimulating factor (GM-CSF) to augment the activity of the innate immune system has proven to be helpful in the treatment of various pathologies including CGD **[35]**. IFN-γ is a major activator of monocytes, macrophages and PMNs but its activity extends to many other cell types

including endothelial and epithelial cells. This cytokine is recognized to play a critical role in macrophage-mediated killing of important intracellular pathogens including *Mycobaterium*, *Rickettsia*, *Legionella* and *Chlamydia* species and it has been approved by the FDA for the treatment of individuals with CGD. **[35]**. The GM-CSF stimulates the proliferation, differentiation and enhances the bactericidal activity of PMNs, monocytes and macrophages **[35]**. Contradictory results have been reported when considering the effect of GM-CSF during staphylococcal infections. When administered prophylactically, GM-CSF significantly improved survival in a neonatal rat model of staphylococcal sepsis **[28]**. In contrast GM-CSF failed to improve survival in murine arthritis **[87]**.

In former publication from our laboratory, it was demonstrated that gamma interferon IFN-γ and GM-CSF impair the intracellular growth of *L. monocytogenes* **[16,62]** by an enhanced production of nitric oxide (NO) and hydrogen peroxide (H_2O_2). Hence it would be interesting to test the effect of these two cytokines on the development of intracellular infection due to *S. aureus*, particularly with *S. aureus* SH1000 that shows a higher resistance to cellular defense mechanism.

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ANNEXES

Participation to congress.

18th ECCMID 2007 - Poster session P1060, Sunday, April 20

Identification of a Staphylococcus aureus strain with increased intracellular growth and reduced intracellular susceptibility to gentamicin

A. Olivier, F. Van Bambeke, M.P. Mingeot-Leclercq, P.M. Tulkens

12th International Symposium on Staphylococci & Staphylococcal infections _ ISSSI 2006 - Poster no. P-021

Intracellular infection of THP-1 human macrophages by Staphylococcus aureus and role of hemolysins

A. Olivier, S. Lemaire, M.P. Mingeot-Leclercq, F. Van Bambeke, P.M. Tulkens

3ème Colloque Francophone de Bactériologie Vétérinaire (Liège 11/07/06 – 13/07/06)

Modèle d'infection intracellulaire de macrophages THP-1 par Staphylococcus aureus

A. Olivier, S. Lemaire, M.P. Mingeot-Leclercq, F. Van Bambeke, P.M. Tulkens

Publications

- **Role of rsbU in the intracellular fate of *Staphylococcus aureus*. Comparison between human phagocytic (THP-1 macrophages) and non-phagocytic (HUVEC) cells.**

  Aurélie C. Olivier, Françoise Van Bambeke, Marie-Paule Mingeot-Leclercq, and Paul M. Tulkens

>> In preparation for the *Journal of infectious diseases*

- **[review] Role of *Staphylococcus aureus* virulence factors in infections.**

  Aurélie Olivier, Françoise Van Bambeke, Marie-Paule Mingeot-Leclercq and Paul M. Tulkens

>> In preparation for *the Journal of Antimicrobial Chemotherapy*

- **Restoration of susceptibility of intracellular methicillin-resistant *Staphylococcus aureus* to beta-lactams: comparison of strains, cells, and antibiotics.**

  Lemaire S, Olivier A, Van Bambeke F, Tulkens PM, Appelbaum PC, Glupczynski Y. Antimicrob Agents Chemother. 2008 Aug;52(8):2797-805. Epub 2008 Jun 2.