NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF LIFE SCIENCES

ANA RITA LUAZES SEQUEIRA

BSc in Cellular and Molecular Biology

# UNRAVELLING THE FUNCTION OF THE SKF SYSTEM OF *STAPHYLOCOCCUS AUREUS*

MASTER IN MOLECULAR GENETICS AND BIOMEDICINE

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"Do unto others as you would have them do unto you."

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"You cannot teach a man anything; you can only help him discover it in himself." (Galileo).

# ABSTRACT

*Staphylococcus aureus* colonizes humans asymptomatically but can also be a world-leading nosocomial opportunistic pathogen, namely methicillin-resistant *S. aureus* (MRSA), able to also cause infections in the healthy community. MRSA can acquire and maintain several resistance mechanisms and presents a high assortment of virulence factors. Since the beginning of utilization of antibiotics, new antibiotic-resistant bacteria, like MRSA, are continuously emerging. Finding new strategies to fight antimicrobial-resistant pathogens is a major priority and antimicrobial peptides (AMPs) are receiving much attention as alternatives to antibiotics. Transcriptome analysis of a *S. aureus* mutant in an essential cell wall gene (*murF*) – that presented a peptidoglycan defect described to be lethal – revealed the overexpression of two small ORFs encoding for the small proteins SkfA and SkfB. These two ORFs have unknown function and are exclusive to *S. aureus*. The results obtained in previous studies indicated that the SkfA and SkfB proteins may act as AMPs, possibly being excreted to the external environment by the putative transmembrane transporter, SkfC. The aim of this project consists of deepening the existing knowledge to functionally characterize the SkfAB system by validating the hypothesis that SkfA and SkfB are AMPs. To achieve this objective, the activity of SkfA and SkfB in inter-species interaction was analyzed. Promoter fusion assays showed that the *skf* promoter is upregulated in inter--species interactions, the upregulation being more evident for co-cultures with Gram-negative bacteria. Assays were also performed to understand how SkfA and SkfB impact interaction with the host. Macrophage infection assays showed that the SkfA and SkfB proteins enhance macrophage internalization and survival.

The results of this thesis showed that the Skf proteins are involved in inter-species interactions and interactions with the host.

**Keywords**: Methicillin-resistant *Staphylococcus aureus*; Antimicrobial Peptides; Peptidoglycan Biosynthesis; Inter-species Interaction; Macrophage Infection.

# RESUMO

*Staphylococcus aureus* coloniza humanos assintomaticamente, mas consegue ser também um patógeno oportunista nosocomial global, nomeadamente *S. aureus* resistente à meticilina (SARM), capaz também de causar infeções na comunidade saudável. SARM consegue adquirir e manter mecanismos de resistência, e apresenta uma grande variedade de fatores de virulência. Desde o início da utilização de antibióticos, que se assiste ao surgimento contínuo de novas bactérias resistentes a antibióticos. Descobrir novas estratégias contra patógenos resistentes a antibióticos é uma prioridade e os péptidos antimicrobianos (PAMs) estão a receber muita atenção como abordagens alternativas a antibióticos. A análise transcriptómica de um mutante de *S. aureus* num gene essencial da parede celular (*murF*) – que apresentava um defeito descrito como letal – revelou a sobreexpressão de duas pequenas ORFs que codificam para as proteínas SkfA e SkfB. Estas duas ORFs são de função desconhecida e exclusivas de *S. aureus*. Os resultados obtidos em estudos anteriores indicaram que SkfA e SkfB possam atuar como PAMs, sendo possivelmente excretadas para o exterior pelo seu transportador transmembranar putativo, a SkfC. O objetivo deste projeto consiste em aprofundar o conhecimento existente de maneira a caraterizar funcionalmente o sistema SkfAB, ao validar a hipótese que SkfA e SkfB são PAMs. Para atingir este objetivo, a atividade de SkfA e SkfB em interações inter-espécies foi analisada. Ensaios de fusão de promotores mostraram uma regulação positiva do promotor *skf* em interações inter-espécies, sendo esta mais evidente quando *S. aureus* está em co-cultura com bactérias Gram-negativas. Também foi explorado o papel de SkfA e SkfB na interação com o hospedeiro. Ensaios de infeção de macrófagos mostraram que as proteínas SkfA e SkfB potenciam internalização e sobrevivência em macrófagos.

Os resultados desta Tese demonstraram que as proteínas Skf estão envolvidas em interações inter-espécies e em interações com o hospedeiro.

**Palavras chave:** *Staphylococcus aureus* Resistente a Meticilina; Péptidos Antimicrobianos; Biossíntese de Peptidoglicano; Interações Inter-espécie; Infeção de Macrófagos.

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# ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| Acetyl-CoA | Acetyl coenzyme A |
| *agr* | Accessory gene regulator |
| Amp | Ampicillin |
| AMP | Antimicrobial peptide |
| ATP | Adenosine triphospahte |
| AU | Arbitrary units |
| $CdCl_2$ | Cadmium chloride |
| CFU | Colony forming unit |
| Cm | Chloramphenicol |
| $Cm^r$ | Chloramphenicol resistant |
| Da | Dalton |
| D-Ala | D-alanine |
| D-iso-Gln | D-iso-glutamine |
| DNA | Deoxyribonuleic acid |
| DME | Drug/metabolite exporter |
| DMSO | Dimethyl sulfoxide |
| F-6-P | Fructose 6-phosphate |
| GFP | Green fluorescent protein |
| GlmM | Phosphoglucosamine mutase |
| GlmS | Glucosamine-6-phosphate synthase |
| GlmU | Glucosamine-1-phosphate acetyltransferase/N-acetylglucosamine-1-phosphate |
| G-6-P | Glucosamine-6-phosphate |
| G-1-P | Glucosamine-1-phosphate |
| GT | Glycosyltransferase |
| $H^+$ | Hydrogen |
| $H_2O_2$ | Hydrogen peroxide |
| HS | Horse serum |
| IgG | Immunoglobulin G |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| Km | Kanamycin |
| $Km^r$ | Kanamycin resistant |
| L-Ala | L-alanine |
| LB | Lysogeny Broth medium |
| L-Lys | L-lysine |
| LTA | Lipoteichoic acids |

| | |
|---|---|
| MOI | Multiplicity of infection |
| MraY | Phospho-N-acetylmuramoyl-pentapeptide transferase |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| NaCl | Sodium chloride |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NAG | N-acetylglucosamine |
| NAM | N-acetylmuramic acid |
| Neo | Neomycin |
| $Neo^r$ | Neomycin resistant |
| NFκB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NMR | Nuclear magnetic resonance |
| NO | Nitric oxide |
| $O_2^-$ | Superoxide ion |
| OD | Optical density |
| O/N | Over night |
| $ONOO^-$ | Peroxynitrite |
| ORF | Open reading frame |
| PBP | Penicillin binding protein |
| PBS | Phosphate-buffered saline |
| PBS-Tx | PBS-triton |
| PFA | Paraformaldehyde |
| PMA | Phorbol-12-myristate-13-acetate |
| PRR | Pathogen recognition receptor |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RPMI | Roswell Park Memorial Institute Medium |
| rpm | revolutions per minute |
| RT | Room temperature |
| SCC*mec* | Staphylococcal cassette chromosome *mec* |
| SCV | Small-colony variant |
| SDS-PAGE | Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis |

| | |
|---|---|
| SERAM | Secretable expanded repertoire adhesive molecules |
| Skf | *Staphylococcus* killing factor |
| TA | Teichoic acid |
| TCS | Two component system |
| TLR | Toll-like receptor |
| TP | Transpeptidase |
| TSA | Tryptic Soy Agar medium |
| TSB | Tryptic Soy Broth medium |
| TSST-1 | Toxic shock syndrome toxin-1 |
| UNAG | UDP-N-acetylglucosamine |
| UNAGEP | UDP-N-acetylglucosamine enolpyruvate |
| UNAM | UDP-N-acetylmuramic acid |
| UP | Undecaprenol-phosphate |
| WTA | Wall teichoic acid |

# 1

# INTRODUCTION

## 1.1 An overview on *Staphylococcus aureus*

The Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) was first isolated by the surgeon Alexander Ogston in 1881 from the pus of surgical wounds [1–5]. In the study conducted by Ogston, *S. aureus* was initially referred to as micrococci and later on as *Staphylococcus* [1,4]. Having embraced the name *Staphylococcus* from Ogston, Anton Rosenbach argued that *Staphylococcus* was a group of organisms rather than merely one organism, something that Ogston had already hypothesized, since he managed to isolate two *Staphylococci* colonies with distinct colors – one golden, which Rosenbach named *S. aureus*, and one white, which received the name *Staphylococcus albus*, later renamed *Staphylococcus epidermidis* [1,4–6].

*S. aureus* are nonmotile, spherical shaped bacteria belonging to the Firmicutes phylum [1–3,7,8]. These bacteria are facultative anaerobes, coagulase and catalase positive, with a cell diameter that ranges from 0.5 to 1.0 µm [2–4,7,8]. The optimal growth temperature of *S. aureus* is 37 ºC and it can grow at temperatures ranging from 10 to 45 ºC. *S. aureus* grows well in 10% NaCl conditions, tolerating saline conditions up to 15% NaCl, and grows optimally at pH 7.4 [3,7].

Being both a commensal bacterium and an important human opportunistic pathogen, *S. aureus* presents itself as the most clinically relevant *Staphylococcus* of the 52 species and 28 subspecies of the *Staphylococcus* genus [2]. Thus, learning more about this bacterium is of high importance.

## 1.2 Methicillin Resistant *Staphylococcus aureus*

Due to its commensal nature, *S. aureus* can be found in about 30% of the healthy adult population, mainly colonizing the skin and mucous membranes, with its main ecological niche being the anterior

nares [2–4,9–12]. It is, however, also an opportunistic pathogen, exhibiting the potential to cause infections, mostly skin related, and a wide range of infectious diseases, such as endocarditis, toxic shock syndrome, bloodstream infections, necrotizing fasciitis, osteomyelitis, sepsis and pneumonia [3,4,8–11,13–16]. These are responsible for considerable morbidity and mortality worldwide [10,11,13].

*S. aureus* is particularly known for causing hospital related infections, having become the world-leading nosocomial pathogen, as well as for causing infections in the healthy community [3,8]. The nosocomial infections are due to a variety of circumstances usually found in hospitals, namely very low birth-weight neonates, patients with compromised immune systems and/or disrupted cutaneous and/or mucosal barriers, which allow *S. aureus* to penetrate underlying tissues or the bloodstream, causing acute, recurrent or chronic and persistent infections [2,4,11,14].

Methicillin-resistant *S. aureus* (MRSA) was first isolated in 1961 in the United Kingdom, not long after methicillin was introduced in medical practice (introduced in 1959 to treat penicillin-resistant *S. aureus* infections), being identified as a nosocomial pathogen [2,3,10,15,17]. MRSA epidemiology changed in the 1990s when MRSA infections were reported in healthy individuals that had no previous contact with hospitals [2,10]. MRSA is currently a major cause of hospital-acquired infections and a leading cause of human bacterial diseases worldwide, being also increasingly found in the community and in nursing homes [3,10,17]. The most challenging issue is the increasing difficulty to combat MRSA-acquired infections, since new MRSA strains continuously emerge with resistance to virtually all current antibiotics [8,13,17]. It is, therefore, critical to continue with efforts to discover new antibiotics and non--antibiotic (vaccines and immune therapeutics) approaches to deal with MRSA infections [4,8].

*S. aureus* develops into MRSA strains by the uptake via horizontal gene transfer of a mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*), encoding the gene *mecA*, responsible for conferring resistance to the antibiotic methicillin and the majority of β-lactam antibiotics [2,3,9,11,15,17,18]. The details of the mechanism of horizontal gene transfer of this genetic element are still not fully grasped. However, it is thought that this resistance mechanism is to be found in the majority of *S. aureus* clones, including animal and human pathogenic strains [2].

### 1.3 *Staphylococcus aureus* pathogenicity

*S. aureus* presents a complex regulatory network to control the production of virulence factors, which allow it to adapt to changing conditions and dominate the host, contributing to its pathogenicity [9,18]. It

is due to these virulence factors that *S. aureus* can adhere to the host cells, evade the host immune system, and degrade and acquire nutrients [9,15].

After a *S. aureus* invasion, the host immune system reaction starts with macrophages that release cytokines to gather neutrophils (phagocytosis is the main process by which the immune system deals with the presence of *S. aureus*) [14]. *S. aureus* fights this immune response through the production of toxins and virulence factors that allow it to escape local infection sites, disseminate to other organs through the bloodstream and survive intracellularly within macrophages and neutrophils, avoiding being captured by phagocytes. Pore-forming toxins, polysaccharide capsules, antibody-binding proteins and cytolytic peptides protect *S. aureus* from phagocytosis, namely protein A, a surface protein that binds and neutralizes the Fc region of the immunoglobulin G (IgG), hindering opsonization (opsonization is the coating of pathogen surfaces by phagocytic immune cells with the antibody IgG, which allows immune cells to recognize and eliminate pathogens) [14,15,19,20]. The corresponding genetic elements, which are not essential for *S. aureus* normal growth, are located on the chromosome or on mobile elements, such as phages, pathogenicity islands or plasmids. They are considered to be one of the major contributes to the strain-to-strain variation in *S. aureus* [9]. Bacteriophages can carry genes that encode virulence factors such as Panton-Valentine leucocidin, the immune evasion cluster, staphylokinase, exfoliative toxin A, cell wall-anchored virulence factor SasX; pathogenicity islands can include genes that encode pyrogenic toxins, also known as superantigens, such as the toxic shock syndrome toxin-1 (TSST-1), the enterotoxin B and enterotoxin-like protein Q; plasmids allow the acquisition of antibiotic resistance and can also contain genes that encode certain toxins [9,15]. The virulence factors that allow for *S. aureus* to adhere are, for example, collagen-, elastin- and fibronectin-binding proteins [15].

*S. aureus* virulence factors are mostly regulated by two component systems (TCSs). TCSs can recognize environmental changes and convert them to a regulatory program. The majority of *S. aureus* strains encode sixteen TCSs, WalKR being the only essential TCS [9]. The accessory gene regulator (*agr*) is the best studied regulatory system, encoding a quorum-sensing system, which performs as a master virulence regulator [9]. *agrAC*, *saeRS*, and *arlRS* are TCSs associated to *S. aureus* virulence [9,19]. In addition to the TCSs, *S. aureus* uses cytoplasmic regulators like the alternative sigma factors (SigB and SigH) and the SarA protein family of transcriptional regulators (such as SarA, Rot, MgrA) to survive in the host [9,18,19].

## 1.4 Antimicrobial peptides

Since the beginning of utilization of antibiotics, new antibiotic-resistant bacteria are continuously emerging, like MRSA [19,21,22]. Antimicrobial resistance has spread worldwide, leading to a global health crisis [21,22]. Finding new strategies to take action against antimicrobial-resistant pathogens is a major priority. Antimicrobial peptides (AMPs) are causing a ruckus in clinical research, due to their potential therapeutic properties and their advantages in comparison to antibiotics, such as having different mechanisms of action and presenting a slower emergence of resistance [19,21,22]. However, designing AMPs that prove to be less susceptible to evolutionary resistance mechanisms than the antibiotics in use is arduous [22].

AMPs are antimicrobial agents that are produced by all organisms (microorganisms, mammals, plants, fungi, insects and protozoan), mainly found in eukaryotes, that can act against both Gram--positive and Gram-negative bacteria, as well as viruses, parasites and human pathogenic fungi. They can also attack some eukaryotic cells [19,21,23,24]. AMPs contribute to innate immunity [19,21–24]. Plants and insects utilize AMPs to defend themselves against pathogens, since they lack an adaptive immune system. Bacteria and other microorganisms produce AMPs with the objective of gaining an advantage in their environmental niche [21,22]. In humans, AMPs help the host to defend itself against pathogenic microbes, being components of the innate immune system [19,21,22,24]. They are able to disrupt bacterial cell membranes, regulate inflammation and modulate the immune response [22]. Monocytes, neutrophils and macrophages release AMPs, which act as chemoattractants for recruiting innate immune cells to an infection site [19,21,22]. It is, however, essential for humans to maintain a balanced expression of AMPs, since over or under expression can cause diseases [19].

Chemically diverse in nature, AMPs are small molecules that commonly contain less than 100 amino acids, are usually cationic as they tend to include more positively charged residues such as lysine, arginine and histidine, and about half of the residues are hydrophobic [19,21,23,24]. They present structural characteristics that set them apart from conventional antibiotics [21]. Regarding their structure, AMPs are classified into four groups – α-helical, β-sheet, cyclic, and extended – and can consist fully of either a single helix or sheet, or present a more complex structure [21,23,24]. Besides presenting diverse structures, AMPs also have different sequences and goals [23]. To date, in an attempt to find clinically applicable AMPs, more than 3000 have been discovered from different species [19,21,22,24].

The potential clinical relevance of AMPs relies on their different mechanisms of action and vast properties in comparison to antibiotics [19,23]. The mode of action of AMPs can be divided into two main categories: membrane disruptive AMPs and non-membrane disruptive AMPs [19,24]. AMPs of the first category target the cell membrane, producing lesions on the cell membrane or leading to cell death by

permeabilizing the phospholipid bilayer, being the most prevalent mechanism of action [19,21,24]. AMPs of the second category, translocate to the cytosol, where they cause disruption of universal cellular processes, targeting proteins, nucleic acids or organelles [19]. An example of non-disruptive AMPs is one where they first interact with the bacterial cell envelope and then translocate to the cytosol, where – unlike antibiotics – they do not impede synthesis of peptidoglycan through protein binding, but rather, can specifically target conserved substrates. Targeting conserved substrates makes it improbable for bacteria to be able to develop resistance mechanisms [22,24].

One advantage that AMPs have over antibiotics, is the fact that many AMPs act through multiple modes of actions, in contrast to antibiotics that usually have one or two target sites. Antibiotics usually target metabolic pathways or enzymes, while AMPs act toward destroying the cell structure, usually targeting the membrane. Naturally, if the target is e.g. an enzyme, the occurrence of a mutation can easily change the target, thus facilitating the development of resistance to an antibiotic that targets such enzyme. Acting through distinct pathways and multiple mechanisms raises AMPs' antimicrobial efficacy and diminishes the development of resistance, since it is less likely for a bacterium to simultaneously acquire multiple mutations [19,21]. Due to these advantages, AMPs are receiving much attention as alternatives to antibiotics in the struggle to fight antimicrobial resistant pathogens [19,22]. Utilizing AMPs as a single therapeutic agent has proven a promising approach, and combining AMPs with antibiotics is an option that is being explored [19,21,22]. Some AMPs have proven to be effective against multi-drug resistant bacteria – teixobactin and the antibiotic malacidin both target lipid II, having low cytotoxicity and diminished susceptibility to create resistance, making them great drug candidates against MRSA [19,22]. In fact, it is important to consider toxicity against eukaryotic cells when trying to find AMPs acceptable for clinical use, since some AMPs have proven to be highly nephrotoxic [21].

These peptides are already being implemented in many areas such as agriculture, food industry, and aquaculture, since they have, as aforementioned, several advantageous properties (e.g. immune--modulatory, anti-inflammatory, anti-parasitic, anti-fungal, anticancer, antiviral, anti-biofilm properties) [22,23]. Nonetheless, the vast potential these peptides present for therapeutic uses has still to be further explored [19,21–24].

## 1.5 Macrophage infection by *Staphylococcus aureus*

### 1.5.1 Macrophages and their line of action in *Staphylococcus aureus* infection

Macrophages are pivotal immune cells of the innate immune system [16,25–27]. These professional phagocytes play an important role not only in the innate but also the adaptive immune response,

participating additionally in a large number of physiological processes (e.g. repairing and remodeling tissue, clearing cellular debris, playing a part in homeostasis) [16,25–28]. Macrophages are capable of fighting and killing pathogens by sensing and migrating to infection sites and engulfing the microorganisms through phagocytosis [16,26]. They participate in immunomodulation through secretion of pro-inflammatory cytokines, such as IL-6, IL-1 and TNF-α [16,26,28]. There are tissue-resident macrophages and macrophages that derive from monocytes. Regardless of their disparate origins, macrophages can switch phenotypes in accordance with local environmental stimuli (e.g. cytokines) [16,25,27,28] . Tissue macrophages are typically found in a "M2" phenotype (anti-inflammatory classification) under homeostatic conditions, being efficient at healing and repairing tissue. When in the presence of an infection, macrophages adopt a "M1" phenotype (pro-inflammatory classification), becoming able to clear pathogens [16]. In spite of existing heterogeneous macrophage populations, macrophages have only one objective, which is fighting infection through their innate ability to phagocyte particles and microorganisms [27].

Normally, macrophages are able to destroy pathogens with little signs of infection. However, some pathogens like *S. aureus* are able to evade the host immune responses [27]. Nonetheless, professional phagocytes are the host's first line of defense and crucial in the clearance of *S. aureus* [16,29].

For macrophages to clear an infection, they must be able to find and phagocyte the invading bacteria. The host's initial response to *S. aureus* occurs through the cells present in the infected sites, which tend to be epithelial cells at mucosal surfaces. These cells can detect various staphylococcal molecules (e.g. peptidoglycan, protein A, toxins, lipoteichoic acids [LTA]) through pathogen recognition receptors (PRRs). Phagocyte recruitment is achieved through epithelial PRR signaling, since it induces pro--inflammatory chemokine and cytokine production. *S. aureus* can also deliver signals to recruit phagocytes [16]. *S. aureus* can activate macrophages via Toll-like receptors (TLRs), which recognize bacterial components [16,26]. TLR2 is activated by the Gram-positive cell wall polymer LTA [26]. This receptor is crucial in the upregulation of inflammatory gene expression, since it activates the MYD88 innate immune signal transduction adaptor and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling pathways, leading to the transcription of pro-inflammatory cytokines, enhancing the immune response (**Figure 1.1**) [16,26]. For macrophages to phagocyte *S. aureus*, NFκB activation is also necessary [16].

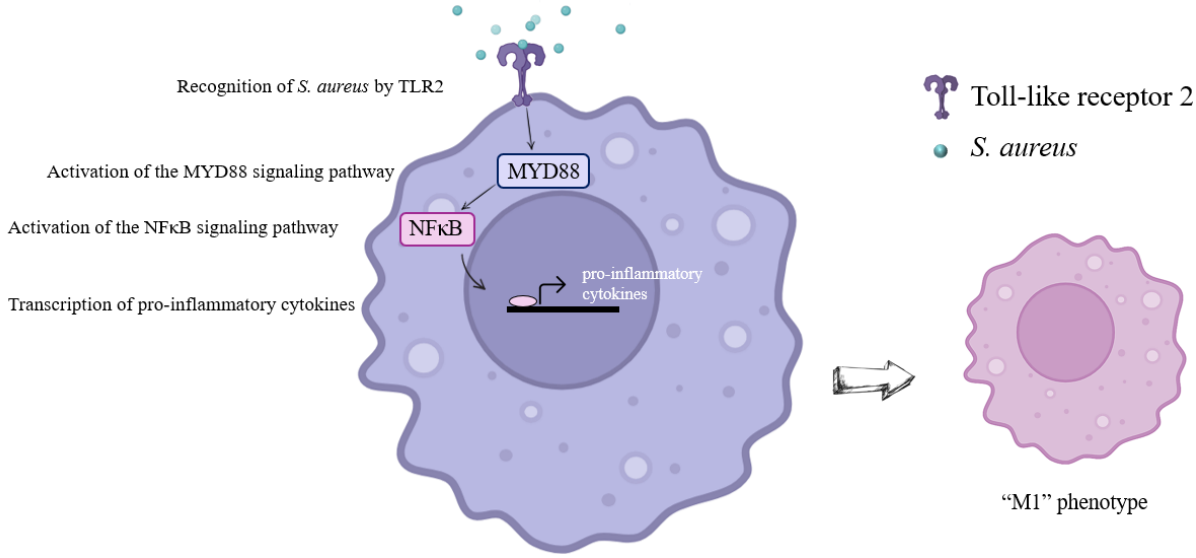


**Figure 1.1. Schematic representation of macrophage response to *S. aureus*.** The recognition of the *S. aureus* by the Toll-like receptor 2 leads to activation of the MYD88 and NFκB signaling pathways, which in turn leads to the transcription of pro-inflammatory cytokines, making the macrophages adopt a pro-inflammatory "M1" phenotype. Adapted from [16]. Image created with BioRender.com.

Phagocytosis of *S. aureus* by macrophages is achieved through the engagement of complement, scavenger and Fc receptors found on its surface [16,27]. Complement receptors initiate a cascade important in the innate immune system that targets pathogens and is able to lyse them, recruiting more phagocytes to the infection site and opsonizing the pathogens for elimination by phagocytes. Scavenger receptors can recognize various molecules of the pathogens (e.g. LTA). Fc receptors bind to the antibody's Fc region, thus facilitating the phagocytosis of invading pathogens [16]. Through rearrangements of the actin cytoskeleton, the bacteria are engulfed into a membrane-bound vacuole, named the phagosome [16,27,30]. The initial phagosome must mature to become microbicidal. This maturation results from several fusion events between endosomes and the phagosome that lead to the formation of the phagolysosome, which presents microbiocidal properties (**Figure 1.2**) [27,30].

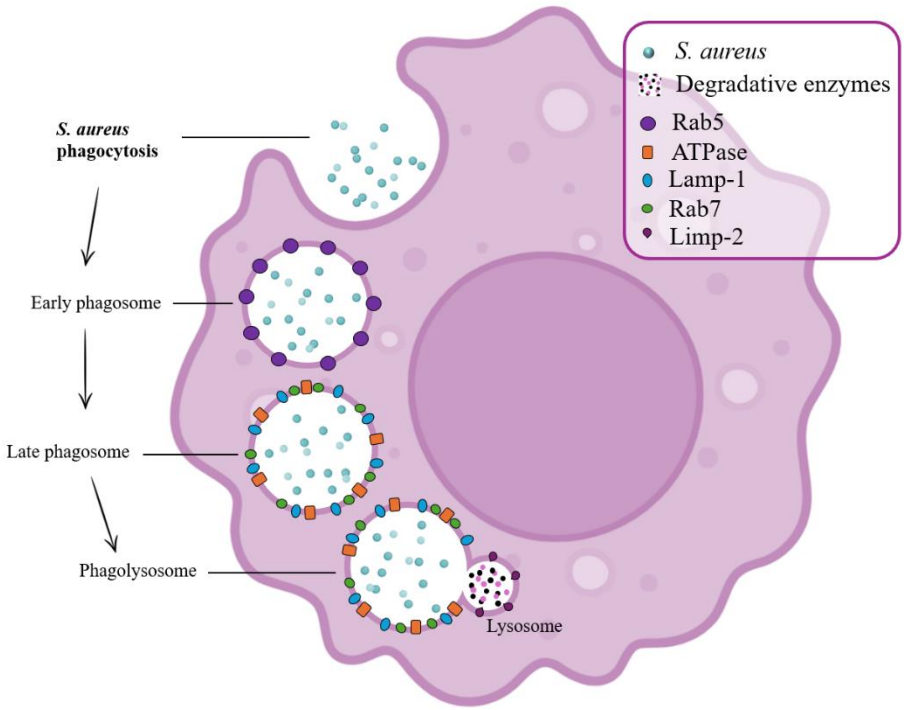


**Figure 1.2. Schematic representation of *S. aureus* receptor-mediated phagocytosis and phagosome maturation.** After phagocytosis, achieved through the engagement of complement, scavenger and Fc receptors found on the macrophage's membrane [16], the early phagosomes (with early endosomal markers, like the small GTPase Rab5) quickly mature into late phagosomes (with the markers Lamp-1 and Rab7), where pH is continuously decreasing via ATPase activity. Phagolysosomes are formed through the fusion of a late phagosome with lysosomes [30]. Abbreviations: Lamp-1, lysosome-associated membrane protein-1; Limp-2, lysosomal integral membrane protein-2. Adapted from [16,30]. Image created with BioRender.com.

The microbiocidal properties of phagolysosomes include enzymes, antimicrobial proteins and AMPs, reactive oxygen species (ROS) and reactive nitrogen species (RNS), being an acidic environment, restricting nutrients, and autophagy [16,27,30]. Hydrolytic enzymes such as lipases, proteases, glycosidases and phosphatases are usually found in phagolysosomes and can kill bacteria like *S. aureus*. Phagosomes may also contain lysozyme, an enzyme that cleaves peptidoglycan, although *S. aureus* peptidoglycan is resistant to lysozyme action [16,31]. As AMPs are usually positively charged, they possess the capacity to inflict damage in bacterial membranes [16]. The release of ROS and RNS, initially activates the NADPH oxidase, which leads to the formation of the unstable super oxide ($O_2^-$) ion [27,28]. This ion triggers multiple chemical reactions that give rise to ROS that damage DNA, proteins and lipids. When in acidic conditions, $O_2^-$ dismutates into hydrogen peroxide ($H_2O_2$), or forms peroxynitrite ($ONOO^-$), after reacting with nitric oxide (NO), generating two powerful cell damaging agents. The acidic environment of the phagolysosome (pH of around 5) is achieved through the vacuolar ATPase, that pumps hydrogen ($H^+$) to the interior of the phagosome [27]. Nutrient restriction is accomplished by the host actively sequestering nutrients, e.g. metal ions that are necessary for bacterial metabolism, reproduction and for bacteria to defend themselves against oxidative stress – macrophages can diminish serum iron concentrations when there is a bacterial infection, removing iron from the blood [16,26,27].

Components of autophagy can target and degrade pathogens [16,29]. Macrophages can also act against pathogens using extracellular traps, which are formed by chromatin protrusions, proteases, DNA, histone proteins and AMPs [16,27].

### 1.5.2 *S. aureus* evading mechanisms

Regardless of all the features that the phagolysosomes present, *S. aureus* is still known to survive in the intracellular niche of phagocytes, being able to eventually escape and further disseminate in the host [16]. This is possible, since *S. aureus* manipulates macrophage responses in its favor, applying strategies that enable it to block macrophage recruitment, phagocytosis and the degrative abilities of phagolysosomes [16,30].

*S. aureus* manipulates macrophage responses due to the expression of its multiple virulence factors in response to environmental cues. It can inhibit the activation of the complement through secretion of the extracellular fibrinogen-binding protein that binds to the complement protein C3, avoiding, for example, opsonization (in the context of infection, C3 is cleaved, generating the bioactive components C3a and C3b; C3a induces a conformational change in the C3b fragment, which is then capable of opsonizing the infecting cell) [16,27,32]. *S. aureus* can also escape phagocytosis mediated by antibodies through the expression of protein A. Both TLR2 and NFκB signalling may be weakened in *S. aureus* infections [16]. To oppose a host immune attack, *S. aureus* can secrete membrane-damaging and pore-forming toxins with the objective of killing professional phagocytes, since dying macrophages do not have microbiocidal properties [27]. *S. aureus* can reduce the negative charge of its cell surface to protect itself against the action of AMPs [16,27]. To counter the effects of ROS and RNS, *S. aureus* uses enzymes such as superoxide dismutase A and superoxide dismutase M (enzymes that undermine superoxide radicals), a catalase encoded by the gene *katA* (converts $O_2^-$ to $H_2O_2$ to $H_2O$ and $O_2$), lipoic acid (limits macrophage production of ROS and RNS), and the carotenoid pigment staphyloxanthin (besides protecting *S. aureus* against oxidative stress, this pigment also grants resistance to AMPs, since it decreases membrane fluidity) [16,27,30]. The resistance to oxidative stress is also mediated by transcriptional regulators like peroxide regulator, responsible for controlling numerous antioxidant genes [16]. *S. aureus* can replicate and survive in an acidic environment due to the activity of the GraRS two component system, the upregulation of the *agr* quorum-sensing system and its multiple mechanisms to deacidify the microenvironment of the bacteria (e.g. generating ammonia) [16,30]. It also manages to undermine and manipulate autophagy to survive intracellularly through the *agr* regulatory system [16,29]. To avoid extracellular traps, the enzymes adenosine synthase and nuclease are secreted, leading to non-inflammatory macrophage apoptosis, allowing *S. aureus* to form abscesses after eliminating phagocytic cells from the site of infection [16].

The persistence that *S. aureus* achieves intracellularly, often responsible for chronic infections, is also associated with the formation of small-colony variants (SCVs) [13,33–35]. These persistent and often recurrent infections are connected with the ability of SCVs to avoid immune clearance by limiting the production of toxins necessary to elicit this clearance (achieved through the reduction of Agr activity) and antimicrobial resistance [11,33–35]. These immune evasion phenotypes show reduced membrane potential and metabolism (slow-growing populations that produce small colonies, usually about one-tenth of the parent colony size), can easily invade neighboring cells following cell lysis, have altered metabolic pathways and/or mutations in global regulatory genes and, as said before, present decreased virulence factor production [11,13,34,35]. SCV infections are therefore not easily cleared, even when combined antimicrobial therapies are applied [34].

It is crucial that new mechanisms to fight this successful pathogen are developed. A starting point would be to know and understand its structure, specifically, the characteristics of its cell wall, which will be described hereinafter.

### 1.6 Cell wall of *Staphylococcus aureus*

Surrounding *S. aureus* is a thick cell wall, of about 20 to 40 nm thick, which is responsible for the maintenance of *S. aureus* cell shape, viability and integrity and provides protection against osmotic pressure caused by the large internal osmolyte concentration (which can reach up to 20 atm in Gram-positive bacteria) [36–40]. It is mainly composed of a highly dynamic peptidoglycan network that permits cell growth and division and provides for the intake of nutrients, excretion of metabolic wastes and manages to exclude toxic substances from entering the cell [36–41]. Peptidoglycan serves as a scaffold to anchor other components of the cell envelope like teichoic acids (TAs) and proteins [36–39]. TAs, which are anionic glycopolymers, also help in protecting the cell against the turgor pressure, as well as aid in roles associated to cell wall maintenance, like defining cell shape, bacterial fitness and antibiotic resistance [36–38]. TAs can be found covalently bound to peptidoglycan, being called wall teichoic acids (WTAs), or anchored to the cell membrane, being named lipoteichoic acids (LTAs) [36,38]. WTAs are crucial in bacterial pathogenesis, since they play a part in cell adhesion and forming biofilm, and are known for protecting *S. aureus* against AMPs and heat, for example [36]. The proteins found on the cell wall play important roles in adhering to surfaces, participating in bacterial virulence and acquiring nutrients [36,37].

The cell wall encompasses the cell membrane, that functions as a selectively permeable barrier, impeding the entrance of toxic substances, like some antibiotics, maintaining the correct nutrient and

waste concentrations inside the cell. It is also crucial in generating ATP, cell to cell communication and other vital processes [37].

Since *S. aureus* cell wall and cell membrane are necessary components for these bacteria to infect and survive the human host, understanding fundamental cell cycle processes such as peptidoglycan synthesis can lead to determining new ways to deal with these pathogens [37,38,40].

### 1.6.1 Peptidoglycan structure of *Staphylococcus aureus*

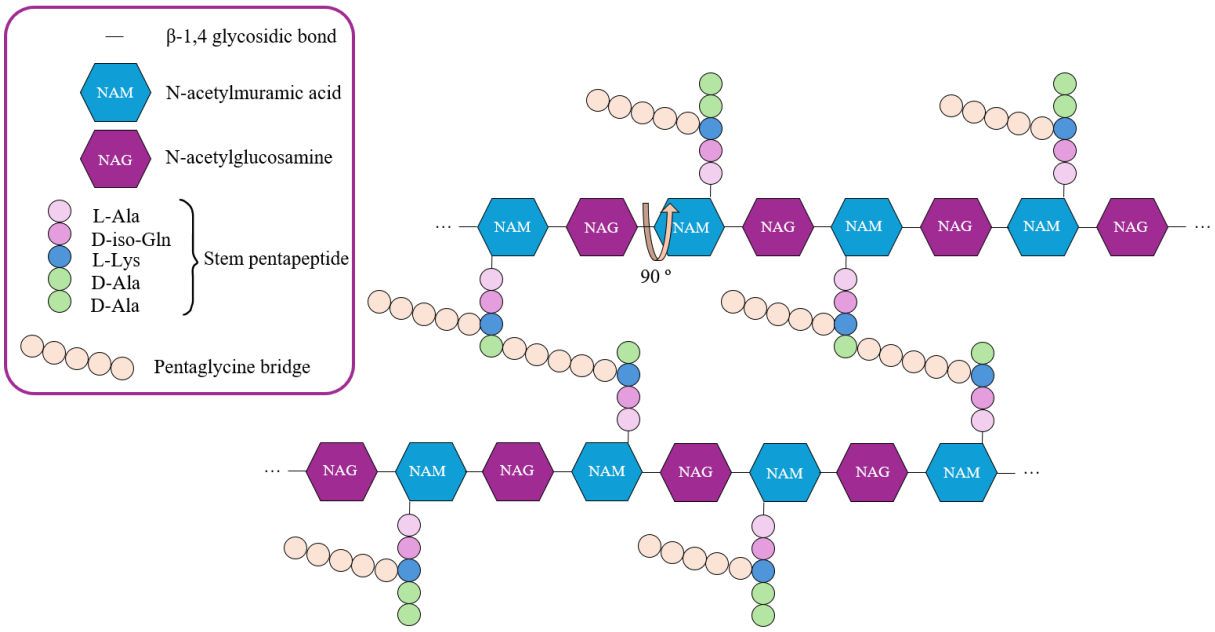


**Figure 1.3. Schematic representation of the peptidoglycan structure of *S. aureus*.** Glycan strands are composed of alternating N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) residues connected by a β-1,4 glycosidic bond; glycan strands are cross-linked by a stem pentapeptide and a pentaglycine bridge. Abbreviations: L-Ala, L-alanine; D-iso-Gln, D-iso-glutamine; L-Lys, L-lysine; D-Ala, D-alanine. Based on [36,38,42].

The main cell wall component of *S. aureus* is peptidoglycan [43,44]. This polymer originates a flexible mesh-like structure, essential to maintain cell integrity by providing the mechanical strength necessary to support the intracellular osmotic pressure, and to maintain cell shape during cell growth, division and infection, granting protection against environmental threats [39,42,44–49]. The peptidoglycan mesh is constituted by cross-linked glycan strands of various lengths, achieving a porous structure that enables the diffusion of various molecules, such as proteins, virulence factors, nutrients and chemical signals [37,39,40,42,48]. *S. aureus* presents a high proportion of peptidoglycan cross-linking, namely 80 – 90%, that influences its physical strength and integrity [36,37]. Its relatively short glycan strands (mean of 6 – 10 disaccharides), which contribute to cell wall flexibility, are composed of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues, connected by a β-1,4 glycosidic bond [36–40,44,45,47,48,50]. The glycan strands are cross-linked by a stem pentapeptide and a

pentaglycine bridge (**Figure 1.3**) [36–40,42,45,47]. The pentapeptides consist of L-alanine, D-iso-glutamine, L-lysine, and two D-alanine residues (L-Ala-D-iso-Gln-L-Lys-D-Ala-D-Ala) [36–38,42,46,49] and are connected to the carboxyl group of each NAM residue. The pentaglycine bridges connect two pentapeptides linking the lysine component of one stem peptide and the penultimate D-alanine of an adjacent stem peptide [46].

### 1.6.2 Peptidoglycan biosynthesis in *Staphylococcus aureus*

The process of peptidoglycan biosynthesis (**Figure 1.4**) occurs in different cell compartments, namely the cytoplasm, and the inner and outer side of the cytoplasmic membrane, dividing the process in three stages [49,51,52].

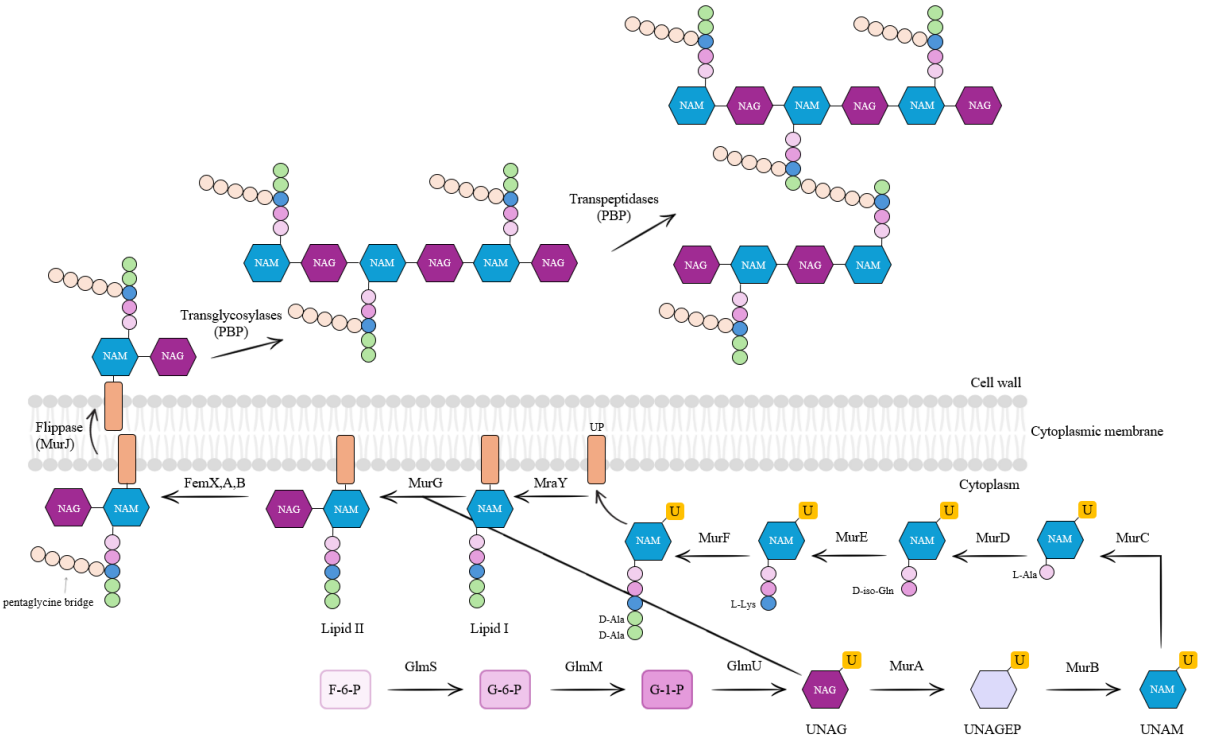


**Figure 1.4. Schematic representation of peptidoglycan biosynthesis pathway in *S. aureus*.** In the cytoplasm and on the inner side of the cytoplasmic membrane the peptidoglycan monomeric building block is built. This peptidoglycan precursor is then flipped to the outer side of the cytoplasmic membrane, becoming available to be used as substrate in polymerization reactions to form mature peptidoglycan. Abbreviations: F-6-P, fructose 6-phosphate; G-6-P, glucosamine-6-phosphate; G-1-P, glucosamine-1-phosphate; NAG, N-acetylglucosamine; U, UDP; UNAG, UDP-N-acetylglucosamine; UNAGEP, UDP-N-acetylglucosamine enolpyruvate; NAM, N-acetylmuramic acid; UNAM, UDP-N-acetylmuramic acid; L-Ala, L-alanine; D-iso-Gln, D-iso-glutamine; L-Lys, L-lysine; D-Ala, D-alanine; UP, undecaprenol-phosphate; PBP, Penicillin binding protein; GlmS, glucosamine-6-phosphate synthase; GlmM, phosphoglucosamine mutase; GlmU, glucosamine-1-phosphate acetyltransferase/N-acetylglucosamine-1-phosphate; MraY, phospho-N-acetylmuramoyl-pentapeptide transferase. Based on [49,51,52]. Image created with BioRender.com.

#### 1.6.2.1 Cytoplasmic stage

The cytoplasmic stage involves building the peptidoglycan monomeric building block, the N-acetylglucosamine-N-acetylmuramyl pentapeptide (NAG-NAM pentapeptide), starting with the

biosynthesis of UDP-N-acetylglucosamine (UNAG) and following with the biosynthesis of both UDP-N-acetylmuramic acid (UNAM) and the UNAM-pentapeptide [52].

UNAG biosynthesis is achieved by converting fructose 6-phosphate (F-6-P) to glucosamine-6-phosphate (G-6-P), G-6-P to glucosamine-1-phosphate (G-1-P), and G-1-P to UNAG by the enzymes glucosamine-6-phosphate synthase (GlmS), phosphoglucosamine mutase (GlmM), and the bifunctional glucosamine-1-phosphate acetyltransferase/N-acetylglucosamine-1-phosphate (GlmU), respectively [45,48,50–52].

UNAM is synthesized from UNAG by the MurA and MurB enzymes [45,48,50–52]. UNAG first receives an enolpyruvyl group, forming UDP-N-acetylglucosamine enolpyruvate (UNAGEP), a step catalyzed by MurA. UNAGEP is then reduced by MurB, leading to the formation of UNAM [49,50,52].

To build the L-Ala-D-iso-Gln-L-Lys-D-Ala-D-Ala pentapeptide, the amino acids are added to UNAM one by one by the Mur ligases MurC, MurD, MurE and MurF [48–52]. MurF adds the dipeptide D-Ala-D-Ala [49,50].

#### 1.6.2.2 Second stage – inner side of the cytoplasmic membrane

The second stage leads to the formation of lipid II, the final monomer unit of the peptidoglycan polymer [52]. To achieve this, the UNAM-pentapeptide becomes bound to the cytoplasmic membrane by undecaprenol-phosphate (UP), a membrane-bound lipid carrier molecule and, through the action of the integral membrane protein phospho-N-acetylmuramoyl-pentapeptide transferase (MraY), undecaprenyl-pyrophosphoryl-NAM-pentapeptide (lipid I) is formed [45,49,51,52]. From here, the NAG moiety of UNAG is transferred to lipid I by N-acetylglucosaminyl transferase (MurG), leading to the formation of lipid II (undecaprenyl-pyrophosphoryl-NAM-[pentapeptide]-NAG) [45,48,49,51,52]. GatD/MurT is the complex that acts on the membrane-bound lipid II and is responsible for the amidation reaction, in which the second amino acid in the stem pentapeptide (D-glutamic acid) is catalyzed into D-iso-glutamine. This modification contributes to *S. aureus*'s resistance to e.g. lysozyme and methicillin [53]. Five glycine residues are then added to the third amino acid of the pentapeptide of lipid II by the FemX, FemA and FemB proteins to form the pentaglycine bridges necessary for peptidoglycan crosslinking [38,49,51].

#### 1.6.2.3 Third stage – outer side of the cytoplasmic membrane

In this stage, the translocation of lipid II through the cytoplasmic membrane occurs, mediated by the MurJ flippase, making this peptidoglycan precursor available to be used as substrate in polymerization reactions to form mature peptidoglycan [36,38,45,49,51,52]. This polymerization is achieved through the action of glycosyltransferases (GTs) and transpeptidases (TPs) [43,45,48,49,52]. The first are responsible for forming

linear glycan chains, and the latter for forming the peptide cross-bridges found between two adjoining glycan chains [36,52]. Penicillin binding proteins (PBPs) are enzymes responsible for transglycosylation and transpeptidation reactions [38,41,49,51,52].

### 1.6.3 Peptidoglycan biosynthesis pathway – a target for antibiotics

Since the biosynthetic pathway of peptidoglycan is essential for bacterial viability, and is not present in eukaryotes, it is the target of many antibiotics, namely the β-lactams and glycopeptides. It continues to receive much attention, since it can lead to the development of new antibiotics [36,38,45,48,49,51].

PBPs are the main target of β-lactam antibiotics [49]. β-lactam antibiotics all have a β-lactam ring[54] that structurally mimics the dipeptide D-Ala-D-Ala, the original substrate of the PBPs. Thus β-lactam antibiotics are recognized and bind to PBPs, impairing their ability to execute their functions of synthetizing the cell wall peptidoglycan, leading to the disruption of the cell [48,49,51].

MRSA strains have found a way to counter the action of these antibiotics by producing an altered PBP (encoded by *mecA*, a mobile genetic element acquired via horizontal gene transfer, responsible for conferring resistance to the majority of β-lactam antibiotics [2,3,9,11,15,17,18]), namely PBP2a, which presents lower affinity for β-lactam antibiotics, allowing for the biosynthesis of peptidoglycan, even in the presence of such antibiotics [55,56].

There are more enzymatic targets of antibiotics in the peptidoglycan biosynthesis pathway other than the PBPs. A few examples are the antibiotics fosfomycin (inactivates MurA), teixobactin (binds to the UNAM moiety of lipid II, hindering polymerization into peptidoglycan) and vancomycin, a glycopeptide (binds to the dipeptide D-Ala-D-Ala, making it inaccessible to the TPs, impeding peptidoglycan crosslinking) [45,49]. In regards to vancomycin-resistant MRSA strains these have developed a way to alter the C-terminal D-Ala-D-Ala of the pentapeptide to variations such as D-Ala-D-Lac or D-Ala-D-Ser (made possible by uptake of genes via horizontal gene transfer) [49].

## 1.7 Identification of the SkfA, SkfB and SkfC proteins

The *murF* gene is quite important in peptidoglycan biosynthesis, since MurF adds the dipeptide D-Ala-D-Ala, necessary for the crosslinking of peptidoglycan [46,49,50,57]. The product of the reaction catalyzed by MurF is recognized by PBSs, resulting in the correct cross-linking of the peptidoglycan structure [48,49,51].

Previous studies indicated that *murF* is involved in the *mecA*-dependent mechanism of resistance to β-lactam antibiotics. In these studies, two mutants were constructed in the background of the early MRSA strain COL, namely: COLspac*murF*, a conditional mutant with *murF* under the control of the inducible P*spac* promotor; and the F9 mutant, in which the *murF* gene is disrupted by the insertion of an integrative plasmid [57,58]. The conditional mutant proved that *murF* is an essential gene, since in the absence of the inducer no growth was detectable. Also, its suboptimal expression leads to the incorporation of abnormal tripeptide, as well as a lower oligomerization of peptidoglycan, destabilizing its structure. In addition, this mutant presents decreased antibiotic resistance of β-lactam antibiotics when low inducer concentrations are provided [57].

However, although the F9 insertion mutant presented a similar decrease in β-lactam antibiotic resistance, and similar levels of tripeptide incorporation in the cell wall, the viability of this mutant was not compromised, which was an unexpected result [58]. Therefore, transcriptomic analysis of the F9 insertion mutant was performed to understand how the strain is able to survive with a mutation that should be lethal. This approach led to the discovery of the overexpression of two small ORFs located sequentially in the chromosome, encoding for the small proteins SkfA and SkfB (Skf meaning Staphylococcal killing factor) of 7.6 and 8.2 KDa, respectively. These two ORFs are still of unknown function and only exist in *S. aureus*. The *skfA* gene was overexpressed 140 fold in F9 and the *skfB* gene 210 fold, when compared to the parental strain COL. These two genes most probably form an operon and a putative promoter was found only upstream of SkfA. Downstream from the *skfA* and *skfB* genes, the *skfC* gene is located, encoding a transmembrane protein (**Figure 1.5**) and was also found slightly overexpressed in the F9 mutant (2.38 fold) [59].

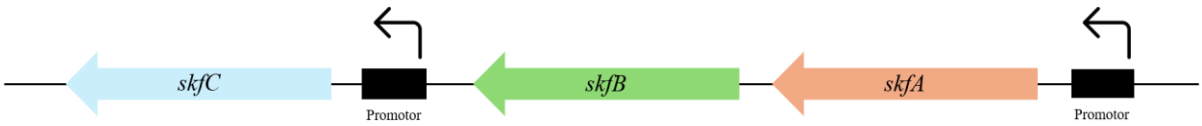


**Figure 1.5. Schematic representation of the relative position of the *skfA*, *skfB* and *skfC* genes in *S. aureus* COL strain.** DNA is represented as a black line. Adapted from [59].

### 1.7.1 The role of Rho in the overexpression of the *Staphylococcus* killing factor proteins

Through whole genome sequencing of the F9 insertion mutant, the single-nucleotide substitution mutation of a glycine for a valine in the position 76 of the Rho protein was found, leading to the impairment of the function of this protein in the F9 mutant [59].

Rho is a transcription terminator factor responsible for releasing the nascent mRNA transcript from the transcription elongation complex for a DNA sequence containing a Rho-dependent termination motif [59–61]. It is also known for repressing pervasive antisense transcription in *S. aureus* and being important

in regulating virulence gene expression dependent from the two-component system SaeRS [59,62]. Various virulence factors are controlled in *S. aureus* by the SaeRS two-component system, including the activation of the production of secreted proteins, essential components of *S. aureus* pathogenicity. It has been indicated that *S. aureus* with an impaired Rho activity show activation of the SaeRS system [62].

Combining the results from different works, it is known that virulence factors are secreted in larger amounts in Rho-deficient strains, as well as that a Rho-deficiency triggers massive transcription of the *skfA* and *skfB* genes [59,62].

### 1.7.2 SkfA and SkfB proteins

In a previous study, it was determined that the proteins SkfA and SkfB evidenced no signs of physical interaction between one another. It is however believed that they are functionally related considering their transcriptional pattern and relative location [59].

Overexpression mutants were constructed, COL+pBCB8*skfA*, COL+pBCB8*skfB* and COL+pBCB8*skfA/skfB*, in which the expression of *skfA*, *skfB* or both *skfA* and *skfB*, respectively, was regulated by the presence/absence of the inducer cadmium chloride ($CdCl_2$) – higher inducer concentrations lead to an increase in gene expression [59].

Making use of the previously mentioned overexpression mutants, to understand how the overexpression of both ORFs (*skfA/skfB*) and each ORF independently (*skfA*; *skfB)* affects *S. aureus*, growth curves where determined using different inducer concentrations (**Figure 1.6**) [59]. Increasing the overexpression of both *skfA* and *skfB* (through the increase in inducer concentration) resulted in a continuous decrease in the growth rate. The overexpression of the genes independently also led to a decrease in the growth rate, even though less prominent. In light of these results, the overexpression of SkfA and SkfB was shown to negatively affect *S. aureus* cell fitness when no cell wall damage was present [59].

To understand if the overexpression of the SkfA and SkfB proteins causes a diminished cell fitness only when in the absence of cell wall damage, the overexpression of *skfA* and *skfB* was conducted for the conditional COLspac*murF* mutant at a suboptimal inducer concentration (IPTG sub-inhibitory concentration – 5 μM) to present incorporation of the abnormal tripeptide in the cell wall and a diminished growth rate. This demanded the construction of an overexpression mutant COLspac*murF*+pBCB8*skfA/skfB*. The growth of the mutants was measured in the presence of different IPTG (*murF* expression inducer) and $CdCl_2$ (*skfA/skfB* expression inducer) concentrations (**Figure 1.7**) [59].

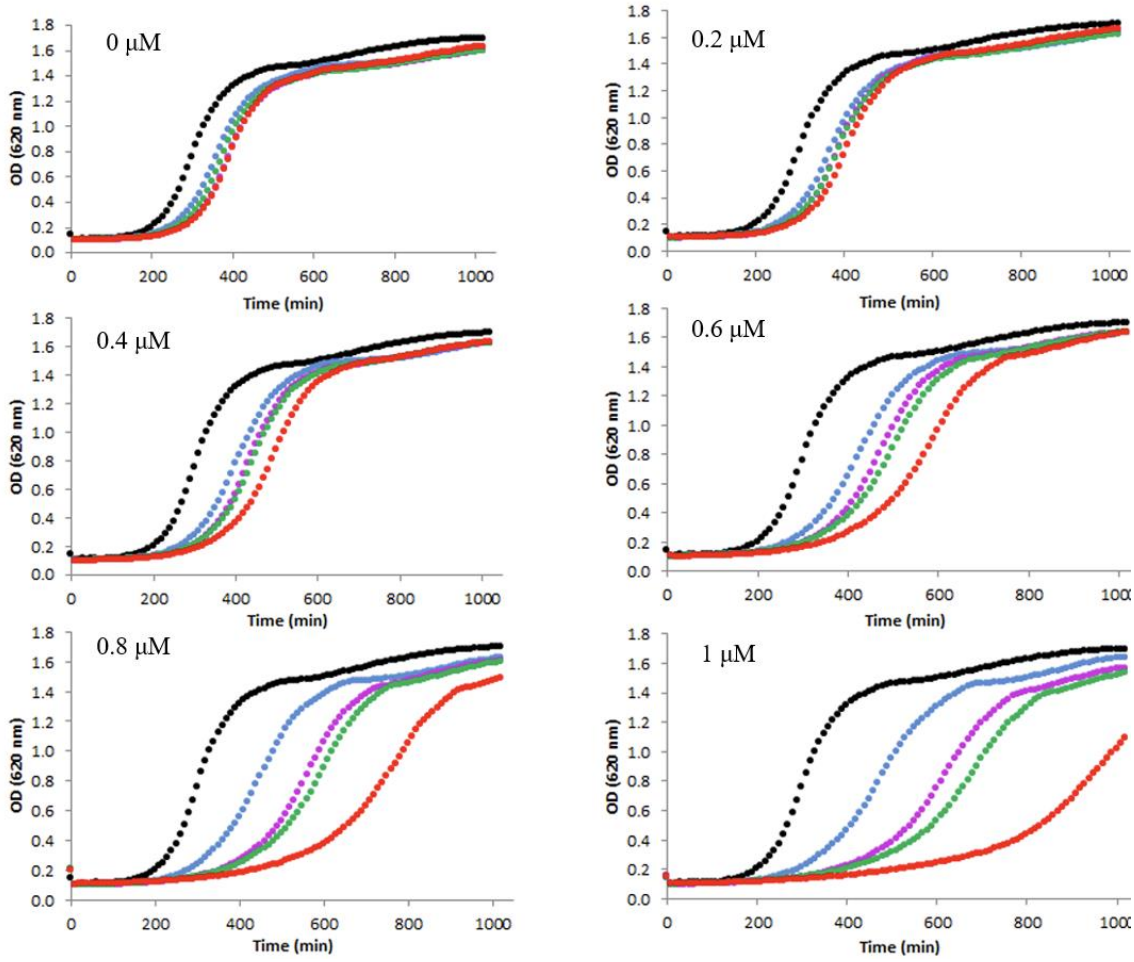


**Figure 1.6. Growth curves of the overexpression mutants COL+pBCB8*skfA*, COL+pBCB8*skfB* and COL+pBCB8*skfA/skfB* in the presence of different inducer ($CdCl_2$) concentrations** – 0; 0.2; 0.4; 0.6; 0.8 and 1 µM. Color representation for the strains utilized is as follows: COL [■]; COL+pBCB8 [■]; COL+pBCB8*skfA* [■]; COL+pBCB8*skfB* [■]; and COL+pBCB8*skfA/skfB* [■]. Image obtained from the study [59].

Upon the inhibition of *murF* expression (Figure 1.7 – grey line), growth was greatly reduced, a behavior also seen in the COLspac*murF*+pBCB8*skfA/skfB* mutant when the *skfA* and *skfB* genes are not being overexpressed (Figure 1.7 – light blue line). The presence of 0.2 and 0.4 µM $CdCl_2$, leading to moderate expression of *skfA* and *skfB* (Figure 1.7 – dark blue and light green lines), resulted in a higher growth rate. An exceedingly increased lag phase was observed for 0.6 µM $CdCl_2$ (Figure 1.7 – dark green line). Higher $CdCl_2$ concentrations (Figure 1.7 – pink and red lines) led to no growth. This suggests that even though higher overexpression of the *skfA* and *skfB* genes is proven unfavorable or lethal, lower overexpression levels of these genes have a positive impact on growth when the cell wall is impaired [59].

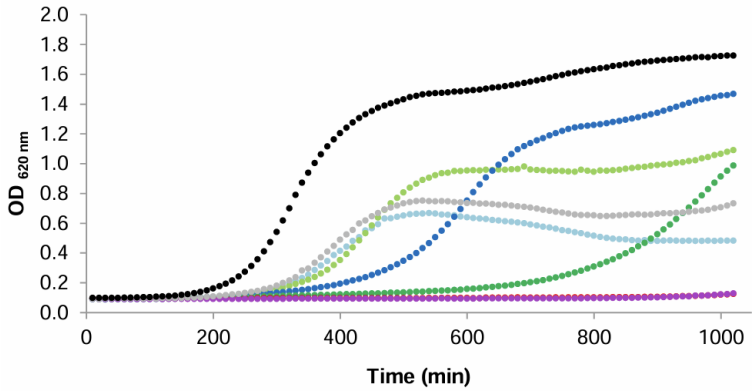


**Figure 1.7. Growth curves of the overexpression of mutant COLspac*murF*+pBCB8*skfA/skfB*.** COLspac*murF*+pBCB8*skfA/skfB* with 100 mM IPTG (optimal concentration) – [■]; COLspac*murF* with 5 mM IPTG (suboptimal concentration) – [■]; COLspac*murF*+pBCB8*skfA/skfB* with 5 mM IPTG and 0 µM $CdCl_2$ – [■]; COLspac*murF*+pBCB8*skfA/skfB* with 5 mM IPTG and 0.2 µM $CdCl_2$ – [■]; COLspac*murF*+pBCB8*skfA/skfB* with 5 mM IPTG and 0.4 µM $CdCl_2$ – [■]; COLspac*murF*+pBCB8*skfA/skfB* with 5 mM IPTG and 0.6 µM $CdCl_2$ – [■]; COLspac*murF*+pBCB8*skfA/skfB* with 5 mM IPTG and 0.8 µM $CdCl_2$ – [■]; COLspac*murF*+pBCB8*skfA/skfB* with 5 mM IPTG and 1 µM $CdCl_2$ – [■]. Image obtained from the study [59].

A knock-out mutant for both the *skfA* and *skfB* genes was constructed (COLΔ*skfA/skfB*). Its growth pattern was very similar to the COL parental strain, indicating the *skfA* and *skfB* genes are not essential and their absence does not affect *S. aureus* growth under normal growth conditions, neither affects antibiotic resistance (β-lactams and other classes of antibiotics). Their overexpression (of both *skfA* and *skfB*), however, resulted in a decrease in resistance to β-lactams [59].

### 1.7.2.1 Structural analysis of the SkfA and SkfB proteins

Structural analysis can provide important information for the understanding of a protein function. Structural elucidation of SkfB was performed with nuclear magnetic resonance (NMR), and the results indicated the protein is found unstructured in solution. The SWISS-MODEL secondary structure prediction tool identified a single alpha helix of amphipathic nature, accommodating on the surface only hydrophilic residues and hydrophobic residues on opposite sides, something commonly found in proteins that can associate with membranes. Additional experiments were conducted to determine if SkfA and SkfB could bind to DNA, since during the purification steps of these proteins, the co-elution of DNA was observed. These experiments indicated that the SkfA and SkfB proteins can, in fact, bind to DNA [59].

The structure of SkfA and SkfB has several common features with bacteriocins, generally defined as being a subgroup of AMPs, since they are small cationic proteins of amphipathic structure and often found unstructured in solution, adopting an α-helical conformation when membrane-associated [63–65]. The possibility that these proteins act as bacteriocins/AMPs against pathogenic bacteria is being exploited.

Since it is thought that SkfA and SkfB can bind to membranes [59], the possibility that they can bind to the extracellular membrane of *Escherichia coli* was exploited. Results revealed that, when overexpressed in *E. coli*, these proteins can, in fact, be found in the membrane fraction after performing a cellular fractionation method. SkfA and SkfB were, however, not found in the cellular membrane fraction of *S. aureus* (various *S. aureus* strains and mutants were studied).

#### 1.7.2.2 SkfA and SkfB proteins in the context of macrophage infection

Given that the SkfA and SkfB proteins may be secreted to the extracellular environment, their capability of infecting human cells was explored through human macrophage infection assays. In conditions of overexpression of the studied proteins, an increase of 4.6 fold was observed for macrophage internalization, when compared to the parental COL strain [66]. It was hypothesized that the SkfA and SkfB proteins could have a similar function as the "secretable expanded repertoire adhesive molecules" (SERAM) family [66,67]. The accepted SERAM features include their mediation of bacterial adhesion to the host and their capacity of host ligand interaction, interfering with host defense mechanisms [67]. If this hypothesis is real, it would mean that the SkfA and SkfB proteins are secreted to the extracellular environment (possibly by the action of the SkfC transmembrane protein) to interact with the macrophages and aid in the adhesion and infection by *S. aureus*. Additionally, taking into account that the SkfA and SkfB proteins can bind DNA, these could be acting as transcription regulators, upregulating the expression of genes involved in macrophage adhesion, explaining the higher internalization percentage observed [66].

### 1.7.3 SkfC protein

*In silico* analysis of SkfC revealed that it is composed of 10 transmembrane α-helixes, as well as two EamA domains that fold in opposite directions, characteristics commonly found in proteins that act as transporters [59,68]. Additionally, the formation of a putative transmembrane pore composed of uncharged amino acids was discovered. This putative pore has residues prone to phosphorylation on the cytoplasmatic side, suggesting that the SkfC protein can act as a sensor for external stimuli and transfer a cascade signal to the cytoplasmic side for the SkfA and SkfB partners to take action. It is therefore hypothesized that SkfC could be a transporter of the drug/metabolite exporter (DME) family [59].

It has been shown that *skfC* is not an essential gene, as its expression was unneeded for *S. aureus* growth in normal conditions. Its expression is, however, relevant when in antibiotic stress conditions. This further corroborates the hypothesis that SkfC can sense external stimuli and translocate the signal to the cytoplasmic side, triggering the response of effector proteins, allowing for cell adaptation [59].

## 1.8 The aim of this project

The results obtained through the discovery of the overexpression of *skfA*, *skfB* and *skfC* in F9 cell wall mutant, indicated that the SkfA, SkfB and SkfC proteins appear to be important for *S. aureus* cell adaptation to lethal stress. Various results suggest that SkfA and SkfB may act as AMPs, possibly being excreted to the external environment by what is thought to be their transmembrane partner, SkfC.

The aim of this project consists of deepening the existing knowledge to functionally characterize the SkfAB system by validating the hypothesis that SkfA and SkfB are AMPs. To achieve this objective, the following tasks were performed:

1. Analysis of the activity of SkfA and SkfB in inter-species interaction:
    a. Making use of *skfA* and *skfB* promotor fusion mutants and exploring the behavior of *skfA* and *skfB* expression when in the presence of other Gram-positive and Gram-negative bacteria.
    b. Making use of overexpression mutants and *E. coli* with built in fluorescence.
2. Analysis of how SkfA and SkfB affect macrophage infection:
    a. Through *skfA* and *skfB* overexpression mutants.
    b. By evaluating how the activity of the *skfA* and *skfB* promoter varies in a macrophage infection experiment.

# 2

# MATERIALS AND METHODS

## 2.1 Bacterial strains

*S. aureus* strains and mutants utilized are presented in **Table 2.1**. The *S. aureus* strains were grown with agitation at 37 ºC in liquid mediums – Tryptic Soy Broth (TSB) medium (Difco Laboratories, Le Pont de Claix, France) or Lysogeny Broth (LB) medium (NZYTech, Lisboa, Portugal; Labchem, Santo Antão do Tojal, Portugal) – or grown in solid medium – Tryptic Soy Agar (TSA) medium (Difco Laboratories, Le Pont de Claix, France). The plasmid pBCB8 is a *S. aureus* replicative vector with the inducible pcad promoter, presenting resistance to Kanamycin (Km, 50 μg/mL) (Gibco, New York, USA) and Neomycin (Neo, 50 μg/mL) (Gibco, New York, USA). The promoter fusion plasmid pIG4 carries the reporter gene *gfp*, that encodes the green fluorescent protein (GFP). The mutants COL+pIG4::p*pta* and COL+pIG4::p*skfA/skfB* have the *gfp* reporter gene under the transcriptional control of either the promoter of the *pta* housekeeping gene, used as a positive control, or the promoter of *skfA/skfB* genes, respectively. This allows for monitorization of the transcription of *pta* and *skfA/skfB* through the expression of GFP, which can relatively quantified through fluorescence reading, not affecting the native expression of the chromosomal genes. The pIG4 plasmid presents resistance to Chloramphenicol (Cm, 10 μg/mL) (NZYtech, Lisboa, Portugal).

For the purpose of conducting this study, strains of *Escherichia coli* (*E. coli*), *Klebsiella oxytoca* (*K. oxytoca*), *Micrococcus luteus* (*M. luteus*) and *Bacillus subtilis* (*B. subtilis*) were utilized (**Table 2.2**), all grown with agitation at 37 ºC in TSB medium, except for *E. coli* DsRed (DH5α), which was grown in LB medium supplemented with Ampicillin (Amp, 10 μg/mL) (Sigma, St. Louis, MO, USA), to which it presents resistance.

**Table 2.1.** *S. aureus* strains and mutants.

| Strain/Plasmid | Description | Source or Reference |
|---|---|---|
| COL cured | MRSA strain resistant to methicillin and susceptible to erythromycin. | Rockefeller University |
| COLΔ*skfA/skfB*+pBCB8 | COL strain with *skfA/skfB* genes deleted from the chromosome, with the empty pBCB8 plasmid, $Km^r$ and $Neo^r$. | Portela, R. (unpublished) |
| COLΔ*skfA/skfB*+pBCB8*skfA/skfB* | COL strain with *skfA/skfB* genes deleted from the chromosome with the pBCB8 plasmid overexpressing *skfA/skfB* in the presence of $CdCl_2$, $Km^r$ and $Neo^r$. | Portela, R. (unpublished) |
| COL+pBCB8*skfA* | COL strain with the pBCB8 plasmid overexpressing *skfA* in the presence of $CdCl_2$, $Km^r$ and $Neo^r$. | Portela, R. (unpublished) |
| COL+pBCB8*skfB* | COL strain with the pBCB8 plasmid overexpressing *skfB* in the presence of $CdCl_2$, $Km^r$ and $Neo^r$. | Portela, R. (unpublished) |
| COL+pIG4::p*pta* | COL strain, with the pIG4 plasmid expressing *gfp* under the transcriptional control of the promoter of the housekeeping gene *pta*, $Cm^r$. | A. Carvalho et al. (unpublished) |
| COL+pIG4::p*skfA/skfB* | COL strain, with the pIG4 plasmid expressing *gfp* under the transcriptional control of the promoter of *skfA/skfB*, $Cm^r$. | A. Carvalho et al. (unpublished) |

**Table 2.2.** Strains used in this study.

| Strain/Plasmid | Description | Source or Reference |
|---|---|---|
| *E. coli* ATCC35218 | A clinical *E. coli* strain. | D. L. Butler et al. [69] |
| *E. coli* DsRed (DH5α) | Strain that produces red fluorescent protein DsRed (exc. 558 nm – em. 583 nm); *recA endA1 gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA1* Φ80 Δ*lacZ*ΔM15; | Invitrogen |
| *K. oxytoca* | Strain isolated from natural samples. | Miragaia, M. (unpublished) |
| *M. luteus* ATCC4698 | A clinical *M. luteus* strain. | Tenreiro, R. (unpublished) |
| *B. Subtilis* 168 | A clinical *B. subtilis* strain | Daniel R. Zeigler et al. [70] |

## 2.2 Promoter expression assays in co-culture

The overnight inocula were grown either in a rotor at 50 rpm at 37 ºC, in test tubes, or in a rotating platform at 180 rpm at 37 ºC, in Erlenmeyer flasks. The inocula of the mutant strains COL+pIG4::p*skfA/skfB* and COL+pIG4::p*pta* were prepared using TSB medium supplemented with 10 µg/mL of Cm. The inoculums of each microorganism tested, namely *E. coli* (ATCC35218), *K. oxytoca*, *M. luteus* and *B. subtilis* were prepared using TSB medium.

The co-culturing experiments were performed in TSB medium.

Firstly, strains COL+pIG4::p*skfA/skfB* and COL+pIG4::p*pta* were inoculated at an optical density ($OD_{600nm}$) = 0.05 and grown at 180 rpm at 37 ºC for 4 hours. Then, the $OD_{600nm}$ of the cultures was determined and the competing microorganism (*E. coli*, *K. oxytoca*, *M. luteus* or *B. subtilis*) was co-inoculated at a 1:10, 1:5 or 1:1 ratio (competing microorganism : *S. aureus*). The co-cultures were grown at 180 rpm at 37 ºC for 3 hours. In parallel, monocultures of COL+pIG4::p*skfA/skfB* and COL+pIG4::p*pta* were grown in the same conditions.

A 1 mL aliquot of the co-cultures and monocultures was collected hourly (3 time points) and the $OD_{600nm}$ was read. The cells were centrifuged at 13 000 rpm for 3 minutes, and the pellet resuspended in 500 µL of phosphate-buffered saline 1x (PBS 1X). This process was repeated (centrifugation and resuspension in PBS 1X) and duplicates of each sample were placed in a black 96 well-plate (NUNC, Roskilde, Denmark). Fluorescence was then read in a microplate reader (TECAN, Spark, Switzerland) at an excitation wavelength of 485 nm, emission wavelength of 535 nm with optimal gain. Negative control was PBS 1X.

For each microorganism and ratio studied, three biological replicates were performed.

### 2.3 *E. coli* growth inhibition assays

The overnight inocula were grown on a rotor at 50 rpm at 37 ºC. Two co-culture experiments were conducted: i) strains COLΔ*skfA/skfB*+pBCB8*skfA/skfB* and *E. coli* DsRed (DH5α); ii) COL+pBCB8*skfA* or COL+pBCB8*skfB* and *E. coli* DsRed (DH5α). COLΔ*skfA/skfB*+pBCB8 strain was used as a control for both assays. All strains were grown in LB medium. *E. coli* DsRed was supplemented with Amp (10 µg/mL) and the strains containing the pBCB8 plasmid with Km and Neo (50 µg/mL).

For both assays, the co-cultures (COLΔ*skfA/skfB*+pBCB8 and *E. coli* DsRed; COLΔ*skfA/skfB*+pBCB8*skfA/skfB* and *E. coli* DsRed; COL+pBCB8*skfA* and *E. coli* DsRed; COL+pBCB8*skfB* and *E. coli* DsRed) were inoculated with a 1:1 ratio of the two strains at an $OD_{600nm}$ = 0.05, with different inducer concentrations, namely 0 µM and 0.4 µM of $CdCl_2$. All monocultures (COLΔ*skfA/skfB*+pBCB8, COLΔ*skfA/skfB*+pBCB8*skfA/skfB*, COL+pBCB8*skfA*, COL+pBCB8*skfB* and *E. coli* DsRed) were inoculated at an $OD_{600nm}$ = 0.05 also with 0 µM $CdCl_2$ and 0.4 µM $CdCl_2$. Following inoculation, all cultures were grown at 180 rpm at 37 ºC for 4 hours. After the initial growth time of 4 hours, the following procedure was repeated for each culture condition for three timepoints (each with 1 hour difference): the $OD_{600nm}$ was measured and 1 mL was collected and centrifuged at

13 000 rpm for 3 minutes. The cells were washed in PBS 1X and subsequently resuspended in 500 µL PBS 1X and 200 µL of each sample (duplicates were performed) were placed in a black 96-well plate (NUNC). Fluorescence was read in a microplate reader (TECAN) at an excitation wavelength of 560 nm, emission wavelength of 610 nm using optimal gain. As negative control, PBS 1X was used.

For the experiment with the strains COLΔ*skfA/skfB*+pBCB8*skfA/skfB* and *E. coli* DsRed, biological duplicates were obtained. Three biological replicates were performed for the experiment with the strains COL+pBCB8*skfA*, COL+pBCB8*skfB* and *E. coli* DsRed.

### 2.4 Evaluation of Small Colony Variants formation

Overnight inocula of the mutants COLΔ*skfA/skfB*+pBCB8 and COLΔ*skfA/skfB*+pBCB8*skfA/skfB*, supplemented with 100 µg/mL Km, were grown on a rotor at 50 rpm at 37 ºC.

The cultures were supplemented with different inducer concentrations (0 µM $CdCl_2$; 0.2 µM $CdCl_2$; 0.4 µM $CdCl_2$) and grown at 180 rpm for 2 hours. The cultures were plated on TSA supplemented with 100 µg/mL Km, at two timepoints with a 2-hour interval, and $OD_{600nm}$ values were measured. The plates were incubated at 37 ºC for 24h, 48h or 72h.

To analyze the colony size, the program ImageJ Fiji editing tool (National Institutes of Health, New York, USA) was utilized.

### 2.5 Impact of *skfA* and *skfB* expression in macrophage internalization and survival

#### 2.5.1 THP-1 cell line culture and growth conditions

The human monocytes cell line THP-1 was maintained in RPMI 1640 (1x) culture medium (Gibco, New York, USA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Gibco), 2 mM glutamine (Gibco), 10 mM HEPES buffer (Gibco), 1 mM sodium pyruvate (Gibco) and 0.05 mM 2-Mercaptoethanol (Gibco). The RPMI medium was maintained at 4 ºC.

The THP-1 cell line is stored in liquid nitrogen with dimethyl sulfoxide (DMSO), which is toxic to the cells [71]. To thaw and initiate the cell line culture, the cryopreserved cells were quickly heated at 37 ºC in a water bath, then resuspended in 5 mL of RPMI medium and centrifuged at 750 rpm for 5 minutes to remove the DMSO. The cell pellet was resuspended in 2.5 mL of RPMI medium and added to a T25

cell culture flask with an additional 10 mL of RPMI medium and incubated (Memmert IC0150 $CO_2$ incubator, Richmond Scientific, United Kingdom) at 37 ºC, 5 % $CO_2$ and 85 % humidity. Cell passages were performed every other day or every three days, adding new RPMI medium, to maintain the cell line. Cell density was determined with a Neubauer chamber. These procedures were performed in a biosafety cabinet (Euroclone S.p.A, model S@FEMATE EZ 1.2, Italy). To maintain the THP-1 cell line culture, it needs to be re-initiated after reaching 30 passages.

### 2.5.2 THP-1 cell line differentiation

The cell density of THP-1 cells was determined using a Neubauer chamber. The THP-1 cells were diluted in previously heated (37 ºC) RPMI medium to achieve a $1 \, x \, 10^6$ cell density and homogenized by vortex. Phorbol-12-myristate-13-acetate (PMA) was added to a final concentration of 0.125 µg/mL to stimulate the differentiation of the monocytes so they mimic human macrophages in terms of morphology, surface markers and cytokine production, as well as to fixate the cells [72,73]. A volume of 1 mL of the diluted THP-1 cells with PMA was placed in each well of a 24-well plate (SARSTEDT). One 24-well plate was used for each timepoint and duplicates of each strain and condition were performed. The 24-well plates were then incubated (Memmert) at 37 ºC, 5 % $CO_2$ and 85 % humidity for 22 hours.

After incubation, the medium was removed by vacuum from each well and replaced by fresh previously heated (37 ºC) RPMI medium. The 24-well plates were again incubated (Memmert) at 37 ºC, 5 % $CO_2$ and 85 % humidity for 22 hours.

### 2.5.3 THP-1 cell line infection

The overnight inocula of the several strains and conditions (COLΔ*skfA/skfB*+pBCB8, COLΔ*skfA/skfB*+pBCB8*skfA/skfB*, COL+pBCB8*skfA*, COL+pBCB8*skfB*; 0 µM $CdCl_2$ and 0.4 µM $CdCl_2$) were grown at 180 rpm at 37 ºC. The inocula of the strains containing the pBCB8 plasmid were supplemented with Km and Neo (50 µg/mL).

A 1 mL aliquot of each pre-inoculum was centrifuged at 13 000 rpm for 3 minutes (for the cultures that were supplemented with antibiotics, cells were previously washed with TSB medium through 2-minute centrifugations at 13 000 rpm). The cells were resuspended in 2.5 mL of previously heated (37 ºC) RPMI medium. The $OD_{600nm}$ of these suspensions was measured to determine the volume of culture needed to inoculate the THP-1 macrophages with a multiplicity of infection (MOI) of 5.

The RPMI medium was removed from the macrophage suspensions in 24-well plates that were incubated for 22 hours (from 2.5.2) and replaced with 0.5 mL of the bacterial suspension (MOI of 5) in RPMI medium. The bacterial suspensions used were inoculated on TSA plates for the initial time

(t = 0h). The 24-well plates were centrifuged at RT for 5 minutes at 250 x g and subsequently incubated (Memmert) at 37 ºC, 5 % $CO_2$ and 85 % humidity for 1 hour. All wells were then washed 3 times with previously heated (37 ºC) RPMI medium supplemented with 100 µg/mL of gentamicin (BioWhittaker, Walkersville, USA) to remove the bacteria that failed to be internalized by the macrophages. The three 24-well plates were incubated in the same conditions as before for 1 hour, 6 hours and 24 hours (t = 1h; t = 6h; t = 24h). After the first hour, the RPMI medium with 100 µg/mL gentamicin was removed for the 24-well plates regarding the timepoints (t = 6h) and (t = 24h) and replaced with RPMI medium with 10 µg/mL gentamicin. A lower antibiotic concentration is used for longer timepoints, to prevent gentamicin to penetrate into eukaryotic cells and influence the growth of intracellular bacteria [74]. For the (t = 1h) 24-well plate, after 1-hour growth time, the RPMI medium with 100 µg/mL gentamicin was removed and the wells were quickly washed with autoclaved water. Subsequently, 1 mL of autoclaved water was added to each well and the plate was incubated at RT for 10 minutes to achieve macrophage lysis. The lysed macrophages were serial diluted in autoclaved water and the bacteria were plated on TSA plates, that were incubated for 24 hours at 37 ºC. The same procedure of macrophage lysis, serial dilutions and plating was repeated for the other 24-well plates (t = 6h) and (t = 24h). The colony-forming unit (CFU) number was determined by counting the colonies formed and applying the dilution and volume factor:

$$CFU = \frac{Number\ of\ colonies\ x\ Total\ dilution\ factor}{Volume\ of\ culture\ plated\ in\ mL}$$

For all conditions that required the addition of the inducer, $CdCl_2$ was provided to the cells throughout the experiment. All procedures were performed in a biosafety cabinet (Euroclone S.p.A, model S@FEMATE EZ 1.2).

All macrophage infection assays were performed at least twice. Some were repeated five times.

### 2.5.4 THP-1 cell line infection with *gfp*-promoter fusion mutants

Macrophage infection with strains that carry pIG4 plasmid with a *gfp*-promoter fusion, namely COL+pIG4::p*skfA/skfB* and COL+pIG4::p*pta*, was carried out as above (2.5.2 and 2.5.3), with the exception that the inocula were supplemented with 10 µg/mL of Cm.

Also, for all timepoints (t = 0h, t = 1h, t = 6h and t = 24h), in addition to the serial dilutions and plating in TSA plates, duplicates of each sample (bacterial suspension in RPMI for t = 0h and lysed macrophages for t = 1h, t= 6h and t = 24h) were placed in a black 96-well plate (NUNC). RPMI medium was used as negative control of fluorescence for t = 0h and autoclaved water was used as negative control for the other timepoints.

In addition to calculating the CFUs, fluorescence was read in a microplate reader (TECAN) at an excitation wavelength of 485 nm, emission wavelength of 535 nm with an optimal gain.

#### 2.5.4.1 Fluorescence microscopy of the infected macrophages

Sterile coverslips were placed in the wells of 24-well plates and the THP-1 infection experiment was performed as described above (2.5.4), except for the final macrophage lysis step. The wells with the sterile coverslips, regarding the timepoints t = 1h, t = 6h and t = 24h, were instead washed with 500 µL of PBS 1X and incubated for 15 minutes in paraformaldehyde (PFA), to fixate the cells and conserve their structure [75]. The wells were then washed twice with 500 µL of PBS 1X and placed at 4 ºC until the labelling step.

The labelling solution was prepared by diluting 1:200 the red fluorophore phalloidin 555 in horse serum (HS) at 10% and PBS-triton (PBS-Tx) 0.1%. Phalloidin is a high-affinity filamentous actin probe, that binds to the actin of the macrophages. Phalloidin stained macrophages (red) can thus be visualized by fluorescence microscopy [76]. HS is added to the solution to reduce the noise in fluorescence microscopy, since it binds non-specifically to all biological molecules, in contrast to phalloidin that binds specifically to actin [77]. PBS-Tx at 0.1% was used to create pores in the membrane. The solution was vigorously mixed and a 30 µL drop was placed over parafilm for each coverslip. The coverslips were removed from the 24-well plates and vigorously shaken in PBS-Tx 0.1%. The excess was removed and each coverslip was placed over a drop of the labelling solution, assuring that the macrophages are in contact with the labelling solution. The preparations were incubated at RT in the dark for 1 hour and the coverslips were subsequently vigorously shaken in PBS-Tx 0.1% twice, and then in distilled water, to remove the salts from PBS. The excess was removed and the coverslips were placed face down over a drop of Aqua Poly/Mount that was previously placed on a microscope slide. The microscope slides prepared were incubated in the dark at RT O/N, and were used the next day or stored at -20 ºC.

After obtaining fluorescence microscopy images (microscope ZeissTM Axioscope 5 Upright Microscope with epifluorescent illumination and a Zeiss objective Plan-Neofluar 100x/1.30 Oil M27), these were refined using the program ImageJ Fiji editing tool (National Institutes of Health, New York, USA).

## 2.6 Statistical evaluation

The statistical method implemented to determine the significance of the results obtained for all assays was the student's t-test. This was achieved using the STATISTICA7 software (StatSoft). As this

program utilizes standard error instead of standard deviation, to maintain conformity, the error portrayed in the graphics and tables presented will be regarding the standard error.

# 3

# RESULTS

The discovery of massive overexpression of an operon of two small genes (*skfA* and *skfB*), unannotated in the databases and found to be exclusive of *S. aureus*, encoding proteins of unknown function raised our interest. Previous studies indicated that these proteins seem to play important roles in cell adaptation to lethal stress and are possibly acting as AMPs [59,66]. Given that AMPs display potential therapeutic properties and present advantages in comparison to antibiotics [19,21,22], functionally characterizing the Skf system by validating the hypothesis that SkfA and SkfB are AMPs can potentially improve the biomedicine field.

## 3.1 *skf* promoter expression in the presence of other bacterial species

To assess how the expression of the *skfA* and *skfB* genes varies in the presence of a competing microorganism, the mutants COL+pIG4::ps*kfA/skfB* and COL+pIG4::p*pta*, the latter being used as positive control, were grown in co-culture with the microorganisms *E. coli*, *K. oxytoca*, *M. luteus* and *B. subtilis*. These co-culture assays were performed in different ratios of microorganism to mutant, namely 1:10, 1:5 and 1:1 ratios. The pIG4 plasmid contains a promoterless reporter gene, *gfp*, that encodes the fluorescent protein GFP. The promoter regions of the *skf* operon and of the *pta* gene were cloned upstream of the reported gene, resulting on the promoter fusions mutants COL+pIG4::p*skfA/skfB* and COL+pIG4::p*pta*. In this way, the fluorescence emitted by the GFP provides a relative quantification of the expression of the *skfA* and *skfB* genes, for the COL+pIG4::p*skfA/skfB* mutant, and of the *pta* gene for the mutant COL+pIG4::p*pta*.

The results of the promoter fusion assays in co-culture with the Gram-negative bacteria *E. coli*, for all three evaluated ratios (**Figure 3.1)**, showed that the expression of the *skfA* and *skfB* genes was higher in the presence of *E. coli*, in comparison with the monoculture. This increment in expression increased

with the ratio of *E. coli* vs. *S. aureus* in the culture – higher ratios, such as 1:5 and 1:1, showed an increase in protein expression of 1.87 ± 0.13 fold and 1.71 ± 0.14 fold, respectively (**Table 3.1**). For *pta* expression, the tendency was for a lower expression of the housekeeping gene, when *S. aureus* was grown in the presence of *E. coli*, in comparison with its expression in monoculture. This diminished expression was generally similar for all studied ratios – a decrease of 0.87 ± 0.02 fold, 0.80 ± 0.03 fold and 0.75 ± 0.05 fold for the 1:10, 1:5 and 1:1 culture ratios, respectively (**Table 3.1**).

The results obtained from the promoter fusion assays with the Gram-negative bacteria *K. oxytoca* (**Figure 3.2**) were similar to the ones observed with *E. coli*. A higher expression from the *skf* promoter was observed, for *S. aureus* co-culture with *K. oxytoca*, compared to the expression in monoculture. This increase in expression from the *skf* promoter was not as pronounced as for the assays with *E. coli*, but was still significant, especially for the higher ratio (1:1), with a mean increase of 1.52 ± 0.07 fold (**Table 3.1**). A slight decrease in *pta* expression was observed for the co-culture ratios 1:10 and 1:5. For the 1:1 ratio however, the decrease in *pta* expression was very significant in co-culture, with a mean of 40% lower expression.

The co-culture assays with the Gram-positive bacteria *M. luteus* (**Figure 3.3**) showed no significant alterations in *gfp* gene expression from the *skf* promoter, for the 1:10 and 1:5 ratios, in comparison to the monocultures. The only exception was for the 60 minutes time point of the 1:5 ratio, with a rise of 5% in fluorescence in co-culture with *M. luteus*. However, for the 1:1 ratio a significant 1.75 ± 0.16 fold increment of *gfp* gene expression was observed when comparing the co-culture and monoculture results (**Table 3.1**). Regarding expression from *pta* promoter, no alterations were observed for the 1:10 and 1:5 ratios, except for the first time point of the ratio 1:10. For the 1:1 ratio, however, *pta* expression decreased in co-culture with *M. luteus*.

The results obtained from the co-culturing assays with the Gram-positive bacteria *B. subtilis* (**Figure 3.4)** showed that the expression from the *skf* promoter suffered slight upregulation when comparing the co-cultures and monocultures for the 1:10 ratio (first time point – 60 minutes) and 1:5 ratio (first two time points – 60 and 120 minutes). A significant increase in expression, of 1.58 ± 0.18 fold, was observed for the higher co-culture ratio (1:1) (**Table 3.1**). For the assays of *pta* promoter expression, comparing monocultures and co-cultures with *B. subtilis*, a slight increase was observed for the 1:10 and 1:5 ratios for the first time point. All other measurements resulted in non-statistically significant alterations.

Overall, the promoter fusion assays showed higher expression from the *skf* promoter when in co-culture with competing Gram-negative bacteria, regardless of the ratios at which the microorganisms

were co-inoculated. In contrast, co-culture assays with Gram-positive bacteria, showed that the expression of the *skf* promoter is barely altered except for high co-culture ratios (1:1).

When comparing co-cultures and monocultures, the behavior of promoter expression for the housekeeping gene *pta* and the *skfA* and *skfB* genes is disparate – in co-culture, *pta* promoter expression overall tends to decrease, while for the *skfA* and *skfB* promoter, expression tends to increase.

**Table 3.1. Variation of expression from the promoter fusion assays for the *skfA/skfB* and *pta* promoters.** Values of relative variation of promoter expression when comparing monocultures and co-cultures. Results over 1.00 indicate an increase in expression when *S. aureus* is grown in co-culture; results lower than 1.00 indicate a decrease in expression when *S. aureus* is grown in co-culture. Results are affected by standard error.

| Gram-negative | COL+pIG4::p*pta* | COL+pIG4::p*skfA/skfB* | | COL+pIG4::p*pta* | COL+pIG4::p*skfA/skfB* |
|---|---|---|---|---|---|
| *E. coli* \| 1:10 | 0.87 ± 0.02 | 1.40 ± 0.07 | *K. oxytoca* \| 1:10 | 0.91 ± 0.03 | 1.31 ± 0.05 |
| *E. coli* \| 1:5 | 0.80 ± 0.03 | 1.87 ± 0.13 | *K. oxytoca* \| 1:5 | 0.80 ± 0.03 | 1.37 ± 0.06 |
| *E. coli* \| 1:1 | 0.75 ± 0.05 | 1.71 ± 0.14 | *K. oxytoca* \| 1:1 | 0.60 ± 0.03 | 1.52 ± 0.07 |

| Gram-positive | COL+pIG4::p*pta* | COL+pIG4::p*skfA/SkfB* | | COL+pIG4::p*pta* | COL+pIG4::p*skfA/skfB* |
|---|---|---|---|---|---|
| *M. luteus*\| 1:10 | 0.94 ± 0.04 | 0.93 ± 0.03 | *B. subtilis* \| 1:10 | 1.07 ± 0.03 | 1.06 ± 0.11 |
| *M. luteus* \| 1:5 | 0.99 ± 0.03 | 1.01 ± 0.02 | *B. subtilis* \| 1:5 | 1.12 ± 0.03 | 1.17 ± 0.06 |
| *M. luteus* \| 1:1 | 0.82 ± 0.02 | 1.75 ± 0.16 | *B. subtilis* \| 1:1 | 1.00 ± 0.04 | 1.58 ± 0.18 |

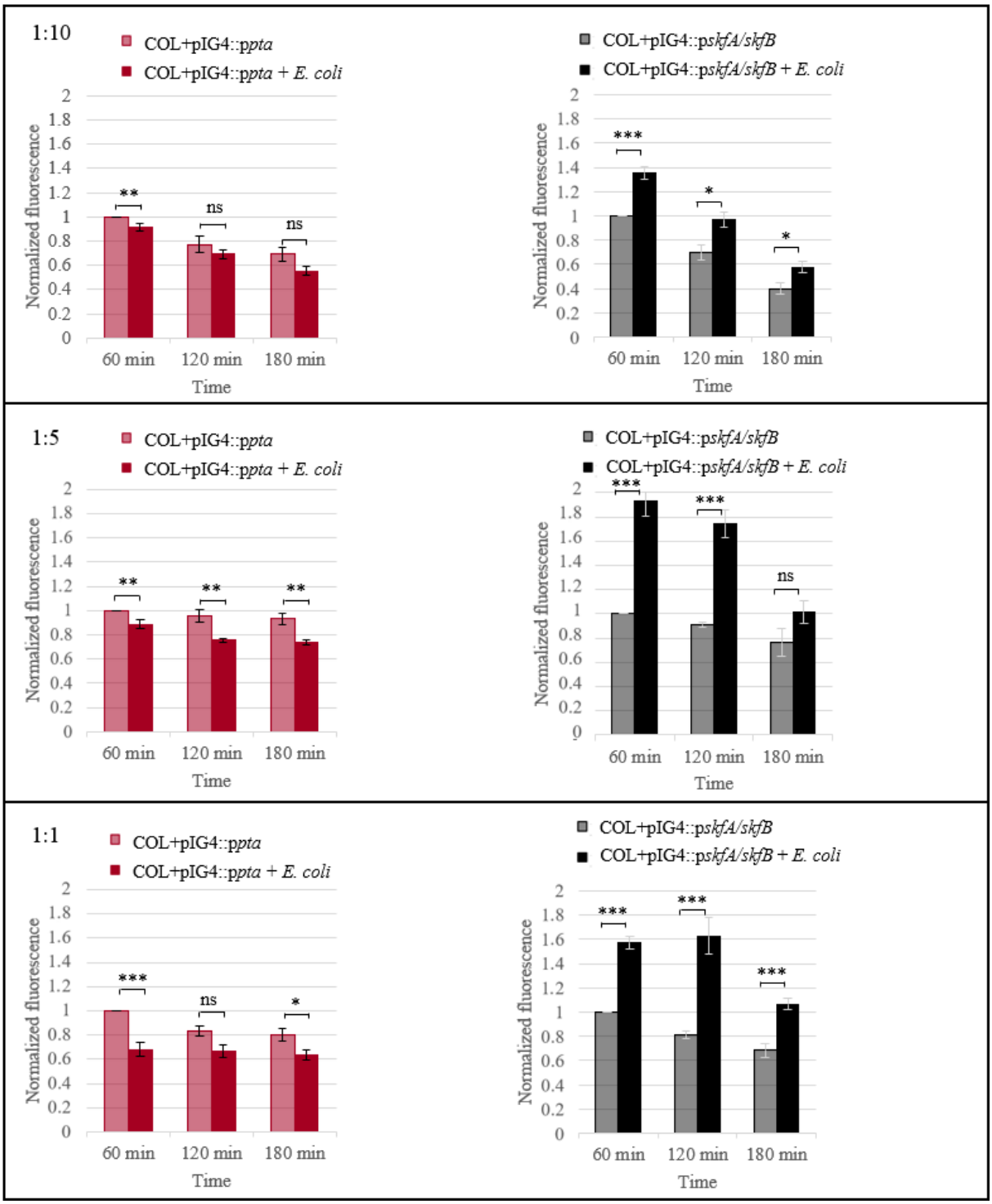


**Figure 3.1. Expression from the *skfA/skfB* and *pta* promoters in the presence of *E. coli*.** In these assays, the *skfA/skfB* and *pta* promoters were fused to the reporter gene *gfp*. The fluorescence emitted by the expressed GFP protein was measured for the *S. aureus* (COL+pIG4::p*pta* or COL+pIG4::p*skfA/skfB*) monoculture (light red/grey colors) and for the *E. coli*/*S. aureus* co-culture (dark red/black colors), for three different ratios 1:10, 1:5 and 1:1, and for three time points. The fluorescence values were normalized against the value of the first time point of the monoculture. ns: no statistical significance; *: significance of $0.01< p \leq 0.05$; **: significance of $0.001 < p \leq 0.01$; ***: significance of $p \leq 0.001$.

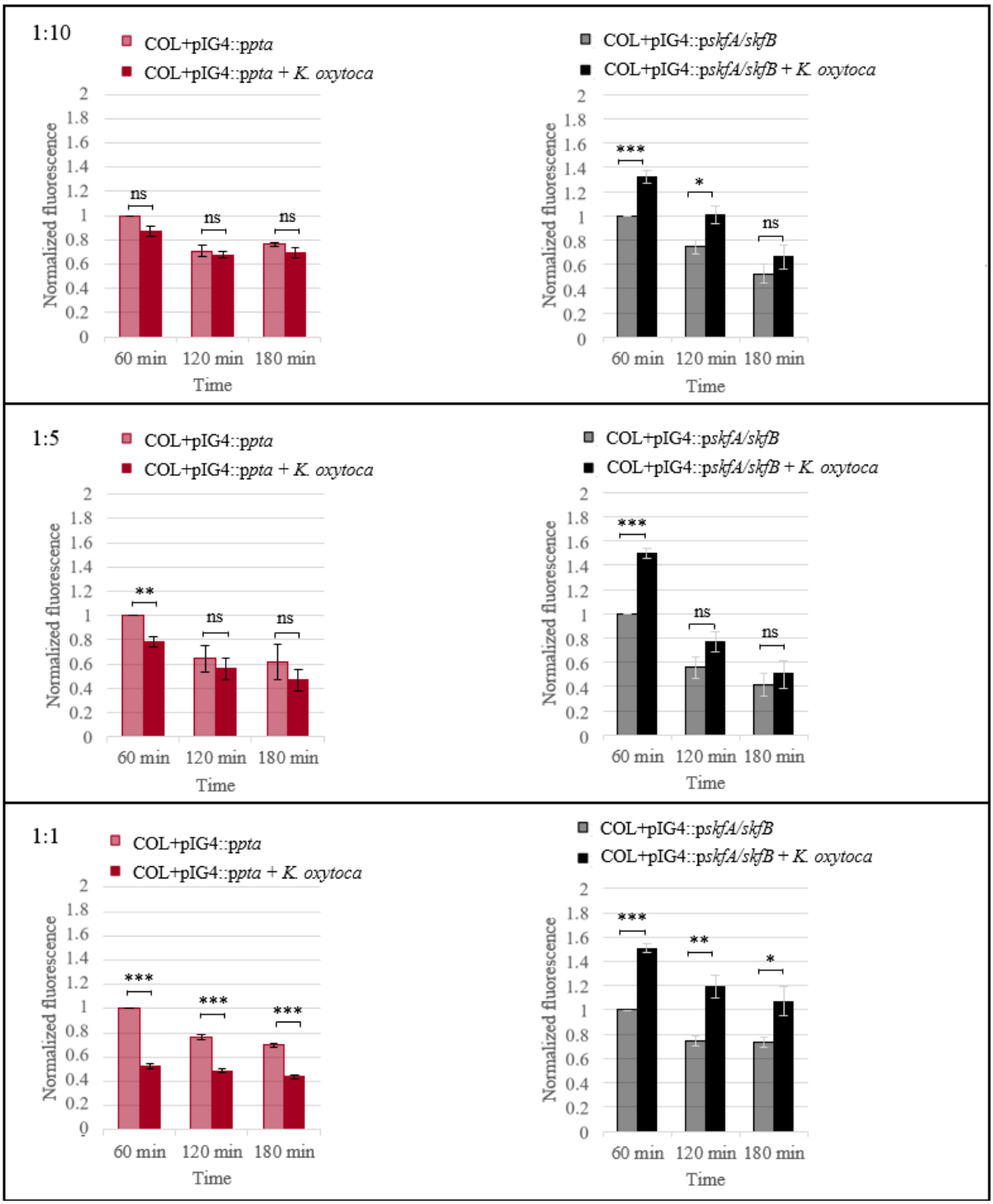


**Figure 3.2. Expression from the *skfA/skfB* and *pta* promoters in the presence of *K. oxytoca*.** In these assays, the *skfA/skfB* and *pta* promoters were fused to the reporter gene *gfp*. The fluorescence emitted by the expressed GFP protein was measured for the *S. aureus* (COL+pIG4::p*pta* or COL+pIG4::p*skfA/skfB*) monoculture (light red/grey colors) and for the *K. oxytoca*/*S. aureus* co-culture (dark red/black colors), for three different ratios 1:10, 1:5 and 1:1, and for three time points. The fluorescence values were normalized against the value of the first time point of the monoculture. ns: no statistical significance; *: significance of $0.01< p \leq 0.05$; **: significance of $0.001 < p \leq 0.01$; ***: significance of $p \leq 0.001$.

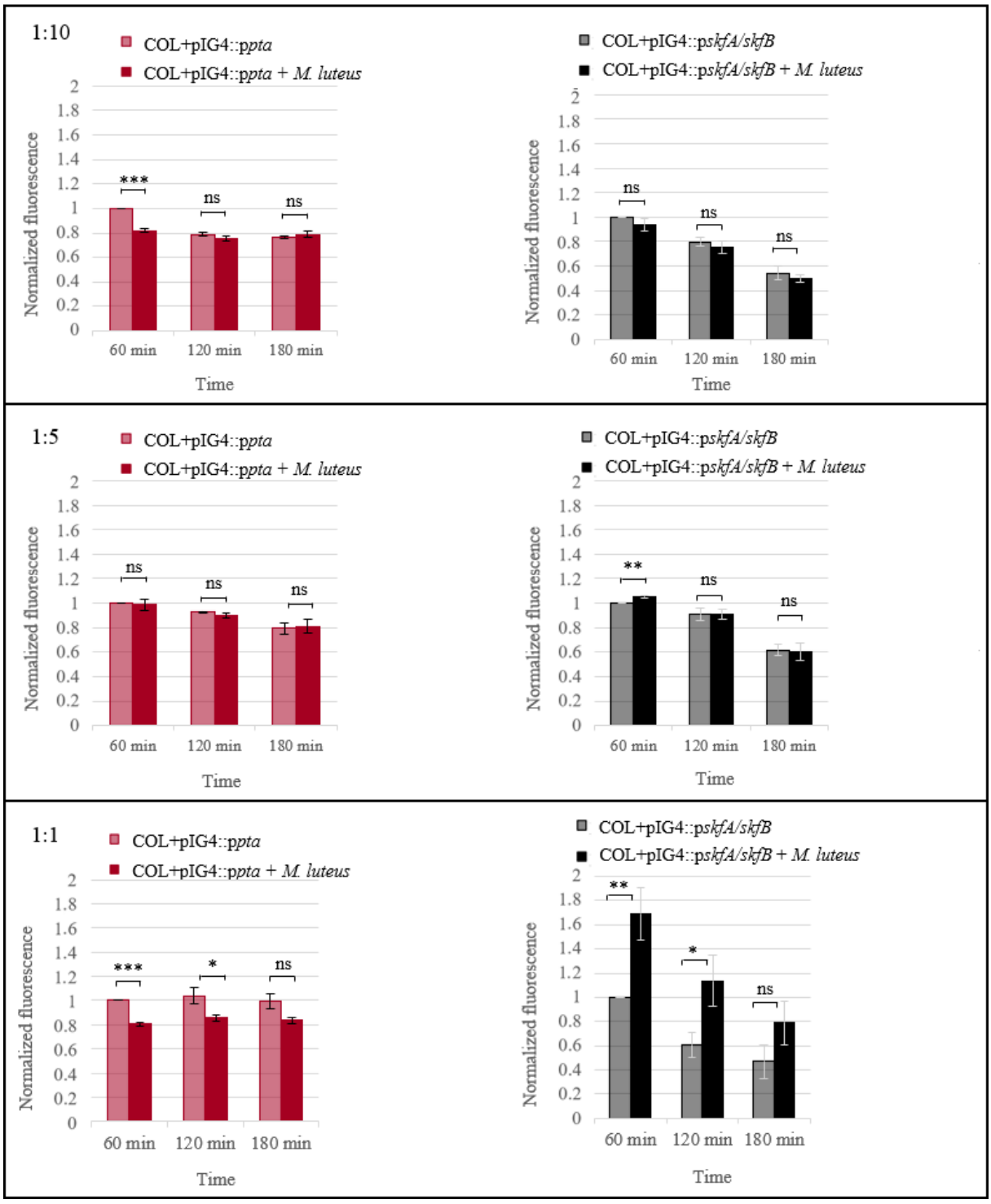


**Figure 3.3. Expression from the *skfA/skfB* and *pta* promoters in the presence of *M. luteus*.** In these assays, the *skfA/skfB* and *pta* promoters were fused to the reporter gene *gfp*. The fluorescence emitted by the expressed GFP protein was measured for the *S. aureus* (COL+pIG4::p*pta* or COL+pIG4::p*skfA/skfB*) monoculture (light red/grey colors) and for the *M. luteus*/*S. aureus* co-culture (dark red/black colors), for three different ratios 1:10, 1:5 and 1:1, and for three time points. The fluorescence values were normalized against the value of the first time point of the monoculture. ns: no statistical significance; *: significance of $0.01< p \leq 0.05$; **: significance of $0.001 < p \leq 0.01$; ***: significance of $p \leq 0.001$.

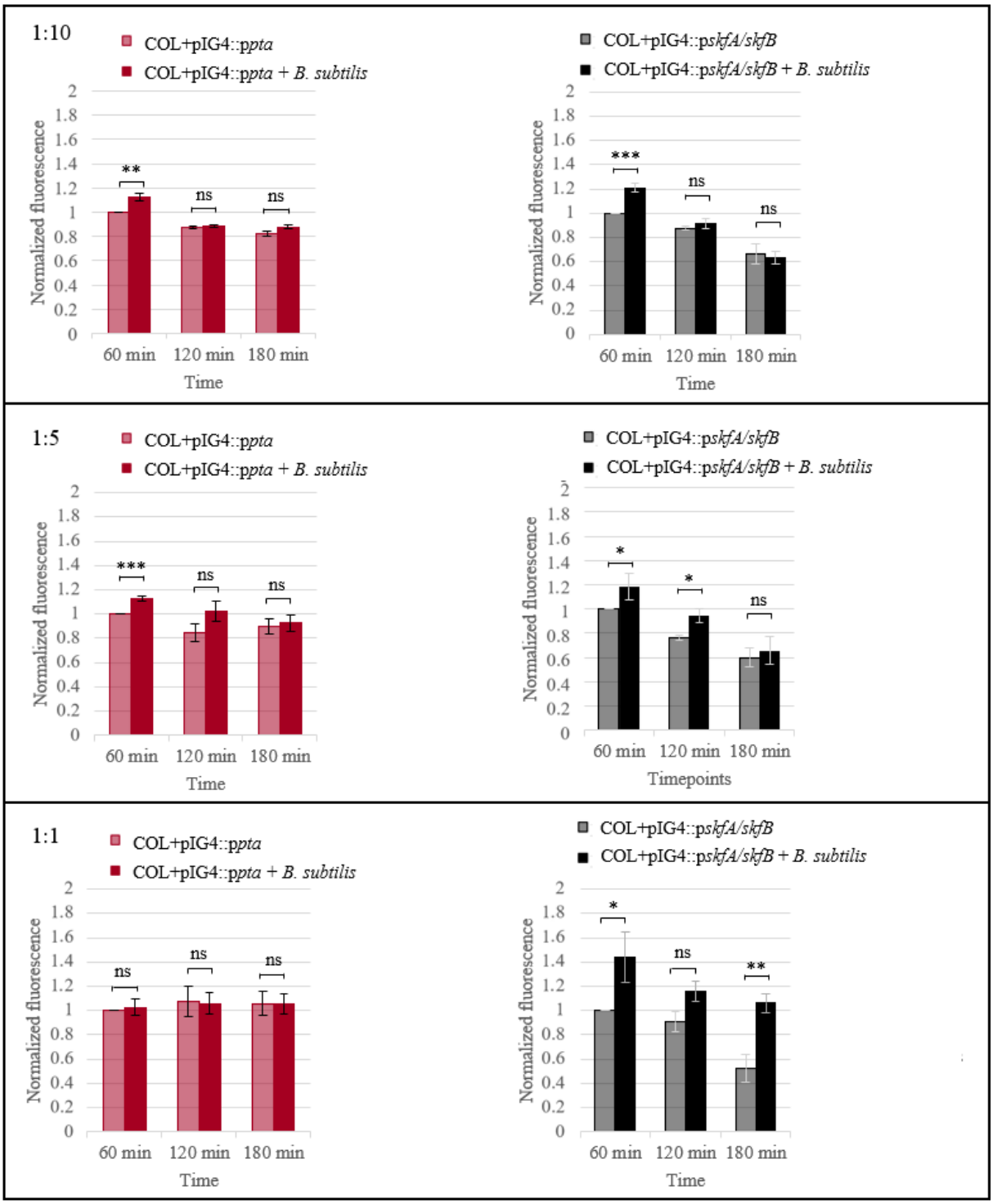


**Figure 3.4. Expression from the *skfA/skfB* and *pta* promoters in the presence of *B. subtilis*.** In these assays, the *skfA/skfB* and *pta* promoters were fused to the reporter gene *gfp*. The fluorescence emitted by the expressed GFP protein was measured for the *S. aureus* (COL+pIG4::p*pta* or COL+pIG4::p*skfA/skfB*) monoculture (light red/grey colors) and for the *B. subtilis*/*S. aureus* co-culture (dark red/black colors), for three different ratios 1:10, 1:5 and 1:1, and for three time points. The fluorescence values were normalized against the value of the first time point of the monoculture. ns: no statistical significance; *: significance of $0.01< p \leq 0.05$; **: significance of $0.001 < p \leq 0.01$; ***: significance of $p \leq 0.001$.

### 3.2 *E. coli* growth in the presence of Skf overexpression mutants

The results from the promoter expression assays in co-culture indicated that the *skfA* and *skfB* genes are overexpressed, when a competitor bacterium is present in the culture. The highest difference in *skfA* and *skfB* overexpression was observed for competitor *E. coli*. As the previous assays only indicated promoter expression (through fluorescence quantification) and did not provide information on the growth of either *E. coli* or the *S. aureus* mutants, co-culture assays using *S. aureus skfA/skfB* overexpression mutants (COLΔ*skfA/skfB*+pBCB8*skfA/skfB*; COL+pBCB8*skfA*; COL+pBCB8*skfB*) and *E. coli* DsRed were performed. The overexpression of the Skf proteins was induced by the presence of $CdCl_2$ at 0.4 µM.

*E. coli* DsRed co-culture assays were performed with the *S. aureus* overexpression mutant (COLΔ*skfA/skfB*+pBCB8*skfA/skfB*) of both proteins SkfA and SkfB simultaneously (**Figure 3.5**) and with the *S. aureus* overexpression mutants (COL+pBCB8*skfA*; COL+pBCB8*skfB*) of each protein SkfA or SkfB (**Figure 3.6**). The monocultures of the COLΔ*skfA/skfB*+pBCB8 (control strain) and Skf overexpression mutants did not emit fluorescence in the detection wavelength of DsRed, confirming that the measured fluorescence resulted only from *E. coli* DsRed. Thus, the fluorescence measured in the assays refers directly to the number of *E. coli* DsRed cells in the culture.

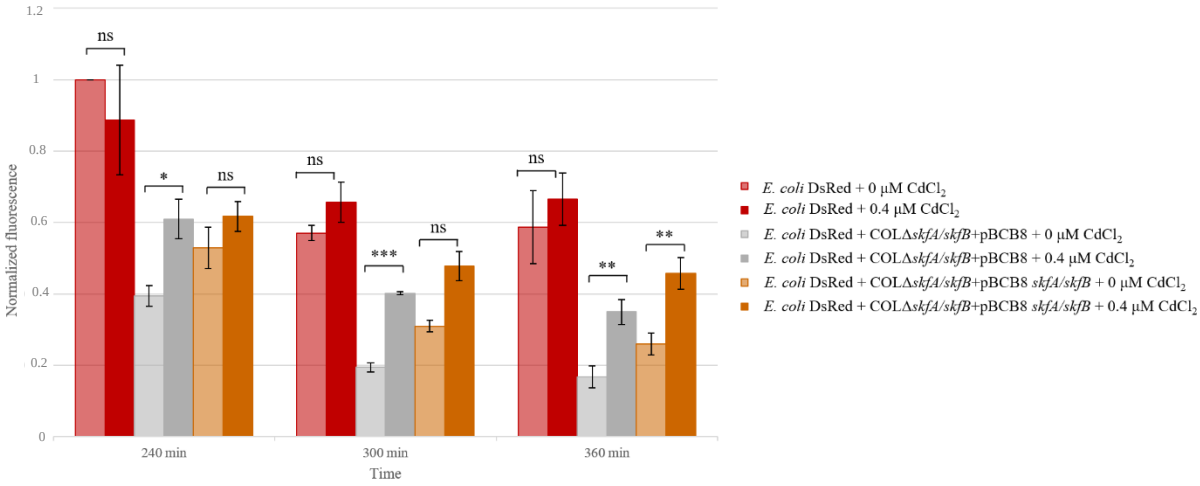


**Figure 3.5. Co-culture assay of *E. coli* DsRed and COLΔ*skfA/skfB*+pBCB8*skfA/skfB*.** Fluorescence emitted by *E. coli* DsRed is normalized against the value of the *E. coli* monoculture grown in the absence of inducer, for the first time point. ns: no statistical significance; *: significance of $0.01 < p \leq 0.05$; **: significance of $0.001 < p \leq 0.01$; ***: significance of $p \leq 0.001$.

The results from **Figure 3.5** showed that the presence of the inducer $CdCl_2$ did not significantly alter the fluorescence emitted by *E. coli* DsRed (light and dark red bars). Since the expression of DsRed is induced by the same amount of arabinose, in both conditions, it can be inferred that the presence of $CdCl_2$ does not alter the growth rate of *E. coli*. The fluorescence measured was higher for *E. coli* DsRed

grown in monoculture, rather than in co-culture with the overexpression mutants (red bars vs. grey/brown bars). An unexpected tendency for fluorescence increase was observed upon overexpression of SkfA and SkfB proteins (dark brown bars) vs. when these proteins were not being overexpressed (light brown bars) – the increase is only statistically significant for the last time point. The same was also observed for the co-cultures with the control COLΔ*skfA/skfB*+pBCB8 mutant (light and dark grey bars).

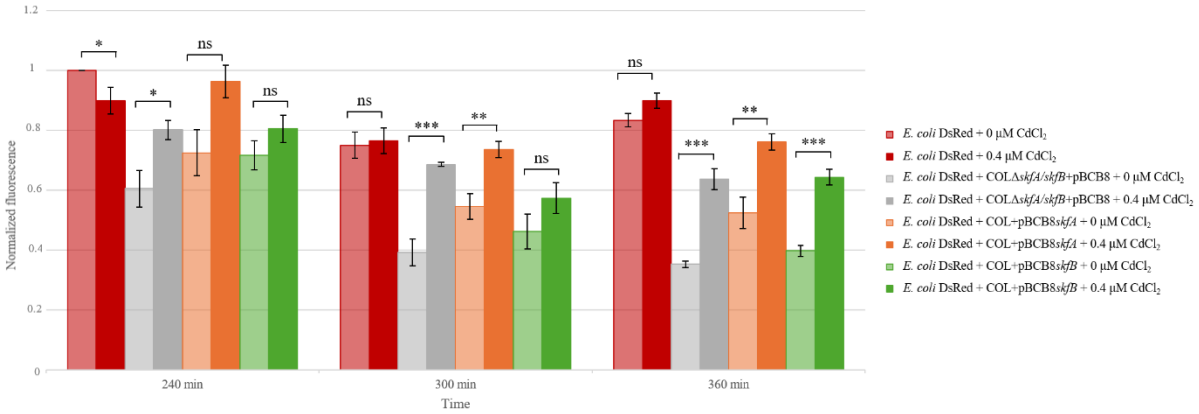


**Figure 3.6. Co-culture assay of *E. coli* DsRed and COL+pBCB8*skfA* and COL+pBCB8*skfB*.** Fluorescence emitted by *E. coli* DsRed is normalized against the value of the *E. coli* monoculture grown in the absence of inducer, for the first time point. ns: no statistical significance; *: significance of $0.01 < p \leq 0.05$; **: significance of $0.001 < p \leq 0.01$; ***: significance of $p \leq 0.001$.

The results of the co-culture assays of *E. coli* DsRed and a *S. aureus* mutant overexpressing only one of the Skf proteins, SkfA or SkfB, are presented in **Figure 3.6.** The overexpression of the SkfB protein (dark green bars) led to lower fluorescence (corresponding to a decreased number of *E. coli* cells) than the overexpression of the SkfA protein (dark orange bars). The fluorescence emitted by *E. coli* when SkfB is being overexpressed (dark green bars) is, however, very similar to the one emitted in co-culture with the COLΔ*skfA/skfB*+pBCB8 control mutant with inducer provided to the medium (dark grey bars). Again, for the co-cultures with the mutant carrying the empty pBCB8 plasmid, in the presence of inducer, higher fluorescence (corresponding to a higher number of *E. coli* cells) is observed, in comparison to the condition with no inducer added to the medium.

Overall, the presence of any *S. aureus* mutant leads to the inhibition of *E. coli* (lower fluorescence). This phenomenon, however, seems independent of Skf protein expression.

### 3.3 Evaluation of Small Colony Variants formation

A previous study demonstrated that for the Skf overexpression mutants, the higher the inducer ($CdCl_2$) concentration provided (0.2, 0.4, 0.6, 0.8, 1 μM), a more prolonged bacterial lag phase of the growth curve was observed [59]. It has been reported that SCVs originate as a consequence of an extended lag time [78]. Thus evaluating the hypothesis that the overexpression of the SkfA and SkfB proteins induces SCV formation in *S. aureus* was explored.

To determine if the proteins SkfA and SkfB can induce *S. aureus* to form SCVs, the overexpression mutant COLΔ*skfA/skfB*+*pBCB8skfA/skfB* and the control strain COLΔ*skfA/skfB*+pBCB8 were grown for 24, 48 and 72 hours, with different inducer concentrations, and the mean colony area after the incubation period was measured (results are shown in **Figure 3.7**).

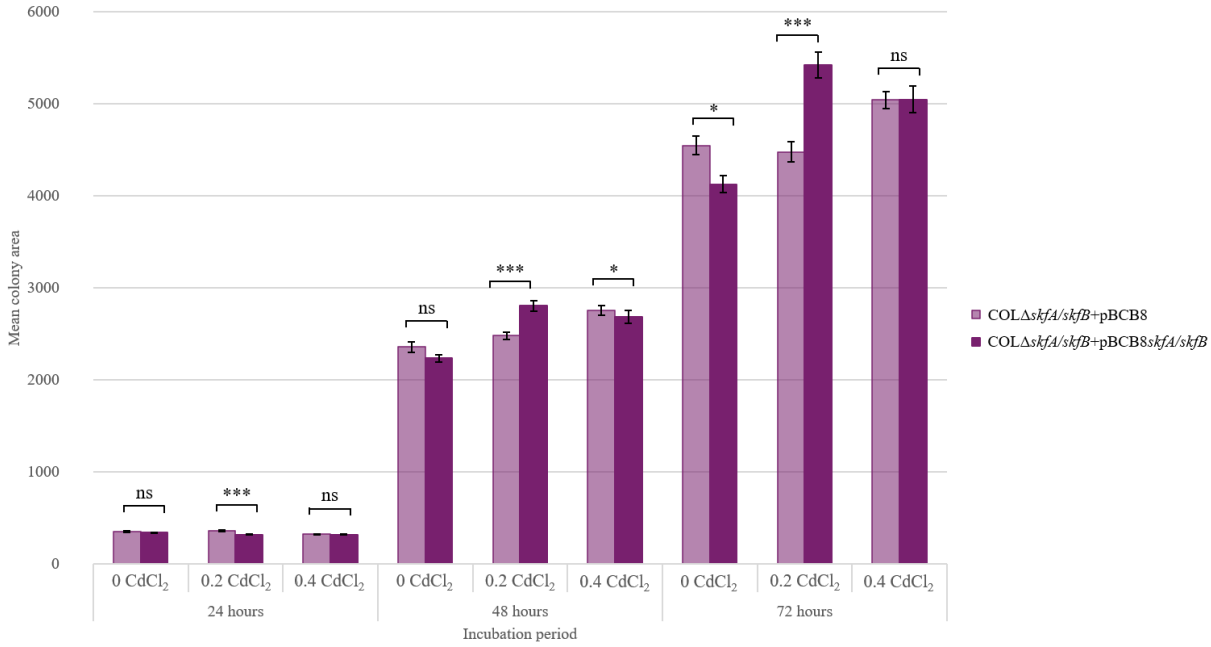


**Figure 3.7. Mean colony area of the mutant COLΔ*skfA/skfB*+*pBCB8skfA/skfB* and the control strain COLΔ*skfA/skfB*+pBCB8.** The mean area of 4430 colonies was determined after different incubation periods and being grown with different inducer concentrations. ns: no statistical significance; *: significance of $0.01 < p \leq 0.05$; **: significance of $0.001 < p \leq 0.01$; ***: significance of $p \leq 0.001$.

For the results regarding an incubation time of 24 hours, in the absence of inducer, no alteration in the colony area was observed when comparing the growth of the control strain, without the *skfA* and *skfB* genes (light purple), and the mutant with the inducible promoter controlling their expression (dark purple). For a low inducer concentration (0.2 μM), a reduction in the colony area was observed for the overexpression mutant, however no longer observed for the 0.4 μM $CdCl_2$ concentration.

Regarding the results of a 48-hour incubation, again no significant changes in the colony area of both strains were observed in the absence of inducer. For the 24-hour incubation, the presence of 0.2 μM of $CdCl_2$ led to an increase in colony area of the mutant overexpressing the proteins. In contrast, for a higher inducer concentration, the overexpression of the proteins caused a slight decrease in colony area.

A higher incubation period of 72 hours showed a decrease in colony area of the COLΔ*skfA/skfB*+*pBCB8skfA/skfB* mutant, when there is no expression of the SkfA and SkfB proteins (0 μM $CdCl_2$). An inducer concentration of 0.2 μM led to an increase in colony area for the mutant overexpressing the proteins, but a higher inducer concentration (0.4 μM) indicated no alteration in the colony area of these mutants.

Overall, the results indicate that the SkfA and SkfB proteins are not involved in forming SCV phenotypes.

## 3.4 Impact of *skfA* and *skfB* expression in macrophage internalization and survival

### 3.4.1 Impact of *skf* genes on macrophage infection

To determine how the overexpression of the SkfA and SkfB proteins affects *S. aureus* infection of the host, assays performed with THP-1 macrophages were conducted, with mutants that overexpress both proteins simultaneously (**Figure 3.8**) and with mutants that are able to overexpress protein SkfA or SkfB, separately (**Figure 3.9**). As control, the COLΔ*skfA/skfB*+pBCB8 mutant was used. Throughout the assays, conditions with and without inducer were tested.

The results in **Figure 3.8** showed that the overexpression of both SkfA and SkfB proteins resulted in a higher internalization (t = 1h) of *S. aureus* in macrophages. A 3.53 fold increase in internalization of the COLΔ*skfA/skfB*+pBCB8*skfA/skfB* mutant into macrophages, when 0.4 μM of inducer was provided in comparison to when no inducer was provided, was observed. There was also an impact on *S. aureus* survival (t = 6h) when the proteins are overexpressed. For the time point t = 6h, roughly the same number of mutant cells were viable as after 1 hour of internalization in macrophages (t = 1h), when 0.4 μM of inducer was provided throughout the assay. When no inducer was provided, and therefore, the SkfA and SkfB proteins were not expressed, a survival percentage similar to the control strain (COLΔ*skfA/skfB*+pBCB8) was observed. For the time point t=24h, no significant difference was observed in the survival percentage of all the strains tested. For the control strain COLΔ*skfA/skfB*+pBCB8, the absence of the trans copies of the *skfA* and *skfB* genes translated in a

survival percentage similar to the mutant COLΔ*skfA/skfB*+pBCB8*skfA/skfB* when the expression of the proteins is not induced, for all time points.

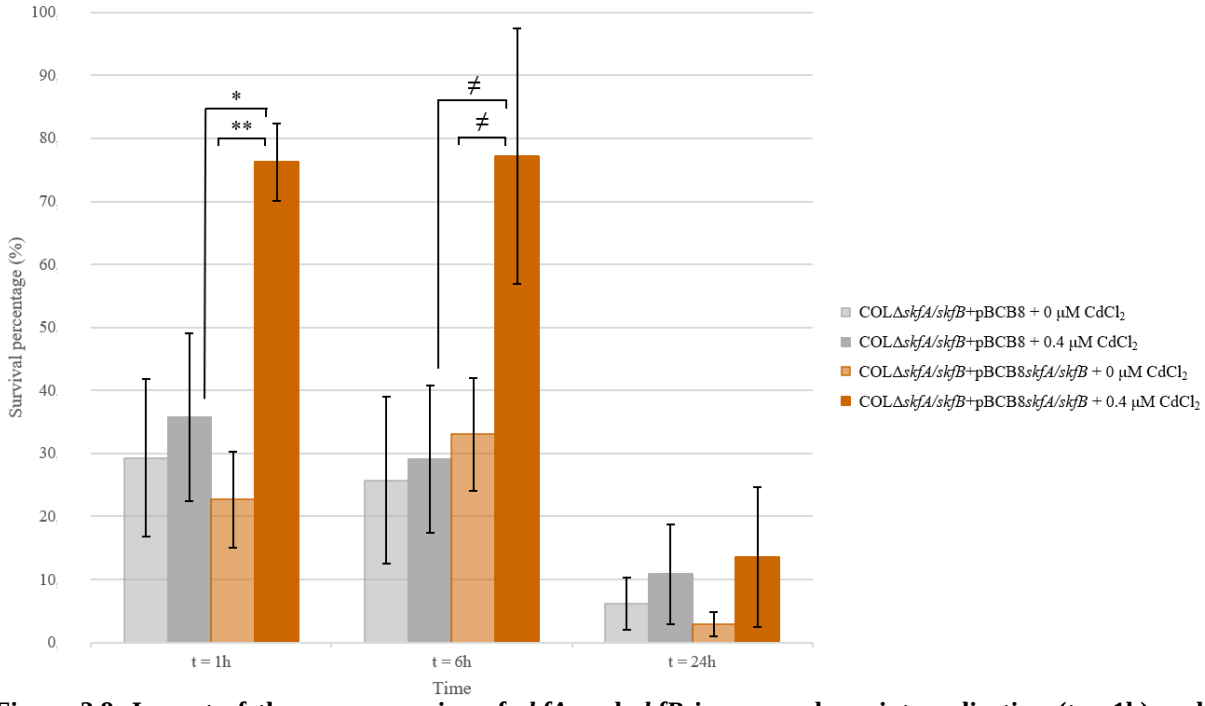


**Figure 3.8. Impact of the overexpression of *skfA* and *skfB* in macrophage internalization (t = 1h) and survival (t = 6h and t = 24h).** For COLΔ*skfA/skfB*+pBCB8*skfA/skfB* in the presence of the inducer $CdCl_2$ for the time points t = 1h and t = 6h, duplicates were performed. For all other conditions, three replicates were performed. For simplification, only the statistically significant differences are displayed. *: significance of $0.01 < p \leq 0.05$; **: significance of $0.001 < p \leq 0.01$; ≠ : significance of $p < 0.06$.

To determine the impact of the individual overexpression of protein SkfA or protein SkfB, macrophage internalization and survival assays were performed with strains COL+pBCB8*skfA* and COL+pBCB8*skfB* (**Figure 3.9**). The individual action of SkfA or SkfB, overexpressed independently (dark orange and green bars respectively), was responsible for a lower survival percentage comparing to the control, either for internalization (t = 1h) or survival (t = 6h and t = 24h). A slight increase was observed, in internalization and survival, for the mutants overexpressing SkfA or SkfB (dark orange and green bars), compared to the same mutants in the absence of inducer (light orange and green bars). The same tendency was observed for the control strains in the presence of inducer. However, these differences were not significant.

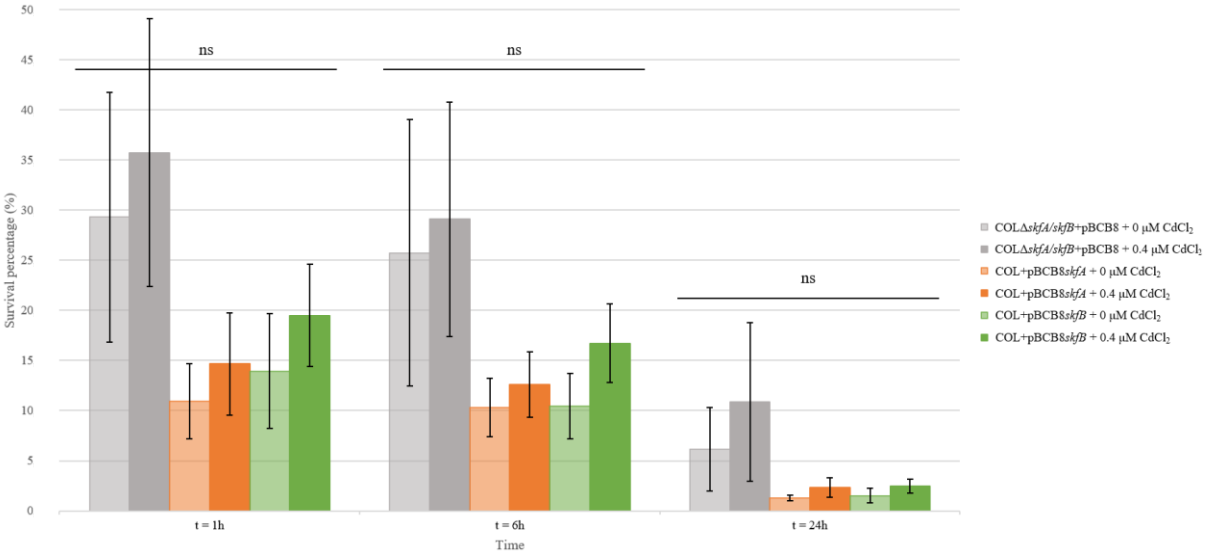


**Figure 3.9. Impact of the overexpression of either *skfA* or *skfB* in macrophage internalization (t = 1h) and survival (t = 6h and t = 24h).** Except for the controls, which were performed in duplicate, all other conditions have five replicates. ns: no statistical significance.

### 3.4.2 Impact of macrophage infection on expression from *skf* promoter

We have demonstrated that the overexpression of the SkfA and SkfB proteins impacts on the *S. aureus* processes of internalization and survival inside macrophages. To determine if the expression of the *skfA* and *skfB* genes is induced when *S. aureus* infects human macrophages, infection assays were conducted with the *gfp*-promoter fusion mutants COL+pIG4::p*pta*, as a control, and COL+pIG4::p*skfA/skfB* (**Figure 3.10)**.

The expression of the *skfA/skfB* promoter, showed a slight increase from t = 1h to t = 6h, and a significant increase in expression for t = 24h. A similar effect was observed for *pta* promoter expression. However, for each time point, the expression from the *skf* promoter appeared higher than the one observed from the *pta* promoter. A comparison of the changes in expression of the *pta* and *skf* promoters between t = 1h and t = 6h and between t =1h and t = 24h is presented in **Figure 3.11**. For *pta* expression, there was an increase of 4.48 fold when comparing between t = 1h and t = 6h and between t =1h and t = 24h, while for *skfA* and *skfB* expression, the increase was of 5.72 fold.

Although the results obtained with fluorescence quantification showed a higher expression from *skf* promoter upon macrophage infection, they were not statistically significant. Thus, the macrophage infection assays were also analyzed by fluorescence microscopy (**Figure 3.12**; **Figure 3.13**). Images were taken of infected macrophages (with both the COL+pIG4::p*pta* and COL+pIG4::p*skfA/skfB*

mutants) for the time points t = 1h, t = 6h and t = 24h. The visualization of the expression of the *pta* and *skfA/skfB* genes was obtained by the green fluorescence emitted by the GFP protein . The definition of the outlines of the macrophages was achieved using the red fluorophore phalloidin 555, which binds to the macrophage's actin.

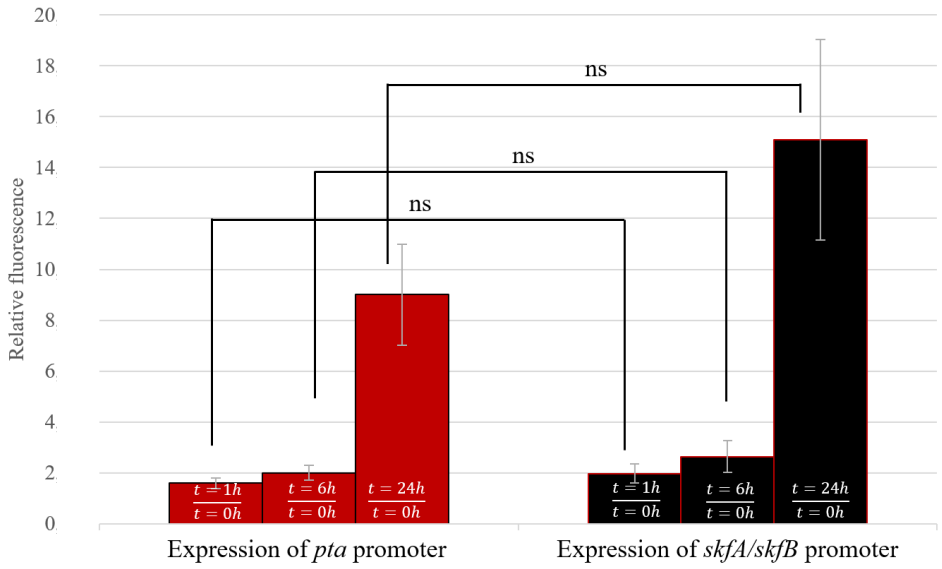


**Figure 3.10. Infection assays of THP-1 macrophage cell line with *gfp*-promoter fusion mutants.** The fluorescence measured resulted from the expression of the reporter gene *gfp*. The expression of the promoters of the *pta* and *skfA/skfB* genes was measured at t = 1h, t = 6h and t = 24h post macrophage infection and compared to the promoter expression at t = 0h, the moment of infection. This experiment was performed thrice. ns: no statistical significance.

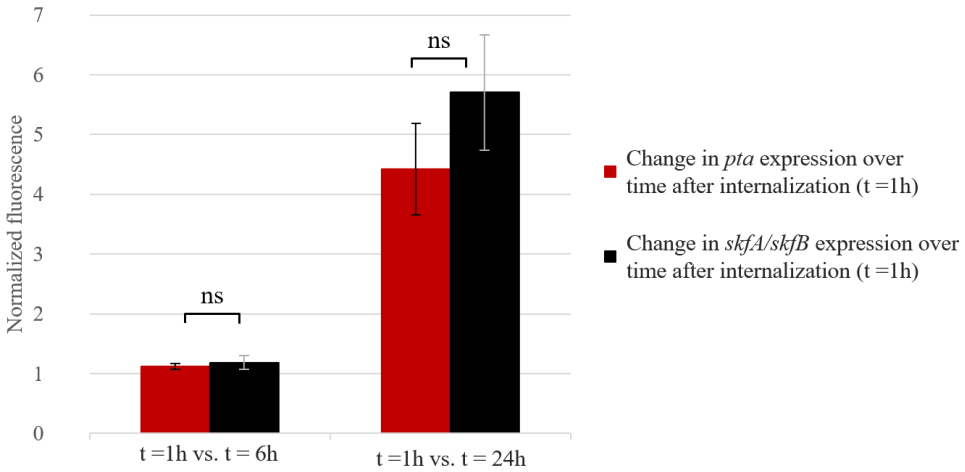


**Figure 3.11. Comparison of the expression of the *pta* and *skfA/skfB* promoters between t =1h and t = 6h and between t =1h and t = 24h post THP-1 cell line infection.** ns: no statistical significance.

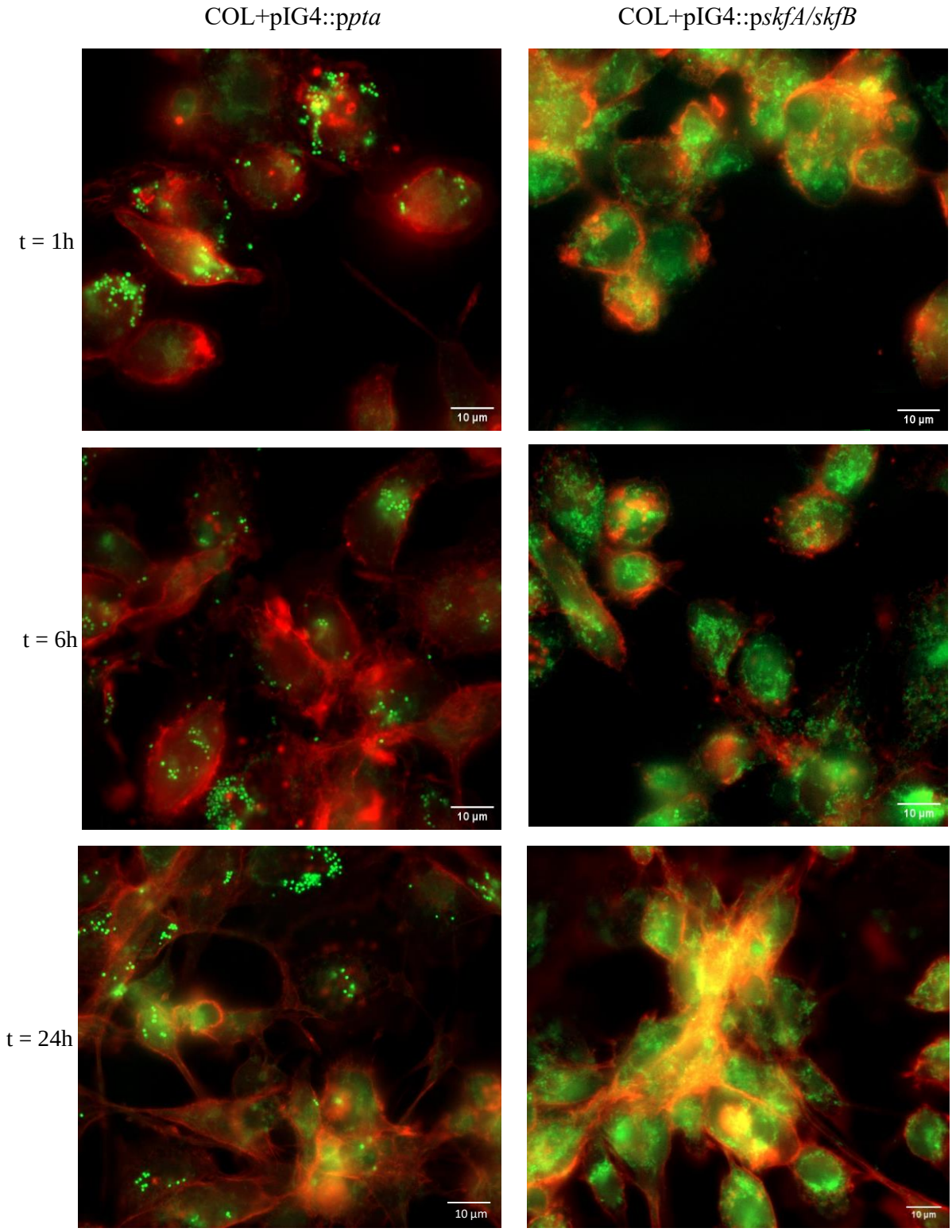


**Figure 3.12. Fluorescence microscopy of THP-1 macrophage cell line infection assays with *gfp*-promoter fusion mutants (COL+pIG4::p*pta* and COL+pIG4::p*skfa/skfB*) for t = 1h, t = 6h and t = 24h – *S. aureus* MOI of 5.** Red fluorescence corresponds to actin from the macrophages and green fluorescence corresponds to *S. aureus* (GFP protein).

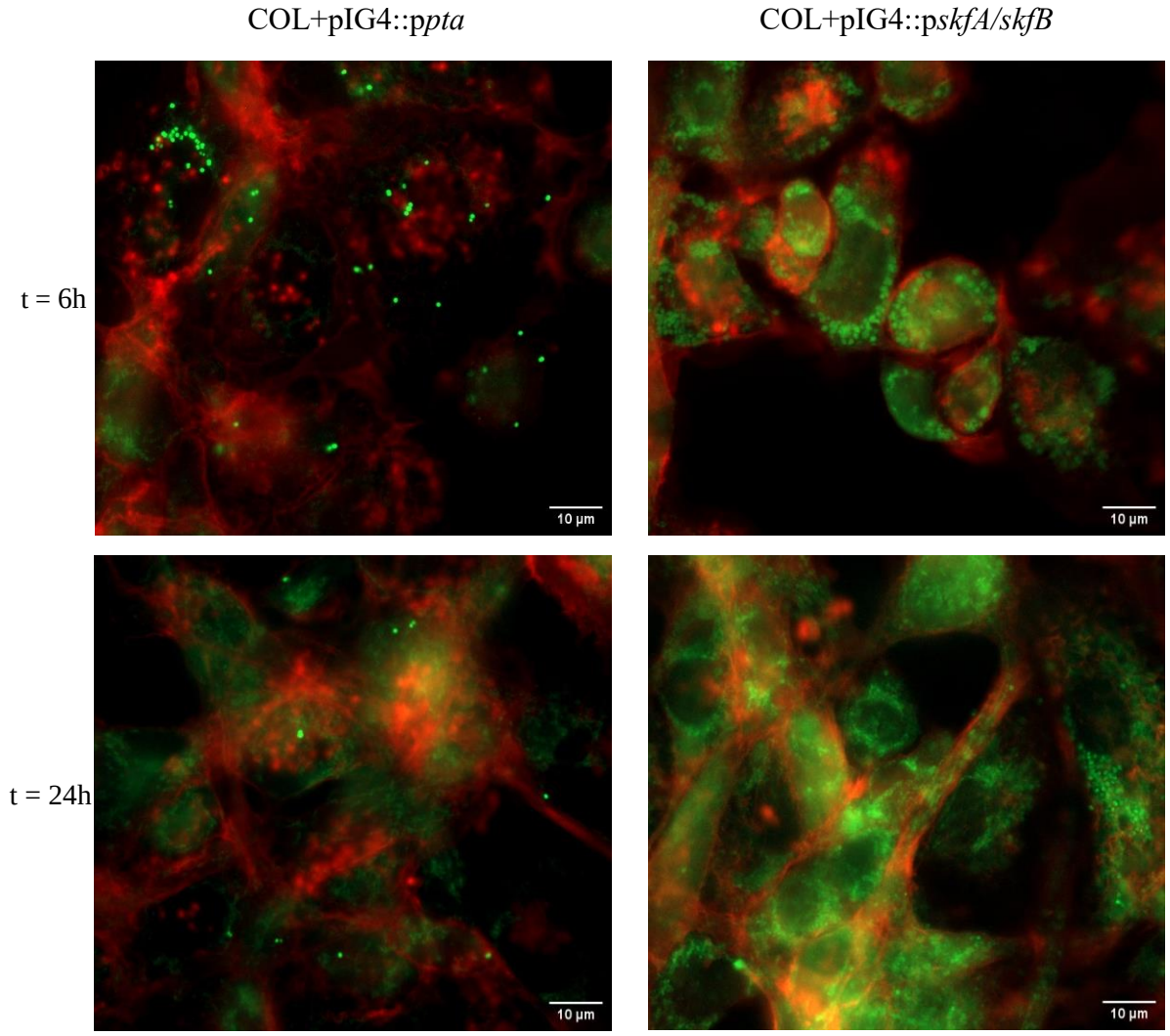


**Figure 3.13. Fluorescence microscopy of THP-1 macrophage cell line infection assays with *gfp*-promoter fusion mutants (COL+pIG4::p*pta* and COL+pIG4::p*skfa/skfB*) for t = 6h and t = 24h – *S. aureus* MOI of 2.5.** Red fluorescence corresponds to actin from the macrophages and green fluorescence corresponds to *S. aureus* (GFP protein).

Higher levels of green fluorescence (caused by the GFP protein) were observed in the microscopy images from the THP-1 infection assays with the COL+pIG4::p*skfA/skfB* mutant than the COL+pIG4::p*pta* mutant (**Figure 3.12)**, performed with the same infection MOI of 5. Also, more GFP fluorescence seemed to be emitted at higher time points for mutant COL+pIG4::p*skfA/skfB*. Visually, the GFP fluorescence emitted by the mutant COL+pIG4::p*skfA/skfB* was cloud-like and diffuse, contrasting with the fluorescence originated by the expression of the housekeeping gene *pta*, which appeared as clear coccus-shaped dots.

To test if this cloud-like fluorescence emission was due to a high MOI (5), the macrophage infection assay was repeated with half the MOI value (**Figure 3.13**). The fluorescence microscopy images still

showed higher and diffuse fluorescence for the COL+pIG4::p*skfA/skfB* mutant, while for the COL+pIG4::p*pta* mutant, the green fluorescence maintains a dot-like appearance. Furthermore, similarly to the microscopy images obtained with a higher MOI (**Figure 3.12)**, higher fluorescence was emitted by GFP protein at t = 24h than at t = 6h.

# 4

# DISCUSSION AND CONCLUSION

To assess the variation of promoter expression of the *skfA* and *skfB* genes in inter-species interaction, promoter expression assays in co-culture with different microorganisms were performed.

When *S. aureus* was grown in co-culture with *E. coli*, the reporter gene controlled by the *skf* promoter was overexpressed, in comparison with the *S. aureus* monoculture condition, indicating that the *skf* genes should be overexpressed in the presence of *E. coli*. This overexpression became more apparent the higher the ratio of *E. coli* versus *S. aureus*. This is due to a higher competition of *E. coli* against *S. aureus*. Since the Skf proteins are overexpressed in response to the presence of *E. coli*, we hypothesized that the SkfA and SkfB proteins could display inhibitory activity against *E. coli* or contribute to *S. aureus* survival when in the presence of the competing microorganism *E. coli*. Under niche competition stress conditions, the promoter of the *skf* genes is stimulated. This stimulus could be a reaction to the presence of a surface-exposed molecule of *E. coli* (e.g. an outer membrane protein, a lipopolysaccharide, an exopolysaccharide, flagella or fimbria) or secreted by *E. coli* (e.g. exotoxins or bacteriocins) [79–84].

The utilization of COL+pIG4::p*pta* as positive control allowed to demonstrate that the presence of the competing microorganism did not contribute to the fluorescence measured, which was solely due to the *S. aureus* mutants carrying the pIG4 plasmid. If the increase in fluorescence observed for the *skf* promoter was caused by *E. coli*, then the same tendency would be seen for co-cultures with COL+pIG4::p*pta*. Moreover, the positive control COL+pIG4::p*pta* allowed to monitor *S. aureus* growth rate alterations, since it was exposing the expression of a housekeeping gene (a decline in fluorescence would indicate a lower *S. aureus* growth rate).

The promoter expression assay in co-culture with *K. oxytoca* also showed overexpression of the *skfA* and *skfB* genes and downregulation of the housekeeping gene *pta* when *S. aureus* was grown in

co-culture with this microorganism. Similarly to the assay with *E. coli*, these expression behaviors became more significant as the inoculation ratio of *K. oxytoca* increased. Since the presence of *E. coli* and *K. oxytoca* triggers the overexpression of the SkfA and SkfB proteins, the trigger to initiate expression of the *skfA/skfB* promoter could be due to the presence of a molecule common to Gram-negative bacteria. Both *E. coli* and *K. oxytoca* are enteric pathogens and virulence genes found in *E. coli* have also been found in *K. oxytoca* (e.g. genes encoding for type VI secretion systems; the heat-labile toxin; the ecotin protein) [85,86]. The Skf proteins may be part of a strategy of *S. aureus* to deal with a specific virulence factor that both *E. coli* and *K. oxytoca* produce. *S. aureus* and *K. oxytoca* are able to infect the same environments (e.g. wounds, food, host environments) and to have a synergistic relationship [85,87]. This synergistic relationship could also be a reason for the upregulation of the *skf* promoter when *S. aureus* is in the presence of *K. oxytoca*.

Just as for the promoter expression assays in co-culture with *E. coli*, the measured fluorescence was solely due to the expression of the GFP protein by *S. aureus* and not from *K. oxytoca*, since the fluorescence of the COL+pIG4::p*pta* mutant decreased in co-culture with *K. oxytoca*. Again, as for *E. coli*, the decrease in fluorescence for the COL+pIG4::p*pta* mutant in co-culture with *K. oxytoca* suggests that *K. oxytoca* impacts the growth of *S. aureus*. The synergistic relationship between *S. aureus* and *K. oxytoca* can be one of enhanced resistance to antimicrobial agents or bacterial invasion of other species and not of biomass enhancement [87].

For *S. aureus* co-cultures with *M. luteus* at 1:10 or 1:5 ratios, no considerable alterations in the expression from the *skf* promoter occurred. This behavior contrasts with the observations for the co-cultures with *E. coli* and *K. oxytoca* and may be explained by the fact that *M. luteus* is a Gram-positive bacterium, while *E. coli* and *K. oxytoca* are Gram-negative bacteria. It is known that the SkfA and SkfB proteins have the ability to bind to lipidic membranes [59]. The non-alteration of the expression of the *skfA* and *skfB* genes, in the presence of *M. luteus* could be a consequence of the mode of action of the Skf system, that may involve binding to the exposed outer membrane of Gram-negative bacteria. In Gram-positive bacteria, that do not have an exposed lipidic membrane, the action of the Skf proteins could be undermined [48,88]. However, for a 1:1 ratio of *S. aureus* and *M. luteus*, upregulation of the *skf* promoter is observed. This suggests the existence of a concentration threshold needed for these proteins to penetrate the cell wall and act in the cytoplasmic membrane of *M. luteus*. A study, in which human skin commensal bacteria, such as *M. luteus*, were co-injected with *S. aureus* in a murine sepsis model, showed that *M. luteus* enhances *S. aureus* virulence [89,90]. For *M. luteus* to be able to alter *S. aureus* virulence factor transcription, as well as to increase the expression of *skf* genes, perhaps it must be present in high ratios.

Regarding *pta* expression in co-culture with *M. luteus*, at 1:10 and 1:5 ratios, no significant changes in its expression were observed in comparison with the monoculture. In a 1:1 ratio, however, the *pta* promoter was downregulated, which could be explained by competition for the available nutrients. *S. aureus* cannot propagate as fast as when in monoculture, when *M. luteus* is also present at the same ratio.

The promoter expression assay in co-culture with *B. subtilis* led to similar results to those with *M. luteus*. Even though an increase in *skfA* and *skfB* expression was already noticeable at ratios 1:10 and 1:5, this increase was more pronounced for the 1:1 ratio. *B. subtilis* is a Gram-positive bacterium that can share a niche with *S. aureus*, such as in epithelial wounds, on skin and in digestive tracts [91]. *B. subtilis* secretes a high number of molecules to control the growth of neighboring microorganisms and has the ability to partly inhibit *S. aureus* virulence factor production (e.g. in *in vitro* assays, on TSB medium), specifically by suppressing the *agr* quorum-sensing signaling system [91,92]. However, as there was an increase in the expression of the *skf* promoter for a 1:1 ratio with *B. subtilis*, this could indicate that the expression of the *skfA* and *skfB* genes is not regulated by this quorum-sensing system.

For *pta* expression, since *B. subtilis* can also inhibit *S. aureus* growth [91], a decrease in fluorescence was expected. Nevertheless, what was observed was a slight increase in *pta* expression, significant for the time point at 60 minutes for 1:10 and 1:5 ratios, while every other measurements indicated no alteration in *pta* expression when comparing to monocultures. A possible explanation is that, when co-cultured with *B. subtilis*, *S. aureus* needs to produce more phosphate acetyltransferase (encoded by the *pta* gene), responsible for the reversible interconversion of acetyl coenzyme A (acetyl-CoA) and acetyl phosphate [93], meaning that *S. aureus* needs to produce more energy when in co-culture with *B. subtilis* (acetyl-CoA delivers the acetyl group to the Krebs cycle, resulting in the production of ATP [94]). This could be a strategy used by *S. aureus* to compete with *B. subtilis*.

In future studies, the variation of the *skf* promoter expression should be evaluated with a broader variety of microorganisms, both Gram-negative and Gram-positive bacteria, contributing to understand the mode of action of the SkfA and SkfB proteins. If the *skfA* and *skfB* genes tend to be overexpressed in the presence of Gram-negative bacteria, this would corroborate the hypothesis that these proteins act on the outer membrane of Gram-negative bacteria or that their expression is triggered by a molecule present in Gram-negative bacteria. It could also help understand if the overexpression at a 1:1 ratio is maintained in other Gram-positive bacteria or if it was specific for *M. luteus* and *B. subtilis*.

The results obtained with the promoter fusion expression assays in co-culture indicated that, in the presence of a competitor, *S. aureus* overexpresses the *skfA* and *skfB* genes, *E. coli* being the bacterium that led to the highest overexpression of these genes. To understand if this overexpression of the *skf*

genes is a competition strategy, we decided to test if these proteins display inhibitory activity against *E. coli*. Thus, *E. coli* DsRed inhibition assays were performed with Skf overexpression mutants.

Regarding the inhibition assays, the fluorescence emitted by *E. coli* DsRed was expected to be higher in monoculture than in co-culture, since in co-culture, the Skf system should be overexpressed according to the promoter fusion assays. Unexpectedly, *E. coli*, grown in co-culture with the *S. aureus skf* overexpression mutant, in the presence of inducer, showed a higher growth rate, translated in higher fluorescence levels. Understanding how $CdCl_2$ affects the growth of *S. aureus* is an important factor. A previous study [59] showed that the presence of the empty pBCB8 plasmid already had a negative impact in the growth of COL, when no inducer was present (**Figure 1.5**). The higher the $CdCl_2$ concentration, the higher the negative impact on the growth rate for the mutant carrying the pBCB8 plasmid. For the COL+pBCB8*skfA/skfB* mutant, as the expression of the SkfA and SkfB proteins increased, the growth rate of the mutant decreased. The same was observed for the COL+pBCB8*skfA* and COL+pBCB8*skfB* mutants, in a less pronounced way. Thus, the presence of the plasmid and the overexpression of these proteins (in the presence of the $CdCl_2$ inducer) is detrimental to *S. aureus* growth rate. The 0.4 μM $CdCl_2$ concentration already has a negative effect in *S. aureus* growth rate, although that delay in growth was deemed acceptable and, therefore, 0.4 μM was the inducer concentration chosen to apply in the assays of this thesis.

The observed higher *E. coli* DsRed survival in co-culture with the control mutant (COLΔ*skfA/skfB*+pBCB8) in the presence of the inducer can be explained by the growth inhibition of *S. aureus* caused by the addition of 0.4 μM $CdCl_2$ to the medium, allowing *E. coli* DsRed to gain some leverage. In the case of the COLΔ*skfA/skfB*+pBCB8*skfA/skfB*, COL+pBCB8*skfA* and COL+pBCB8*skfB* mutants, the presence of inducer, but also of the overexpression of the SkfA and SkfB proteins, result in a decrease of *S. aureus* growth, therefore granting *E. coli* the opportunity to achieve higher growth rates compared to the co-culture in the absence of the inducer. The higher growth rate of *E. coli* in the conditions of overexpression of Skf proteins suggests that these proteins may not display inhibitory activity against *E. coli*. There is also the possibility that *S. aureus* is not able to secrete the overproduced SkfA and SkfB proteins at a sufficient rate, since the putative transporter of these proteins, the SkfC transmembrane protein, is not being overexpressed, and therefore cannot keep up with the high production of the SkfA and SkfB proteins, leading to the accumulation of these proteins inside the cell. This accumulation could be detrimental to *S. aureus* survival, especially because the Skf proteins are thought to be able to associate to cellular membranes and could be binding to *S. aureus* cytoplasmic membrane, possibly disrupting it, or they could be binding to DNA, disrupting normal transcription activity. If this is the case, then this assay is not suitable to demonstrate an eventual inhibitory activity that the SkfA and SkfB proteins may have against *E. coli*. It would be advantageous to explore this

possibility in a future study by repeating these assays, adding a $MgCl_2$ concentration that allows to permeabilize *S. aureus* without impairing *S. aureus* growth and allow the overexpressed SkfA and SkfB proteins to exit to the exterior and not be accumulated inside *S. aureus*. Also, the assays could also be repeated with a lower inducer concentration, such as 0.2 μM $CdCl_2$, as this concentration does not induce such a negative impact on *S. aureus* growth rate and, as the proteins SkfA and SkfB are not being as overexpressed, the SkfC transporter could better manage their export to the exterior.

Regarding the assay performed to evaluate if the SkfA and SkfB proteins are able to lead *S. aureus* to form SCVs, the erratic results obtained indicate that these proteins do not have the capacity to trigger the formation of SCVs in *S. aureus*. Since SCVs usually need 48 to 72 hours to become visible on agar plates, presenting a colony size ten times as small as the wild-type strain [95] and the results obtained from this assay do not indicate a consistent decrease in colony size after a 48- or 72-hour incubation, these proteins most likely do not incite the formation of SCVs in *S. aureus*. To completely rule out this hypothesis, however, it would be interesting to repeat this assay adding $CdCl_2$ to the plate's medium, since at the time only antibiotic was added to the plates.

Since the SkfA and SkfB proteins are probably secreted to the extracellular space and probably interact with lipidic membranes, the hypothesis was raised that they may be involved in the capacity of *S. aureus* infecting host cells. To explore this hypothesis, THP-1 human macrophages cell line infection assays with both overexpression mutants and *gfp*-promoter fusion mutants were conducted.

The assays performed with the overexpression mutants showed that the joint overexpression of both proteins SkfA and SkfB significantly and positively influenced *S. aureus* internalization (t = 1h) and highly influenced *S. aureus* survival, particularly at t = 6h, while the overexpression of SkfA or SkfB individually did not incite any significant alterations in either internalization nor survival of *S. aureus* in human macrophages. These results indicate that the SkfA and SkfB proteins need to physically interact with each other and possibly with other molecules to achieve a positive effect in boosting *S. aureus* capacity to infect and interact with human macrophage cells. These interactions could lead to the transcription or regulation of genes that *S. aureus* needs to successfully infect macrophages and to evade the harsh environment and be able to survive (e.g. help boost the expression of the extracellular fibrinogen-binding protein, protein A, toxins, enzymes that undermine superoxide radicals such as superoxide dismutase A or M, catalase, and so on), since the SkfA and SkfB proteins could be acting as transcription factors, as they can bind to DNA [16,27,30]. The SkfA and SkfB proteins may also be secreted to the extracellular environment and interact with the membrane of the macrophages, as they may present a similar function as the molecules of the SERAM family, aiding in the adhesion of *S. aureus* to the host and in interfering with the host defense mechanisms [67].

$CdCl_2$ could be negatively influencing the viability of the macrophages. A study conducted on the THP-1 cell line explored the possibility that $CdCl_2$ may induce macrophage apoptosis and necrosis, since cadmium is a known toxic, mutagenic and carcinogenic heavy metal. It was ascertained that $CdCl_2$ (with concentrations as low as 5 nM) can indeed reduce macrophage viability by exerting mitochondrial toxicity in THP-1 macrophages and cause apoptosis and necrosis of THP-1 macrophages after a 48-hour incubation period. The mitochondrial toxicity exerted by the presence of $CdCl_2$ increases ROS production in THP-1 macrophages [96]. As the strains overexpressing both the *skfA* and *skfB* genes have such positive impact in the internalization and survival of *S. aureus* in the presence of 0.4 μM $CdCl_2$, this further corroborates the hypothesis that together, the SkfA and SkfB proteins play a part in upregulating the transcription of genes important to counteract the effects of ROS, thus prolonging their survival inside macrophages. The effect of $CdCl_2$ on the macrophages does not explain the higher internalization and survival of *S. aureus*, otherwise we would see the same increase in survival for the control strain COLΔ*skfA/skfB*+pBCB8.

To understand if the *skfA* and *skfB* genes are being overexpressed in the context of macrophage infection, THP-1 cell line infection assays with the *gfp*-promoter fusion mutants were performed.

The *skfA* and *skfB* genes suffered a slight upregulation in expression between t = 1h and t = 6h, followed by a high increase in overexpression for t = 24h (**Figure 3.10**). The expression of the housekeeping gene *pta*, used as control, behaves similarly to that of the *skfA* and *skfB* genes, which are non-essential genes [59]. This indicates that the expression of the *skfA* and *skfB* genes is upregulated in a very similar way to a housekeeping gene such as *pta* when *S. aureus* infects macrophages. This result validated the hypothesis that the SkfA and SkfB proteins are involved in the *S. aureus* mechanism of infection, internalization and survival inside THP-1 macrophages, either by behaving as transcriptional factors or by interacting with the membrane of macrophages. The increase in expression of the *pta* gene may be explained by a cell requirement of energy [93][94] to maintain itself viable inside the macrophages for prolonged periods, since it activates the expression of multiple virulence factors in response to the environmental cues [16,27].

When comparing the expression of the *pta* and *skfA* and *skfB* promoters between t = 1h and t = 6h and between t = 1h and t = 24h post THP-1 macrophage infection, although not statistically significant, the THP-1 macrophages appeared to stimulate the expression of the *skfA* and *skfB* genes at a higher level than of the *pta* gene. The fact that the *skfA* and *skfB* genes were equally expressed between t = 1h and t = 6h, but more expressed than *pta* between t = 1h and t = 24h, indicates that the SkfA and SkfB proteins are important for the prolonged survival of *S. aureus* inside macrophages or that THP-1 macrophages induce a higher expression of these genes at a later stage of infection.

Fluorescence microscopy was performed to visualize the results of the macrophage infection assays with the *gfp*-promoter fusion mutants. The images suggested that the *skf* promoter is more active than the *pta* promoter in both internalization (t = 1h) and survival phases (t = 6h and t = 24h) of infection, since lower GFP fluorescence was observed in the images of *pta* promoter fusion. This corroborated the results obtained from the infection assays with the *gfp*-promoter fusion mutants that the SkfA and SkfB proteins are involved in *S. aureus* infection of THP-1 macrophages and survival inside the host cells. Furthermore, in the images of both infection assays (**Figure 3.12** and **Figure 3.13**), higher fluorescence from GFP protein was observed at t = 24h regarding the *skf* promoter expression. This was in line with the previous results and further corroborated the hypothesis that the SkfA and SkfB proteins are necessary for the prolonged survival of *S. aureus* inside macrophages, and, therefore, their production and expression is more strongly induced at a later stage of infection.

The cloud-like appearance of the fluorescence produced by the expression from the *skf* promoter may imply that there is such high production of GFP protein, that the *S. aureus* cells lyse, dispersing the signal (since GFP is cytotoxic and can lead to cellular death [97]), or that such a high GFP production leads to a more dispersed fluorescence signal in the macrophages.

Before infecting the macrophages, at t = 0h, the fluorescence generated by the expression of the *skf* promoter was already measured. *S. aureus* at t = 0h, just before infection of the macrophages, is suspended in RPMI medium. This suggest that RPMI medium also induces the expression of the *skfA* and *skfB* genes. TSB is known to be the preferred growth medium for *S. aureus* [98] and has a very different composition from the RPMI medium [99], which is frequently used for growing macrophage cell cultures since it has a composition similar to the fluids and extracellular matrix that surrounds macrophages [100]. The fluorescence measured at t = 0h indicates that molecules of the host's extracellular matrix are able to upregulate the expression of the *skfA* and *skfB* genes. This overexpression becomes more prominent the longer the infection period.

While promising, more studies need to be conducted to further understand the mechanism of action of these proteins in the context of macrophage infection and understand how this could be explored to develop new therapies regarding *S. aureus* infections.

Overall, the results of this thesis contributed to the functional elucidation of the proteins encoded by the *skfA* and *skfB* genes of *S. aureus* in the context of inter-species interaction and host infection. It has become clear that these genes are upregulated in the context of inter-species interactions, the upregulation being more evident when *S. aureus* is co-cultured with Gram-negative bacteria, although Gram-positive bacteria also induce upregulation when present in higher ratios. The most probable explanation is that the expression of the *skfA* and *skfB* genes is triggered by the presence of a common

virulence factor of *E. coli* and *K. oxytoca*. The co-culture promoter expression assay with *B. subtilis* showed that there is a high probability that the expression of the *skfA* and *skfB* genes is not regulated by the *agr* quorum-sensing signaling system. In the context of macrophage infection, it has been shown that the SkfA and SkfB proteins need to physically interact with one another to aid in *S. aureus* infection and that they significantly and positively influence *S. aureus* internalization and survival in THP-1 macrophages. These proteins appear to be more vital to the prolonged survival of *S. aureus* inside macrophages, possibly by playing a part in upregulating the expression of genes that help deal with oxidative stress (or by upregulating the expression of virulence factors *S. aureus* needs to infect the host) by acting as transcription factors. These proteins could also act as molecules of the SERAM family and, after being secreted to the extracellular environment, bind to the macrophage's membrane, aid in *S. aureus* adhesion to the host and interfere with the host's defense mechanisms.

Adding to the future perspectives already suggested above, the following studies should be conducted:

1. Deepen the knowledge of how the overexpression of the SkfA and SkfB proteins is connected to *S. aureus* cell wall damage. One strategy could involve employing the *gpf*-promoter fusion mutants in assays where *S. aureus* is grown in medium supplemented with cell wall disrupting agents and understand if cell wall disruption upregulates the *skfA* and *skfB* genes.
2. To determine if the SkfA and SkfB proteins are being excreted to the extracellular environment or remaining inside the cell, assays in which there is production and fractionation of whole cell extracts from the overexpression mutants should be performed. The cytoplasmic-, membrane- and supernatant- fractions should be purified and separated by SDS-PAGE and transferred into a nitrocellulose membrane to detect the presence of the Skf proteins in each fraction by Western Blotting, using anti-SkfA and anti-SkfB polyclonal antibodies.
3. To understand if the SkfA and SkfB proteins could indeed work as transcription factors connected to *S. aureus* virulence, the structure of the proteins should be further elucidated to understand to which molecules or DNA sequence these proteins could bind to. This could be achieved through an electrophoretic mobility shift assay to detect protein–nucleic acid interactions, utilizing growing concentrations of both proteins and discern if these interact with DNA fragments of known virulence factor sequences of *S. aureus*.
4. Test the response of *S. aureus* (by utilizing the *gpf*-promoter fusion mutants) to virulence factors common to *E. coli* and *K. oxytoca* to understand which, if any, trigger the overexpression of the SkfA and SkfB proteins. Knock-out mutants of said virulence factors could be constructed for *E. coli* and *K. oxytoca* to understand if these bacteria continue to induce *skf* promoter expression.

# BIBLIOGRAPHIC REFERENCES


1. Ogston, A. Report upon Micro-Organisms in Surgical Diseases. *BMJ* **1**, 369–375 (1881).
2. Lee, A. S. *et al.* Methicillin-resistant *Staphylococcus aureus*. *Nat. Rev. Dis. Primer* **4**, 18033 (2018).
3. Guo, Y., Song, G., Sun, M., Wang, J. & Wang, Y. Prevalence and Therapies of Antibiotic-Resistance in *Staphylococcus aureus*. *Front. Cell. Infect. Microbiol.* **10**, 107 (2020).
4. Thomer, L., Schneewind, O. & Missiakas, D. Pathogenesis of *Staphylococcus aureus* Bloodstream Infections. *Annu. Rev. Pathol. Mech. Dis.* **11**, 343–364 (2016).
5. Namvar, A. E. *et al.* Clinical characteristics of *Staphylococcus epidermidis*: a systematic review. *GMS Hyg. Infect. Control 93Doc23 ISSN 2196-5226* (2014) doi:10.3205/DGKH000243.
6. Rosenbach, F. J. & Rosenbach, A. J. F. *Mikro-organismen bei den Wund-Infections-Krankheiten des Menschen*. (J.F. Bergmann, 1884).
7. De Vos, P. *et al. BERGEY'S MANUAL OF Systematic Bacteriology (Second Edition)*. vol. The Firmicutes (Springer).
8. Gnanamani, A., Hariharan, P. & Paul-Satyaseela, M. *Staphylococcus aureus*: Overview of Bacteriology, Clinical Diseases, Epidemiology, Antibiotic Resistance and Therapeutic Approach. in *Frontiers in Staphylococcus aureus* (eds. Enany, S. & Crotty Alexander, L. E.) (InTech, 2017). doi:10.5772/67338.
9. Jenul, C. & Horswill, A. R. Regulation of *Staphylococcus aureus* Virulence. *Microbiol. Spectr.* **7**, 7.2.29 (2019).
10. De Jong, N. W. M., Van Kessel, K. P. M. & Van Strijp, J. A. G. Immune Evasion by *Staphylococcus aureus*. *Microbiol. Spectr.* **7**, 7.2.20 (2019).
11. Howden, B. P. *et al. Staphylococcus aureus* host interactions and adaptation. *Nat. Rev. Microbiol.* **21**, 380–395 (2023).
12. Laux, C., Peschel, A. & Krismer, B. *Staphylococcus aureus* Colonization of the Human Nose and Interaction with Other Microbiome Members. *Microbiol. Spectr.* **7**, (2019).
13. Cheung, G. Y. C., Bae, J. S. & Otto, M. Pathogenicity and virulence of *Staphylococcus aureus*. *Virulence* **12**, 547–569 (2021).
14. Pollitt, E. J. G., Szkuta, P. T., Burns, N. & Foster, S. J. *Staphylococcus aureus* infection dynamics. *PLOS Pathog.* **14**, e1007112 (2018).
15. Kong, E. F., Johnson, J. K. & Jabra-Rizk, M. A. Community-Associated Methicillin-Resistant *Staphylococcus aureus*: An Enemy amidst Us. *PLOS Pathog.* **12**, e1005837 (2016).
16. Pidwill, G. R., Gibson, J. F., Cole, J., Renshaw, S. A. & Foster, S. J. The Role of Macrophages in *Staphylococcus aureus* Infection. *Front. Immunol.* **11**, 620339 (2021).
17. Enright, M. C. *et al.* The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc. Natl. Acad. Sci.* **99**, 7687–7692 (2002).
18. Richardson, A. R. Virulence and Metabolism. *Microbiol. Spectr.* **7**, 7.2.39 (2019).
19. Nayab, S. *et al.* A Review of Antimicrobial Peptides: Its Function, Mode of Action and Therapeutic Potential. *Int. J. Pept. Res. Ther.* **28**, 46 (2022).
20. Kelley, J. M., Edberg, J. C. & Kimberly, R. P. Pathways: Strategies for susceptibility genes in SLE. *Autoimmun. Rev.* **9**, 473–476 (2010).
21. Browne, K. *et al.* A New Era of Antibiotics: The Clinical Potential of Antimicrobial Peptides. *Int. J. Mol. Sci.* **21**, 7047 (2020).
22. Magana, M. *et al.* The value of antimicrobial peptides in the age of resistance. *Lancet Infect. Dis.* **20**, e216–e230 (2020).

23. Seyfi, R. *et al.* Antimicrobial Peptides (AMPs): Roles, Functions and Mechanism of Action. *Int. J. Pept. Res. Ther.* **26**, 1451–1463 (2020).
24. Boparai, J. K. & Sharma, P. K. Mini Review on Antimicrobial Peptides, Sources, Mechanism and Recent Applications. *Protein Pept. Lett.* **27**, 4–16 (2019).
25. Zhang, C., Yang, M. & Ericsson, A. C. Function of Macrophages in Disease: Current Understanding on Molecular Mechanisms. *Front. Immunol.* **12**, 620510 (2021).
26. Pandur, E., Tamási, K., Pap, R., Jánosa, G. & Sipos, K. Distinct Effects of *Escherichia coli*,*Pseudomonas aeruginosa* and *Staphylococcus aureus* Cell Wall Component-Induced Inflammation on the Iron Metabolism of THP-1 Cells. *Int. J. Mol. Sci.* **22**, 1497 (2021).
27. Flannagan, R., Heit, B. & Heinrichs, D. Antimicrobial Mechanisms of Macrophages and the Immune Evasion Strategies of *Staphylococcus aureus*. *Pathogens* **4**, 826–868 (2015).
28. Gordon, S. & Martinez-Pomares, L. Physiological roles of macrophages. *Pflüg. Arch. - Eur. J. Physiol.* **469**, 365–374 (2017).
29. Cai, J. *et al. Staphylococcus aureus* facilitates its survival in bovine macrophages by blocking autophagic flux. *J. Cell. Mol. Med.* **24**, 3460–3468 (2020).
30. Moldovan, A. & Fraunholz, M. J. In or out: Phagosomal escape of *Staphylococcus aureus*. *Cell. Microbiol.* **21**, e12997 (2019).
31. Shimada, T. *et al. Staphylococcus aureus* Evades Lysozyme-Based Peptidoglycan Digestion that Links Phagocytosis, Inflammasome Activation, and IL-1β Secretion. *Cell Host Microbe* **7**, 38–49 (2010).
32. Ricklin, D., Reis, E. S., Mastellos, D. C., Gros, P. & Lambris, J. D. Complement component C3 – The "Swiss Army Knife" of innate immunity and host defense. *Immunol. Rev.* **274**, 33–58 (2016).
33. Wong Fok Lung, T. *et al. Staphylococcus aureus* small colony variants impair host immunity by activating host cell glycolysis and inducing necroptosis. *Nat. Microbiol.* **5**, 141–153 (2019).
34. Tuchscherr, L., Löffler, B. & Proctor, R. A. Persistence of *Staphylococcus aureus*: Multiple Metabolic Pathways Impact the Expression of Virulence Factors in Small-Colony Variants (SCVs). *Front. Microbiol.* **11**, 1028 (2020).
35. Melter, O. & Radojevič, B. Small colony variants of *Staphylococcus aureus* — review. *Folia Microbiol. (Praha)* **55**, 548–558 (2010).
36. Wang, M., Buist, G. & van Dijl, J. M. *Staphylococcus aureus* cell wall maintenance – the multifaceted roles of peptidoglycan hydrolases in bacterial growth, fitness, and virulence. *FEMS Microbiol. Rev.* **46**, fuac025 (2022).
37. Nikolic, P. & Mudgil, P. The Cell Wall, Cell Membrane and Virulence Factors of *Staphylococcus aureus* and Their Role in Antibiotic Resistance. *Microorganisms* **11**, 259 (2023).
38. Barbuti, M. D., Myrbråten, I. S., Morales Angeles, D. & Kjos, M. The cell cycle of *Staphylococcus aureus* : An updated review. *MicrobiologyOpen* **12**, e1338 (2023).
39. Vollmer, W., Blanot, D. & De Pedro, M. A. Peptidoglycan structure and architecture. *FEMS Microbiol. Rev.* **32**, 149–167 (2008).
40. Bailey, R. G. *et al.* The Interplay between Cell Wall Mechanical Properties and the Cell Cycle in *Staphylococcus aureus*. *Biophys. J.* **107**, 2538–2545 (2014).
41. Monteiro, J. M. *et al.* Cell shape dynamics during the staphylococcal cell cycle. *Nat. Commun.* **6**, 8055 (2015).
42. Pokhrel, R., Shakya, R., Baral, P. & Chapagain, P. Molecular Modeling and Simulation of the Peptidoglycan Layer of Gram-Positive Bacteria *Staphylococcus aureus*. *J. Chem. Inf. Model.* **62**, 4955–4962 (2022).
43. Kim, S. J., Chang, J. & Singh, M. Peptidoglycan architecture of Gram-positive bacteria by solid-state NMR. *Biochim. Biophys. Acta BBA - Biomembr.* **1848**, 350–362 (2015).
44. Porfírio, S., Carlson, R. W. & Azadi, P. Elucidating Peptidoglycan Structure: An Analytical Toolset. *Trends Microbiol.* **27**, 607–622 (2019).
45. Garde, S., Chodisetti, P. K. & Reddy, M. Peptidoglycan: Structure, Synthesis, and Regulation. *EcoSal Plus* **9**, ecosalplus.ESP-0010-2020 (2021).
46. Szweda, P. *et al.* Peptidoglycan hydrolases-potential weapons against *Staphylococcus aureus*. *Appl. Microbiol. Biotechnol.* **96**, 1157–1174 (2012).
47. Schumann, P. Peptidoglycan Structure. in *Methods in Microbiology* vol. 38 101–129 (Elsevier, 2011).
48. Pazos, M. & Peters, K. Peptidoglycan. in *Bacterial Cell Walls and Membranes* (ed. Kuhn, A.) vol. 92 127–168 (Springer International Publishing, Cham, 2019).
49. Lovering, A. L., Safadi, S. S. & Strynadka, N. C. J. Structural Perspective of Peptidoglycan Biosynthesis and Assembly. *Annu. Rev. Biochem.* **81**, 451–478 (2012).
50. Barreteau, H. *et al.* Cytoplasmic steps of peptidoglycan biosynthesis. *FEMS Microbiol. Rev.* **32**, 168–207 (2008).

51. Shaku, M., Ealand, C., Matlhabe, O., Lala, R. & Kana, B. D. Peptidoglycan biosynthesis and remodeling revisited. in *Advances in Applied Microbiology* vol. 112 67–103 (Elsevier, 2020).
52. Gautam, A., Vyas, R. & Tewari, R. Peptidoglycan biosynthesis machinery: A rich source of drug targets. *Crit. Rev. Biotechnol.* **31**, 295–336 (2011).
53. Nöldeke, E. R. & Stehle, T. Unraveling the mechanism of peptidoglycan amidation by the bifunctional enzyme complex GatD/MurT: A comparative structural approach. *Int. J. Med. Microbiol.* **309**, 151334 (2019).
54. Mora-Ochomogo, M. & Lohans, C. T. β-Lactam antibiotic targets and resistance mechanisms: from covalent inhibitors to substrates. *RSC Med. Chem.* **12**, 1623–1639 (2021).
55. Vestergaard, M., Frees, D. & Ingmer, H. Antibiotic Resistance and the MRSA Problem. *Microbiol. Spectr.* **7**, 7.2.18 (2019).
56. Idrees, M., Sawant, S., Karodia, N. & Rahman, A. *Staphylococcus aureus* Biofilm: Morphology, Genetics, Pathogenesis and Treatment Strategies. *Int. J. Environ. Res. Public. Health* **18**, 7602 (2021).
57. Sobral, R. G., Ludovice, A. M., De Lencastre, H. & Tomasz, A. Role of murF in Cell Wall Biosynthesis: Isolation and Characterization of a murF Conditional Mutant of *Staphylococcus aureus*. *J. Bacteriol.* **188**, 2543–2553 (2006).
58. Sobral, R. G., Ludovice, A. M., Tabei, K., De Lencastre, H. & Tomasz, A. Normally Functioning murF Is Essential for the Optimal Expression of Methicillin Resistance in *Staphylococcus aureus*. *Microb. Drug Resist.* **9**, 231–241 (2003).
59. Pereira Portela, R. New studies in the resistance to β-lactams and in cell wall damage survival in *Staphylococcus aureus*: an integrated approach. (2019).
60. Roberts, J. W. Mechanisms of Bacterial Transcription Termination. *J. Mol. Biol.* **431**, 4030–4039 (2019).
61. Bossi, L., Figueroa-Bossi, N., Bouloc, P. & Boudvillain, M. Regulatory interplay between small RNAs and transcription termination factor Rho. *Biochim. Biophys. Acta BBA - Gene Regul. Mech.* **1863**, 194546 (2020).
62. Nagel, A. *et al.* Inhibition of Rho Activity Increases Expression of SaeRS-Dependent Virulence Factor Genes in *Staphylococcus aureus*, Showing a Link between Transcription Termination, Antibiotic Action, and Virulence. *mBio* **9**, e01332-18 (2018).
63. Zimina, M. *et al.* Overview of Global Trends in Classification, Methods of Preparation and Application of Bacteriocins. *Antibiotics* **9**, 553 (2020).
64. Martínez, B., Rodríguez, A. & Suárez, E. Antimicrobial Peptides Produced by Bacteria: The Bacteriocins. in *New Weapons to Control Bacterial Growth* (eds. Villa, T. G. & Vinas, M.) 15–38 (Springer International Publishing, Cham, 2016). doi:10.1007/978-3-319-28368-5_2.
65. Yount, N. Y. *et al.* Discovery of Novel Type II Bacteriocins Using a New High-Dimensional Bioinformatic Algorithm. *Front. Immunol.* **11**, 1873 (2020).
66. Patinho Silva, M. Â. Caraterização da função do sistema SCWDABC de *Staphylococcus aureus* em interações inter-espécie e com o hospedeiro. (2021).
67. Chavakis, T., Wiechmann, K., Preissner, K. T. & Herrmann, M. *Staphylococcus aureus* interactions with the endothelium: The role of bacterial "Secretable Expanded Repertoire Adhesive Molecules" (SERAM) in disturbing host defense systems. *Thromb. Haemost.* **94**, 278–285 (2005).
68. Liang, X. *et al.* Natural allelic variation in the EamA-like transporter, CmSN, is associated with fruit skin netting in melon. *Theor. Appl. Genet.* **136**, 192 (2023).
69. Butler, D. L., Jakielaszek, C. J., Miller, L. A. & Poupard, J. A. *Escherichia coli* ATCC 35218 as a Quality Control Isolate for Susceptibility Testing of *Haemophilus influenzae* with Haemophilus Test Medium. *Antimicrob. Agents Chemother.* **43**, 283–286 (1999).
70. Zeigler, D. R. *et al.* The Origins of 168, W23, and Other *Bacillus subtilis* Legacy Strains. *J. Bacteriol.* **190**, 6983–6995 (2008).
71. Awan, M. *et al.* Dimethyl Sulfoxide: A Central Player Since the Dawn of Cryobiology, is Efficacy Balanced by Toxicity? *Regen. Med.* **15**, 1463–1491 (2020).
72. Daigneault, M., Preston, J. A., Marriott, H. M., Whyte, M. K. B. & Dockrell, D. H. The Identification of Markers of Macrophage Differentiation in PMA-Stimulated THP-1 Cells and Monocyte-Derived Macrophages. *PLoS ONE* **5**, e8668 (2010).
73. Tedesco, S. *et al.* Convenience versus Biological Significance: Are PMA-Differentiated THP-1 Cells a Reliable Substitute for Blood-Derived Macrophages When Studying in Vitro Polarization? *Front. Pharmacol.* **9**, 71 (2018).
74. VanCleave, T. T., Pulsifer, A. R., Connor, M. G., Warawa, J. M. & Lawrenz, M. B. Impact of Gentamicin Concentration and Exposure Time on Intracellular *Yersinia pestis*. *Front. Cell. Infect. Microbiol.* **7**, 505 (2017).
75. Kiernan, J. A. Formaldehyde, Formalin, Paraformaldehyde And Glutaraldehyde: What They Are And What They Do. *Microsc. Today* **8**, 8–13 (2000).
76. Melak, M., Plessner, M. & Grosse, R. Actin visualization at a glance. *J. Cell Sci.* **130**, 525–530 (2017).

77. Horse Serum for Cell Culture - PT. https://www.thermofisher.com/uk/en/home/life-science/cell-culture/mammalian-cell-culture/other-animal-sera/horse-serum.html.
78. Vulin, C., Leimer, N., Huemer, M., Ackermann, M. & Zinkernagel, A. S. Prolonged bacterial lag time results in small colony variants that represent a sub-population of persisters. *Nat. Commun.* **9**, 4074 (2018).
79. Majeed, H., Gillor, O., Kerr, B. & Riley, M. A. Competitive interactions in *Escherichia coli* populations: the role of bacteriocins. *ISME J.* **5**, 71–81 (2011).
80. Kamenšek, S. & Žgur-Bertok, D. Global transcriptional responses to the bacteriocin colicin M in *Escherichia coli*. *BMC Microbiol.* **13**, 42 (2013).
81. Bosák, J., Hrala, M., Micenková, L. & Šmajs, D. Non-antibiotic antibacterial peptides and proteins of *Escherichia coli* : efficacy and potency of bacteriocins. *Expert Rev. Anti Infect. Ther.* **19**, 309–322 (2021).
82. Newstead, L. L., Varjonen, K., Nuttall, T. & Paterson, G. K. Staphylococcal-Produced Bacteriocins and Antimicrobial Peptides: Their Potential as Alternative Treatments for *Staphylococcus aureus* Infections. *Antibiotics* **9**, 40 (2020).
83. Mainil, J. *Escherichia coli* virulence factors. *Vet. Immunol. Immunopathol.* **152**, 2–12 (2013).
84. Wang, J., Ma, W. & Wang, X. Insights into the structure of *Escherichia coli* outer membrane as the target for engineering microbial cell factories. *Microb. Cell Factories* **20**, 73 (2021).
85. Yang, J. *et al.* Klebsiella oxytoca Complex: Update on Taxonomy, Antimicrobial Resistance, and Virulence. *Clin. Microbiol. Rev.* **35**, e00006-21 (2022).
86. Darby, A. *et al.* Cytotoxic and Pathogenic Properties of *Klebsiella oxytoca* Isolated from Laboratory Animals. *PLoS ONE* **9**, e100542 (2014).
87. Chen, X., Hu, Y., Tian, S. & Han, B. Understanding the Interactions between *Staphylococcus aureus* and the Raw-Meat-Processing Environment Isolate *Klebsiella oxytoca* in Dual-Species Biofilms via Discovering an Altered Metabolic Profile. *Microorganisms* **9**, 672 (2021).
88. Rohde, M. The Gram-Positive Bacterial Cell Wall. *Microbiol. Spectr.* **7**, 7.3.10 (2019).
89. Chiller, K., Selkin, B. A. & Murakawa, G. J. Skin Microflora and Bacterial Infections of the Skin. *J. Investig. Dermatol. Symp. Proc.* **6**, 170–174 (2001).
90. Boldock, E. *et al.* Human skin commensals augment *Staphylococcus aureus* pathogenesis. *Nat. Microbiol.* **3**, 881–890 (2018).
91. Gonzalez, D. J. *et al.* Microbial competition between Bacillus subtilis and *Staphylococcus aureus* monitored by imaging mass spectrometry. *Microbiology* **157**, 2485–2492 (2011).
92. Zhang, F. *et al.* Bacillus subtilis revives conventional antibiotics against *Staphylococcus aureus* osteomyelitis. *Microb. Cell Factories* **20**, 102 (2021).
93. pta - Phosphate acetyltransferase - *Staphylococcus aureus* (strain COL) | UniProtKB | UniProt. https://www.uniprot.org/uniprotkb/Q5HI88/entry.
94. Alabduladhem, T. O. & Bordoni, B. Physiology, Krebs Cycle. in *StatPearls* (StatPearls Publishing, Treasure Island (FL), 2024).
95. Zhou, S., Rao, Y., Li, J., Huang, Q. & Rao, X. *Staphylococcus aureus* small-colony variants: Formation, infection, and treatment. *Microbiol. Res.* **260**, 127040 (2022).
96. Olszowski, T. *et al.* The Effects of Cadmium at Low Environmental Concentrations on THP-1 Macrophage Apoptosis. *Int. J. Mol. Sci.* **16**, 21410–21427 (2015).
97. Ansari, A. M. *et al.* Cellular GFP Toxicity and Immunogenicity: Potential Confounders in in Vivo Cell Tracking Experiments. *Stem Cell Rev. Rep.* **12**, 553–559 (2016).
98. Missiakas, D. M. & Schneewind, O. Growth and Laboratory Maintenance of *Staphylococcus aureus*. *Curr. Protoc. Microbiol.* **28**, (2013).
99. Chair and Department of Medical Microbiology, Medical University, Lublin, Poland, Bogut, A., Magryś, A., & Chair and Department of Medical Microbiology, Medical University, Lublin, Poland. Analysis of the bacterial biofilm formation in different models of the in vitro culture. *Eur. J. Clin. Exp. Med.* **19**, 40–45 (2021).
100. Antonsen, K. W. *et al.* Comparison of culture media reveals that non-essential amino acids strongly affect the phenotype of human monocyte-derived macrophages. *Immunology* **170**, 344–358 (2023).