TITLE

Understanding *Staphylococcus aureus* internalisation and induction of antimicrobial tolerance

Frédéric Goormaghtigh (https://orcid.org/0000-0002-8506-848X) and Françoise Van Bambeke* (https://orcid.org/0000-0002-0052-7991)

Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium

*Corresponding author

Avenue Mounier 73 B1.73.05 -1200 Brussels - Belgium
Francoise.vanbambeke@uclouvain.be
+32.2.764.73.78

ABSTRACT

Introduction: *Staphylococcus aureus,* a human commensal, is also one of the most common and serious pathogens for humans. Over the last years, its capacity to survive and replicate in phagocytic and non-phagocytic cells has been largely demonstrated. In these intracellular niches, bacteria are shielded from the immune response and antibiotics, turning host cells into long-term infectious reservoirs. Moreover, neutrophils carry intracellular bacteria in the bloodstream, leading to systemic spreading of the disease. Despite the serious threat posed by intracellular *S. aureus* to human health, the molecular mechanisms behind its intracellular survival and subsequent antibiotic treatment failure remain elusive.

Area covered: we give an overview of the killing mechanisms of phagocytes and of the impressive arsenal of virulence factors, toxins and stress responses deployed by *S. aureus* as a response. We then discuss the different barriers to antibiotic activity in this intracellular niche and finally describe innovative strategies to target intracellular persisting reservoirs.

Expert opinion: Intracellular niches represent a challenge in terms of diagnostic and treatment. Further research using ad-hoc in-vivo models and single cell approaches are needed to better understand the molecular mechanisms underlying intracellular survival and tolerance to antibiotics in order to identify strategies to eliminate these persistent bacteria.

Areas covered

This review paper describes how antibiotic efficacy is affected by host-pathogen interactions in the case of *Staphylococcus aureus* infections. Three major fields are covered: (i) clinical biology as great care is taken to inform the reader about the clinical relevance and clinical data behind intracellular survival of *S. aureus* in human cells. (ii) Infection biology and more specifically host-pathogen interactions are of major importance to understand the mechanisms allowing *S. aureus* to survive to the host immune response. (iii) Pharmacology is required to describe the mechanisms of action of antibiotics and how cellular pharmacodynamics and pharmacokinetics affect antibiotic activity inside host cells. Altogether, antibiotic activity is affected by host-pathogen interactions when *S. aureus* is internalized within host cells, leading to chronic and potentially systemic infections. The three areas of research previously described are thus intimately linked in this context.

Keywords (4-10 – alphabetical order)

Antibiotic tolerance - Bacterial persistence - Chronic infections - Host-pathogen interactions - Intracellular pathogen - Intracellular survival - Persistent infections - Staphylococcus aureus – Small colony variants – reactive oxygen species

Article highlights

- *S. aureus* survival inside host cells is associated with recurrent infections and systemic dissemination.
- *S. aureus* evolved a large array of mechanisms to elude phagocytosis by macrophages and neutrophils.
- *S. aureus* evolved a diversity of mechanisms to elude killing upon internalization by phagocytes: (i) oxidative stress, (ii) acidic pH and (iii) antimicrobial peptides/proteins.
- Inside phagocytes, poor antibiotic activity results from a combination of factors: (i) poor intracellular pharmacokinetics and pharmacodynamics of antibiotics, (ii) induction of bacterial stress responses and (iii) phenotypic switches to small colony variants or antibiotic persisters.
- Small colony variants and persisters share many similarities. Are the two phenotypes linked?
- Several promising strategies are in the pipeline to target intracellular pathogens.

Introduction

Staphylococcus aureus is the second leading cause of mortality related to infectious diseases in the world according to a recent survey [1], with more than 700,000 deaths recorded in 2019. It is also one of the top priority pathogens on the WHO list for the search for new therapies due to its frequent resistance to multiple antibiotics, with a major interest for Methicillin-Resistant *S. aureus* or MRSA [2]. The eradication of this bacterium is further complicated by its ability to thrive and survive intracellularly. Inside the host cells, it is protected from the immune defences and from most antibiotics, exhibiting a phenotype of antibiotic tolerance. In this review, we discuss the mechanism by which *S. aureus* survives intracellularly and how this intracellular niche is associated with persistence or recurrence of the infection. We then describe persisters characteristics and discuss innovative strategies that are in the pipeline to target this recalcitrant intracellular reservoir.

1. Colonization

Staphylococcus aureus is a Gram-positive bacterium that is intimately associated to humans and a few animals. Although *S. aureus* can be found on the skin and at multiple body sites, its major reservoir is the anterior nares of the nose that it reaches using our hands as main vectors [3]. In this hostile environment, thanks to its extraordinary versatility and adaptability, *S. aureus* evades most of the host defences present in the nasal secretions, including neutrophil recruitment, antimicrobial peptides, immunoglobulins A and G, lactoferrin and lysozyme [4,5]. The nasal microbiota constitutes an equally formidable challenge for *S. aureus* to overcome, especially in the presence of niche competitors such as *Streptococcus pneumoniae*, *Staphylococcus epidermidis* and *Corynebacteria* [6]. As examples, a trial with a pneumococcal vaccine in children led to an increase in *S. aureus* nasal colonization after vaccination [7] and artificial colonization with non-pathogenic *S. aureus* strain 502A has been used in the 60^{s} during outbreaks of *S. aureus* infections to prevent subsequent colonization by pathogenic strains of *S. aureus* [8]. Following initial colonization, most individuals will eliminate *S. aureus*, while a fraction of the individuals will become intermittent (about 30 to 60% of the population) or persistent (about 20% of the population) nasal carriers [5,9]. In the latter case, long term co-evolutionary processes must have shaped the intertwined ballet between bacteria and host cells, establishing a long term, stable relationship with the resident strain of *S. aureus* [10] as shown, for instance, by the extraordinary resilience of the resident strain upon subsequent reinoculation with a mixture of *S. aureus* strains in human volunteers [11].

In addition to the nose, recent research found the gut to constitute another major reservoir of *S. aureus* [12,13]. The group of Otto even found that *S. aureus* colonization could be abolished

in a Thai population using *Bacillus* probiotics as they secrete fengycins, a class of lipopeptides that inhibits quorum-sensing signalling in *S. aureus*, thereby impeding colonization of the pathogen [12].

2. Infection

Importantly and in contrast to most other *Staphylococcus* species, a disturbance of the balanced host-bacterium equilibrium can turn any *S. aureus* strain into a dangerous pathogen, even in otherwise perfectly healthy individuals [14,15]. Infection pathogenesis can be even more dramatic in the presence of mobile genetic elements such as plasmids, pathogenicity islands or prophages that are known to carry virulence factors such as the toxic shock syndrome toxin-1, staphylococcal enterotoxins or the Panton-Valentine leukocidin (PVL) [10]. The large array of virulence factors of *S. aureus* thus accounts for an equally large diversity of diseases, ranging from mild skin infections such as abscesses and impetigo to severe and sometimes life-threatening diseases including deep abscesses, necrotizing pneumoniae, osteomyelitis, endocarditis, *etc*. It is important to notice that nasal colonization is a major risk factor for infections both in the hospital and the community as attested by the shared genotypes and phagotypes of infecting and resident *S. aureus* strains [16].

Infections are initiated when a breach in the skin or mucosal tissue allows bacteria to invade adjoining tissues or the bloodstream. This typically occurs in the presence of open wounds or alternatively, as a consequence of a viral infection that damages the mucosal tissues of the upper airway [17].

3. Extracellular evasion of the immune system

Once beyond the skin or mucosa, or upon reaching the bloodstream, tissue injury and “pathogen-associated molecular patterns” or PAMPs (peptidoglycan, lipoproteins, lipoteichoic acid and formylated peptides) trigger the first line of defense against *S. aureus* infection: the innate immunity [18,19]. However, and most probably as a consequence of hundreds of thousands of years of coevolution with human host cells, *S. aureus* has developed a plethora of virulence factors to evade the immune response. Here is a brief and non-exhaustive overview of the mechanisms at play. Please refer to the following reviews for an exhaustive overview [18,20-22].

Neutrophils play a major role in clearing *S. aureus* infections as shown by the extreme severity of infections in patients with neutrophils dysfunctions [23]. Migration of neutrophils and monocytes to the site of infection should lead to clearance of the pathogen with the help of the

complement system and available antibodies. However, *S. aureus* undermines phagocytosis efficiency of neutrophils or other types of phagocytic cells through several mechanisms.

I. *S. aureus* produces the Staphylococcal superantigen-like protein 5 (SSL5) that interferes with the interaction between PSGL-1 and P-selectin required for adhesion of neutrophils to endothelial cells, thereby preventing neutrophil extravasation to the infection site [24].

II. *S. aureus* secretes a variety of proteins that inhibit chemotaxis as well as neutrophil activation through direct binding or degradation of various neutrophil receptors. SSL10 [25] and CHIPS, the Chemotaxis Inhibitory Protein of *Staphylococcus* [26] are two examples of the former, that antagonize (i) CXCR4 chemokine receptor [27] and (ii) C5a as well as formylated peptide receptor FPR, respectively [28]. In addition, a series of proteases are secreted by *S. aureus*, leading to degradation of many immunity proteins, with, to cite a few, Staphopain A that degrades the CXC chemokine receptor 2 (CXCR2) [29] and the aureolysin metalloprotease from *S. aureus* that cleaves the central complement C3 [29].

III. *S. aureus* produces a variety of surface proteins that interfere with complement activation and antibodies binding. (i) The Staphylococcal Complement Inhibitor protein (SCIN) interferes with complement activation and prevents C3b and C5a opsonization of *S. aureus* [29]. (ii) Clumping factor A or ClfA is produced in stationary phase and binds to the gamma-chain of fibrinogen, generating a fibrinogen coating that restricts deposition of opsonins [30]. (iii) *S. aureus* expresses two immunoglobulin-binding proteins Spa and Sbi that are anchored at the cell wall and bind to the Fc region of IgG. This causes bacterial cell surface to be coated with IgG molecules that are wrongly oriented and cannot be recognized by Fc receptors of neutrophils or macrophages [31-33], inhibits complement activation and opsonin-mediated phagocytosis by macrophages [34].

IV. Most clinical isolates of *S. aureus* produce a polysaccharide capsule that generates a physical barrier allowing complement factors to bind the cell wall but masking them from recognition by phagocytic cells [35,36].

V. *S. aureus* has access to more “active” mechanisms by secreting pore-forming toxins that lyse neutrophils, monocytes and macrophages. These toxins are commonly classified in three groups comprising (i) the (in)famous alpha-toxin (Hla) [37], (ii) the two-component leukocidins [38] among which the PVL associated with MRSA necrotizing pneumonia infections [39] and (iii) phenol-soluble modulins (PSMs) [40].

VI. During infection, *S. aureus* and host immune cells compete for nutrients, leading to entire metabolic reprogramming. Importantly, as recently reviewed by Prince et al., this

rewiring of immune cells can be beneficial or even triggered by *S. aureus*, leading to persistent infections [22]. It is therefore crucial to better understand the complex metabolic interplay between *S. aureus* and host cells as it shapes the pathogenesis of infection.

Altogether, *S. aureus* has access to an impressive toolbox allowing to undermine phagocytosis during infection, most probably indicative of a long-standing co-evolution with human tissues. This is notably why the vast majority of efforts in understanding the pathogenicity of *S. aureus* were strongly focused on its extracellular lifestyle. However, it has now become clear that an important fraction of bacteria can survive and even replicate within the phagolysosomes of phagocytes, turning them into Trojan horses that carry infectious bacteria to distant infection sites, while offering protection against the host immune response and most antibiotics. This intracellular lifestyle and its consequences on disease progression are discussed in the following section.

4. *Staphylococcus aureus*, a facultative intracellular pathogen

4.1. Clinical evidence of intracellular survival

There has been mounting evidence of the intracellular lifestyle of *S. aureus* in human tissues such as nasal polyps [41], the nasal mucosa of patients with recurrent sinusitis [42], human tonsils [43], lung macrophages of patients with cystic fibrosis [44], or periprothetic tissue of patients with bone infection [45]. A recent prospective study in humans revealed that 5.6% patients undergoing ear, nose and throat surgery carried an intracellular niche of *S. aureus* [46] while another study showed that even healthy individuals carried intracellular *S. aureus* within their nasal vestibule [47]. The major concern is that intracellular *S. aureus* could constitute a persistent reservoir of virulent bacteria that can (re)establish infections after antibiotic therapy, including vancomycin that is classically used to treat MRSA infections [48,49]. As an example, a study realized in a murine model showed that within minutes after intravenous infection, 90% of *S. aureus* cells were sequestered by resident liver macrophages called Kupffer cells (KCs) in the liver sinusoids. Following this, a small subpopulation of bacteria survived and even proliferated for days in this intracellular niche, ultimately leading to macrophage lysis and infection relapse [50]. An independent study similarly showed that *S. aureus* can survive in vitro for several days within intracellular vacuoles of KCs without affecting the viability of the host cell [51].

4.2. Survival in professional phagocytes – the Trojan Horse problem

Importantly, survival of *S. aureus* in bloodstream phagocytes has been thought to play an important role in disease progression and dissemination. The analogy to the Trojan horse is often used to illustrate the role of mobile phagocytic cells to serve as vehicles for bacterial dissemination, potentially causing metastatic and life-threatening infections [52]. Several pieces of evidence support this hypothesis:

- A large prospective study showed that neutropenia (reduced neutrophil counts) in clinical patients correlates inversely with *S. aureus* bacteraemia occurrences [53]. In addition, *S. aureus* infections in neutropenic patients is associated with shorter bacteraemia duration and reduced dissemination to other organs [54].
- Mice infected with bacteria sequestered by macrophages or neutrophils led to equivalent or higher infection loads in the kidneys and in the brain [48].
- Internalized *S. aureus* can survive for long periods of time (hours to days) in bloodstream leukocytes without affecting its host viability [51]. It was also shown that following initial gut colonization in mice, MRSA could be isolated within circulating neutrophils and lead to infection of surgical wounds, with the development of visible abscesses in 10% of the mice [55].
- Neutrophil depletion in a murine sepsis model using cyclophosphamide or antiLy6G mouse antibodies led to increased bacterial loads but lack of systemic spread. Conversely, tissue resident macrophage depletion resulted in even greater bacterial loads, but with no impact of the dissemination of pathogens to other organs [56].

Altogether, it seems clear that even though the intracellular lifestyle of *S. aureus* has been overlooked for decades, it poses a formidable threat to human health by generating persistent pathogenic reservoirs that are associated with chronic infections and systemic disease progression. It is therefore important to understand the molecular mechanisms allowing *S. aureus* to thrive inside human phagocytic cells. The following paragraphs provide an overview of the challenges bacteria face upon internalization inside professional phagocytes and the mechanisms that have been selected to overcome them (**Figure 1**).

4.2.1. Phagosomal maturation and acidification

When *S. aureus* is successfully engulfed by a macrophage, a monocyte or a neutrophil, it initially resides in a phagosome. The next step in the phagocytic process is phagosome maturation. Within minutes after uptake, the phagosome recruits the RAB5 GTPase, an early endocytic marker, which is later exchanged for RAB7, followed by the acquisition of lysosome-associated proteins LAMP1 and LAMP2, ultimately triggering the fusion with lysosome structures [57]. While other intracellular pathogens such as *Listeria monocytogenes* or *Mycobacterium tuberculosis* are known to interfere with phagosomal maturation [58,59], this is

still a matter of debate for *S. aureus* with (i) some studies reporting normal acidification and maturation of phagosomes [60,61] and (ii) others reporting that *S. aureus* may perturb recruitment of key enzymes such as cathepsin D and B-glucuronidase and inhibit acidification of the phagosome [62].

Nevertheless, during phagosomal maturation, vacuolar ATPases are trafficked to the phagosome leading to its acidification, a prerequisite for the activity of many essential components of the phagosome such as the cathepsin proteases. While minor acid drops are usually tolerated, strong acidification leads to metabolic disorders or cell death in most bacteria. This is not the case with *S. aureus* for which, similarly to *Salmonella* [63], the low phagosomal pH is even required for survival and replication within phagocytes, which has been attributed to the upregulation at low pH of *agr*, a major regulator of virulence [61], and to the activation of the sensor kinase GraS, involved in antimicrobial peptide resistance in macrophages [64]. In line with this, inhibitors of phagosomal acidification reduce intracellular survival [61] and a deficient mutant for the SLC4A7 bicarbonate transporter in macrophages that perturbs phagosome acidification results in increased survival of intracellular *S. aureus* [65].

4.2.2. Oxidative burst

Concomitant to phagosome acidification, neutrophils unleash an oxidative burst in the forms of reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive chlorine species (RCS), *etc.* NADPH oxidase (NOX) generates superoxide ions ($O_2^{\cdot -}$) that spontaneously dismutate to hydrogen peroxide (H_2O_2) in turn generating highly reactive hydroxyl radical OH^- through the Fenton reaction. Neutrophils in addition express myeloperoxidase (MPO) that converts H_2O_2 or $O_2^{\cdot -}$ to the highly bactericidal hypochlorous acid (HOCl). It is important to notice that MPO-mediated killing efficacy varies from one bacterial pathogen to another and is considered as a dispensable host defense in the context of *S. aureus* infections [66]. ROS usually constitute powerful antimicrobial weapons due to their ability to damage any oxidizable moiety in DNA, peptides and proteins, ultimately leading to DNA mutations, protein carbonylation, enzymatic inactivation and the formation of protein aggregates [67]. Nevertheless, *S. aureus* evolved several oxidative stress resistance mechanisms:

- Staphyloxanthin, a carotenoid pigment responsible for the golden color of *S. aureus* colonies, is a potent antioxidant due to its numerous conjugated double bonds. Non-pigmented mutants disrupted for carotenoid biosynthesis show increased sensitivity to ROS, $OONO^-$ and HOCl, reduced survival in neutrophils and reduced virulence in mice [68-70].

- *S. aureus* encodes for 2 superoxide dismutase genes *sodA* and *sodM.* The protein products of these genes are metalloenzymes that dismutate O_2^- to O_2 and H_2O_2. Hydrogen peroxide can further be detoxified into H_2O and O_2 by the complementary actions of the *katA*-encoded catalase and the *ahpC*-encoded peroxiredoxin, both required for resistance to H_2O_2 stress as well as environmental persistence and nasal colonization [71]. Regulation of the genes is dependent on PerR, a peroxide sensing protein of the Fur family of transcriptional regulators. PerR boxes can be found in the promoter regions of both genes and PerR was shown to be crucial for oxidative stress resistance and virulence of *S. aureus* as a result [72,73]. *katA* expression is additionally regulated by the Ferric uptake regulator Fur.
- *S. aureus* peroxidase inhibitor (SPIN) directly binds human MPO), preventing H_2O_2 access to the MPO active site and formation of the highly bactericidal reactive chlorine species HOCl [74].

4.2.3. Nitrosative burst

Reactive nitrogen species (RNS) are produced in all immune cells by the inducible nitric oxide synthase (iNOS) in the form of nitric oxide ($NO^{\cdot}$) that can react with $O_2^{\cdot -}$ to form a very destructive product, peroxynitrite ($OONO^-$) [67,75]. Additionally, NO^- inhibits oxygen consumption and aerobic respiration by binding to cytochrome hemes, thereby restricting bacterial growth [76]. Nevertheless, while nitric oxide is known to play a critical role in infection control for other intracellular pathogens such as *Salmonella*, it does not affect *S. aureus* growth *in vitro*. The ability to grow in the face of nitric oxide also distinguishes *S. aureus* from other Staphylococcal pathogens including *S. epidermidis* and *S. saprophyticus*. The specificity of *S. aureus* is to encode for the flavohaemoglobin Hmp, a highly efficient detoxification enzyme [77] and to switch its metabolic activity to homolactic fermentation as a response to nitrosative stress by inducing the expression of *ldh1*, a lactate dehydrogenase. Both activities are essential for virulence by allowing *S. aureus* to maintain its redox homeostasis during nitrosative stress. Interestingly, while *hmp* expression is (partially) dependent on the SrrAB 2-component system, *ldh1* expression is independent of it, suggesting other mechanisms of NO-sensitivity in *S. aureus* than SrrAB [76,77].

4.2.5 Antimicrobial peptides and proteins

Apart from oxygen-dependent bactericidal mechanisms described above, neutrophils also deploy oxygen-independent bactericidal mechanisms through the production of antimicrobial peptides and proteins, including lysozyme. They generally have a pore-forming activity, but different inhibitory mode of actions have been observed. They are secreted in the phagosome

but are also present on mucosal surface, in the airways and on the skin, and are an important part of the innate immune response. Different classes of antimicrobial peptides and proteins exist for which *S. aureus* has evolved a variety of antagonistic responses.

- Cationic peptides such as defensins and protegrins are positively charged and rely on electrostatic interactions to bind to bacterial membranes. To elude their pore-forming activity, *S. aureus* partially neutralizes the negative charge of its cell surface. Examples of this are the *dlt* operon and *mprF* genes (Multiple peptide resistance factor) that encode for enzymes involved in the transfer of D-alanine into teichoic acids [78] and modification of phosphatidylglycerol with L-lysine [79], respectively. Both *dlt* and *mprF* genes are transcriptionally regulated by a complex five-component system GraXSR-VraFG that senses cationic antimicrobial peptides and activates GraR-dependent transcription [80,81].
- *S. aureus* secretes proteases that degrade cationic antimicrobial peptides as well as the negatively charged AMP dermcidin [82]. Examples are (i) staphylokinase that degrades IgG and C3, but also binds to and inactivates human defensins [83] and (ii) aureolysin that cleaves both complement C3 and cathelicidin LL-37, one of the few human antibacterial peptides with anti-staphylococcal activity [84,85].
- Lysozyme is a crucial antimicrobial protein that degrades peptidoglycan by cleaving the glycosidic bound between *N*-acetylglucosamine and *N*-acetyl muramic acid, causing cell lysis and leading to rapid killing of gram-positive bacteria, … except for *S. aureus*. The molecular mechanism conferring resistance to lysozyme is attributed to the *O*-acetyltransferase activity of OatA that acetylates the muramic acid parts of peptidoglycan [86].

4.3. Hiding in and escaping from non-professional phagocytes

While *S. aureus* has evolved a myriad of molecular mechanisms allowing its unique survival in the phagosome of professional phagocytes, its intracellular niche is not restricted to such cells. Indeed, intracellular *S. aureus* can be found in a wide range of non-phagocytic cells such as epithelial and endothelial cells, osteoblasts, keratinocytes and fibroblasts [87], notably in a clinical context [88,89]. *In vitro* studies indicate that invasion of keratinocytes, osteoblasts and fibroblasts leads to substantially lower numbers of intracellular *S. aureus* than invasion of epithelial and endothelial cells [87]. The main invasion pathway occurs via a well-described “Zipper mechanism”. *S. aureus* exposes fibronectin-binding proteins A and B (FnBP-A and FnBP-B) of the MSCRAMM family (microbial surface component recognizing adhesive matrix molecule) at its cell surface. Host cells fibronectin (Fn) then acts as a ligand to form a fibronectin bridge that binds *S. aureus* to the host cell integrin α5β1 (also called the fibronectin

receptor). This in turn leads to clustering of integrins which causes endocytic uptake of plasma membrane and internalization of bacteria. For additional information, this processus is detailed in the following reviews [90,91]. It is important to notice that α5β1 integrins are found on the basolateral membrane of epithelial and endothelial barriers, and thus not accessible to pathogens. Nevertheless, α5β1 integrins are ubiquitously expressed on the cell surfaces upon tissue damage, indicating that a disrupted epithelium or endothelium barrier might trigger internalization of *S. aureus* [92]. The FnBP-Fn- α5β1 integrin pathway in not the only internalization mechanism and several secondary adhesins have been identified including Eap (extracellular adherence protein), the *S. aureus* clumping factor A (ClfA) and the autolysin Atl as reviewed elsewhere [90,91].

After host cell invasion, two divergent cell fates have been observed depending on the host cell type and the infectious strain [87]:

(i) Bacteria escape from the endosome, replicate in the cytosol and cause a variety of cytotoxic and immunogenic effects. A variety of mechanisms are involved in phagosomal escape and have been described in previous reviews [49,93]. In particular, the quorum-sensing system *agr* plays a key role as a disrupted mutant is unable to escape from the phagosome [49]. The Agr system controls the expression of a plethora of toxins among which the pore-forming α -toxin and the phenol-soluble modulin α (PSMα) that have been found to play a predominant role in the escape from phagosomes of non-professional and professional phagocytes [94,95], but other factors may be involved, including β or δ toxins as well as the nonribosomal peptide synthetase complex AusAB or its dipeptide products [49]. Induction of the Agr system and phagosomal escape are often accompanied by strong inflammatory and cytotoxic effects in host cells.

(ii) Bacteria downregulate expression of virulence factors (including cytotoxins and the Agr-system) and switch to a reduced metabolic state, typical of SCVs. In this state, bacterial pathogens can persist for long periods of time and withstand otherwise lethal antibiotic treatment including flucloxacillin, clindamycin, linezolid and teicoplanin, but not rifampicin [96]. These bacteria therefore pose a formidable challenge for the immune response and antibiotics and could be the source of chronic and recurrent infections.

It is important at this stage to notice that cell fate is vastly different in professional phagocytes and non-professional phagocytes. As described in the corresponding paragraphs (see above), in professional phagocytes, the vast majority of internalized *S. aureus* remains in the phagosome until cell lysis is triggered. Bacterial replication can also be observed in

phagosomes of macrophages [60]. Conversely, phagosomal escape readily occurs in non-professional phagocytes, followed by bacterial replication in the cytoplasm and host cell death.

5. Why do antibiotics display poor activity inside host cells?

A major challenge with internalized bacteria is their recalcitrance to most antibiotics, including vancomycin, both in professional and non-professional phagocytes [48,49,96]. Although this is of major clinical significance, the molecular mechanisms behind their capacity to survive to antibiotics are not clearly established and are thought to be a combination of poor cellular pharmacokinetics or pharmacodynamics of most antibiotics, induction of bacterial stress responses triggered by harsh environment of the phagosome and phenotypic diversification leading to bacterial persisters and small colony variants (SCVs) (**Figure 2**).

5.1. Cellular pharmacokinetics and pharmacodynamics of antibiotics

The intracellular activities of 16 antibiotics have been studied in THP-1 monocytes [97]. The major conclusions of this study are that antibiotic killing inside monocytes is concentration- and time-dependent (except for macrolides), but always lower than in the extracellular medium. Importantly, intracellular accumulation alone is not a good indicator of intracellular activity as there is no correlation between cellular concentration and activity of antibiotics. Two examples of this are (i) the poor intracellular activity of macrolides despite important accumulation inside cells and (ii) the good intracellular activity of beta-lactams although they poorly accumulate in eucaryotic cells [98-100]. Several hypotheses have been put forward to explain this inconsistency:

(i) Different sub-cellular localization of the antibiotic and the bacteria.

(ii) Reduced bioavailability caused by the interaction of antibiotics with cellular constituents such as lipids and proteins or extrusion out of the cell by efflux pumps.

(iii) Environmental effects such as acidic pH have been shown to affect the activity of antibiotics negatively (gentamicin, macrolides and fluoroquinolones) or positively (beta-lactams and rifampicin).

It therefore appears that efforts should be made to optimize both the cellular pharmacokinetics and pharmacodynamics of antibiotics. An interesting example is that of delafloxacin. Fluoroquinolones are classically poorly active at acidic pH due to the molecules becoming protonated and thereby unable to cross the bacterial membrane. In contrast to other molecules in the class, delafloxacin is anionic at neutral pH and neutral at acidic pH due to the protonation of its carboxylate function, allowing it to accumulate to larger extent in bacteria and cells in an acidic environment. This explains why it shows improved activity at acidic pH and within

phagocytic cells [101]. Such molecule could thus be of great interest to eradicate intracellular persistent *S. aureus*.

5.2. Host-dependent induction of bacterial stress responses and antibiotic persistence

Antibiotic persistence is the property of a small subpopulation of bacteria to transiently switch to an antibiotic-tolerant state. Persisters, on the contrary to resistant bacteria, cannot replicate during antibiotic exposure and revert to a growing and sensitive phenotype after antibiotic removal. This phenotype has been largely studied in *E. coli*, and to a lesser extent in *Salmonella* and *Mycobacterium tuberculosis* and *S. aureus* [102].

Internalization of bacteria in host cells inevitably causes massive transcriptomic reprogramming resulting notably in the induction of a plethora of toxins, in the activation of stress responses and the adjustment of the central carbon metabolism. Previous works have established the central role of stress responses and the growth rate in antibiotic persistence. For instance, a study showed that oxidative stress induced by menadione or paraquat treatment in vitro improved survival to subsequent exposure to ciprofloxacin, oxacillin and vancomycin in correlation with reduced respiration rates [103]. Peroxynitrite, a combined product or ROS and RNS, was shown in a follow-up study by the authors to induce strong ATP depletion and tolerance to rifampicin in a dose-dependent manner, with greater potency than any other ROS or RNS tested. In line with that, inhibition of NOX (ROS production) and iNOS (RNS production) restored rifampicin susceptibility of intracellular *S. aureus*. However, in vivo data remain mostly correlative with no causal link at the single-cell level showing that peroxynitrite or ATP depletion is necessary or sufficient to trigger antibiotic persistence. Nevertheless, these data suggest that the oxidative burst of phagocytes might prime pathogens for antibiotic treatment survival in macrophages [104]. An independent study observed that different host cells yielded different levels of ROS production, with human primary macrophages showing a substantially higher ROS production than J774 murine macrophages. Bacteria recovered from human primary macrophages showed decreased ATP levels and increased lag times when regrown in rich medium (and no growth within 24 on agar plates). However, the overall bactericidal effect of oxacillin was mostly similar in both cell types, highlighting the complexity of the intracellular persistence phenomenon [105]. To conclude, while ROS and RNS seem to play an important antagonistic role to rifampicin activity by decreasing metabolic activity of bacterial pathogens, this remains to be verified with a larger panel of antibiotics and using live microscopy and relevant experimental models. The role of ATP depletion for antibiotic persistence also should be verified as an independent study found no significant difference in ATP content between intracellular persisters and a control sample containing extracellular bacteria mixed with J774 cell lysate [106]. This discrepancy might be

explained by the different experimental models used but also by a current limitation in the antibiotic persistence field that relies on bulk averaging methods to explain single-cell heterogeneity. Among others, single-cell tracking of ATP levels using fluorescent-based biosensors [107,108] during macrophage infection could be realized to monitor ATP in intracellular antibiotic persistence.

In order to get a general picture of intracellular antibiotic persisters, Peyrusson and colleagues characterized the transcriptome of *S. aureus* SH1000 after 24h of exposure to oxacillin in J774 murine macrophages [106]. Bacteria were sorted based on constitutive GFP fluorescence and negative PI staining and CFU counting confirmed that the vast majority of the bacteria were viable. Interestingly, neither of the classical oxidative and nitrosative detoxification enzymes (Soda, SodM, KatA, AhpC and Hmp) were strongly upregulated, indicating that oxidative and nitrosative stresses might only be transient and not required for survival to prolonged antibiotic treatment. The most drastic transcriptomic changes were the induction of the stringent response, the SOS response, the heat shock response and cell wall stress stimulon and a decrease in metabolic activity, especially oxidative phosphorylation as well as a carbon source shift from glucose to lactose. Lastly, persisters could show de novo synthesis of GFP, although at a lower rate than extracellular bacteria, indicating that translation is still functional in these cells.

These data crucially highlight the vast complexity of genetic networks at play in antibiotic persisters and is reminiscent of the “disrupted stress state” as recently described by the Balaban group [109]. The authors postulate that specific pathway activation cannot account for the complexity of persister cells. Instead, antibiotic exposure might trigger a disrupted state of acute stress in persisters that is better described by a random model ignoring the specificities of the underlying molecular mechanisms. The overall result would be high cell-to-cell heterogeneity, thereby highlighting even more the need to develop single-cell methods to interrogate intracellular persisters.

Nevertheless, several stress responses have been shown previously to play an important role in intracellular survival after phagocytosis and might play key roles in antibiotic susceptibility. The stringent response for instance was shown to be activated after uptake of *S. aureus* by neutrophils and led to induction of intracellular expression of phenol-soluble modulins (see corresponding paragraphs for the role of PSMs pathogenesis of *S. aureus*) [110]. Interestingly, *S. aureus* rsh_{syn} mutants defective for ppGpp synthesis (the major alarmone of the stringent response) showed reduced levels of persistence to oxacillin, clarithromycin and moxifloxacin [106]. The SOS response is most often associated with fluoroquinolone survival and was shown to be required only during the recovery of *E. coli* persisters after antibiotic removal

[111,112]. Beta-lactams were also shown to induce the SOS response through the DpiBA 2-component system in *E. coli* [113]. It however remains to be tested if this can be translated to *S. aureus*. The heat shock stress response is a universal response to hostile conditions resulting in production of heat shock proteins, among which several chaperones including GroEL and DnaK and proteases including DegP and ClpXP that either rescue or degrade misfolded proteins and protein aggregates. It is important to notice that heat shock proteins GroEL and DnaK were previously found to be the most abundant proteins synthesized by *Salmonella* inside macrophages [114] and that DegP protease is essential for oxidative stress tolerance in several pathogens including *Streptococcus pyogenes* [115] and *Salmonella* [116]. However, the link with antibiotic survival is currently not established in *S. aureus*. Finally, similar to *E. coli* persisters, toxin-antitoxin systems were shown to have no impact on *S. aureus* persistence [117].

To conclude, intracellular persistence to antibiotics correlates with induction of a large panel of stress responses (**Figure 3**). However, the causality between induction of a specific stress response and antibiotic persistence remains to be tested at the single-cell level, especially in relevant experimental models such as human macrophage cultures or animal models. On the other hand, Balaban and colleagues raised the possibility that persisters heterogeneity caused by a disrupted stress state might account for the difficulties to link this phenomenon to a specific molecular mechanism. More work involving single-cell techniques (microscopy, single-cell RNAseq, FACS, *etc.*) is needed to better characterize intracellular persisters and how such cells recover upon antibiotic removal. It is also important to notice that different antibiotics will most probably be linked to different recovery pathways, further adding to the complexity of this phenomenon.

5.3. Small colony variants

After phagocytosis, a subpopulation of *S. aureus* can reside inside cells for prolonged periods of time by switching to a quiescent lifestyle. In this state, bacteria show a reduced growth rate and form colonies of about 10% of the normal size of *S. aureus* colonies, hence their name, Small Colony Variants or SCVs. Such cell types are not specific to *S. aureus* but have been best studied in this pathogen with major relevance for long-time chronic infections [118]. Two different types of SCVs have been identified: first, dynamic SCVs that quickly revert to their wild-type phenotype when cultured in rich growth medium. They are often generated during the intracellular lifestyle of *S. aureus* and originate from regulatory mechanisms that involve global regulators such as *sigB*, *sarA* and *agr* [119]. While most SCVs isolated from clinical

samples are dynamic/unstable, specific mutations, notably those leading to defects in the electron transport chain, have been associated with formation of stable/permanent SCVs [119].

Nevertheless, both types share many phenotypic traits that are detailed in the following dedicated reviews [120,121] and can be summarized as follows:

(i) Reduced respiration rates and lower capacity for oxidative phosphorylation.
(ii) Downregulation of virulence regulators including *agr* and *sarA* and related virulence genes such as *spa* and *hla*. Attenuated virulence is thought to facilitate intracellular survival and evasion from the immune system.
(iii) Decreased pigmentation yielding non-colored colonies on agar plates.
(iv) Decreased haemolytic, cytotoxic and coagulase activities.
(v) Resistance to aminoglycosides.
(vi) Increased tolerance towards most antibiotics.
(vii) Upregulation of genes involved in biofilm formation and adhesion.

Similar to bacterial persistence, a vast diversity of molecular mechanisms has been found to underline formation of SCVs rather than one common metabolic pathway. Nevertheless, three major types of SCVs have been identified based on the activation mechanism [120,121]:

(i) SCVs altered for electron transport typically contain genetic inactivating mutations in one or several genes of the biosynthesis of the 2 first electron acceptors menaquinone or haemin, and this phenotype is complemented by supplementation with menadione or haemin. Electron transport deficiency results in ATP depletion, slow growth rate and a metabolic switch to fermentation pathways, with lactic acid found as the main product of fermentation.
(ii) SCVs with defective thymidine biosynthesis. They are mostly isolated from cystic fibrosis patients under long-term trimethoprim sulfamethoxazole (SXT) treatment and can be complemented by overexpression of the thymidylate synthase gene *thyA*.
(iii) CO_2-dependent SCVs. CO_2 auxotrophic SCVs are more challenging to recover and identify and therefore less documented.

Importantly, the intracellular environment as well as oxidative stress [122] were shown to be sufficient to induce formation of SCVs, allowing for long-term intracellular persistence without affecting the host cells integrity [123].

In terms of clinical relevance, SCVs of *S. aureus* occur in about 1% of clinical samples [124], a number that rises to 17% among cystic fibrosis patients infected with *S. aureus* [125]. In a recent review, Kahl and colleagues [120] compiled a list of 46 clinical studies dealing with SCVs

and described their occurrence in more than 350 infected patients, including a large diversity of pathologies: device-related infections, skin and soft tissue infections, prosthetic joint infections, osteomyelitis and cystic fibrosis. The main associated risks are failure of the antibiotic treatment and long-term persistence inside host cells (sometimes for years) leading to frequent infection relapses even for infections that have apparently been treated successfully [120]. Of note, although SCVs are generally considered as less virulent, it has been reported that children with cystic fibrosis infected by thymidine-dependent SCVs show reduced lung function and increased risk of respiratory exacerbations than children without SCVs [88].

As a personal thought, although several differences can be observed between SCVs and persisters, they share striking similarities. The biggest difference lies in the switch to the SCV phenotype that can be triggered by nutrient starvation, whereas persisters occur only after antibiotic exposure. However, such difference might in fact not be considered as such for the reasons depicted hereafter. In the seminal work of Balaban in 2004 [126], 2 types of persisters were identified that were later named “triggered” and “spontaneous” persisters. Triggered persisters arise following a stress such as nutrient starvation while spontaneous persisters are generated during steady state exponential state at a constant rate [102]. Therefore, exposure to antibiotics only reveals a persister trait, but their very nature exists even in the absence of it. In this regard, they thus strongly resemble their SCV counterparts. Other important similarities are listed below:

(i) Both phenotypes are transient, allowing pathogens to survive inside host cells and withstand otherwise lethal antibiotic exposure while being able to quickly revert to a rapidly growing and fully virulent state after antibiotic removal.
(ii) Their metabolic activity shows reduced respiration and a switch towards lactic acid fermentation.
(iii) Internalization by host cells as well as oxidative stress seem to play an important role in the formation of both cell types.
(iv) Reduced expression of virulence factors seems to be important for both cell types [127].

Overall, phenotypic heterogeneity constitutes a formidable challenge for researchers, making it difficult to recover, identify and study these phenomena in physiologically relevant conditions. In light of these similarities, efforts might be needed to identify and understand the link between persisters and SCVs or on the contrary, to provide evidence of their differences and subsequently characterize their respective roles during disease progression, notably post antibiotic treatment.

6. Towards novel antimicrobial strategies to eradicate intracellular reservoirs

A recent review describes innovative strategies to act upon intracellular *S. aureus* [128]. In a nutshell, these involve (a) bioconjugates coupling a cell-penetrating peptide to an active molecule (enzyme), or an antibody coupled to an antibiotic that binds to extracellular bacteria and is internalized with them, (b) nano-formulations (liposomes, nanoparticles) that improve the accumulation of antibiotics in the infected compartments, or (c) strategies aiming at increasing cell defence mechanisms. Here we focus on strategies aiming specifically at clearing intracellular persisters.

In recent years, many efforts focused on the search for molecules that specifically target persisters, which could be used alone or in combination with antibiotics. The first molecule described is ADEP4, an acyldepsipeptide antibiotic that activates the bacterial ClpP protease. This causes a massive degradation of a wide variety of proteins, which forces bacterial cells to self-digest and kills persisters [129]. ADEP4 is highly active in-vitro (including in models of biofilms) or in-vivo, especially when combined with rifampicin, but has not been tested against intracellular bacteria. Its penetration inside the cells will probably require ad-hoc formulation as it is a bulky molecule. Smaller, diffusible molecules with anti-persister activity may offer an advantage in this context. For example, JD1, a small aromatic molecule containing a piperidinepropanol core and an adamantyl group, kills *S. aureus* persisters as well as intracellular *S. aureus,* by disturbing bacterial membrane integrity [130]. However, it is also toxic to eukaryotic cells, probably because it lacks specificity for bacterial membranes.

Membrane targeting is an attractive mode of action for killing non-replicative bacteria and is the appanage of many antimicrobial peptides. In fact, depending on their structural characteristics, antimicrobial peptides can disrupt membranes, cell wall, or intracellular functions. Cationic amphiphilic peptides that act by altering membrane integrity are thought to be more active against persisters (see [131] for review). A first challenge for these peptides is to gain access to the intracellular environment, but intracellular activity has nevertheless been shown for plectasin, a defensin-type antimicrobial peptide [132]. Poor cell penetration can be overcome by designing peptidomimetics. A recent example concerns an oligoguanidine-based peptidomimetic that is avidly taken up by host cells via endocytosis and accumulates in phagolysosomes where it eradicates persisters by a membrane/DNA dual-targeting mechanism of action [133]. Another reason for poor intracellular activity of peptides could be their degradation by phagolysosomal enzymes. As an example, two short peptides, WR12 and D-IK8, are both capable of eradicating stationary phase culture of *S. aureus*, but WR12 is less active than D-IK8 intracellularly because it is more susceptible to proteases [134]. A third drawback of peptidic drugs is that they can illicit immune reactions, with production of

antibodies that compromise their efficacy and can be detrimental for the patient. Molecular design tools have been used successfully to engineer a functionally deimmunized active derivative of lysostaphin, the activity of which has however not been tested on persisters or intracellular *S. aureus* [135].

In addition to chemical weapons, biological weapons should also be discussed. Phages are of particular interest against *S. aureus*, which have few anti-phage defense systems. The ability of phages to act on intracellular *S. aureus* is however still controversial [136,137]. Phage-derived peptidoglycan hydrolases, also known as endolysins, may constitute a promising approach to eradicate persister cells with little potential for the evolution of resistance mechanisms. As an example, exebacase, has successfully completed a phase II trial for the treatment of *S. aureus* bacteremia [138]. It also proved useful as an adjuvant to antibiotics in animal models of prosthetic joint infection or infective endocarditis but has not been tested against intracellular *S. aureus*.

Lastly, physical eradication of persisters might be achieved using cold atmospheric plasma, which generates ROS and RNS [139]. Interestingly, cold atmospheric plasma also enhances the oxidant defense mechanisms of macrophages, and therefore their capacity to kill intracellular *S. aureus* [140]. This approach, however, is limited in-vivo to accessible sites of infection, such as the skin.

Conclusion

S. aureus, which colonizes approximately 20% of the population, can also transform into a dangerous pathogen. It produces virulence factors associated with various acute diseases. More recently, it was found to also survive within host cells, including professional phagocytes and non-phagocytic cells. In this context, it expresses a series of enzymes and protective molecules that allow immune evasion and the establishment of a persistent niche. A growing body of evidence also points toward the possibility that internalization by host cells might trigger phenotypic diversification and be at the origin of antibiotic tolerant phenotypes such as SCVs and antibiotic persisters.

Importantly, intracellular bacteria, confined within phagolysosomes in phagocytic cells, are protected from humoral host defenses and, to some extent, antibiotics. Antibiotics must reach this subcellular compartment at a sufficient concentration and express their activity in this acidic environment. Moreover, the stress induced by host cell attacks trigger profound transcriptomic changes in intracellular *S. aureus*. Activation of global stress responses and reduced metabolic activity could notably underly the poor responsiveness to antibiotics.

Given these complexities, novel approaches are required to interrogate the specificities of intracellular bacteria at the single-cell level. Understanding how phenotypic heterogeneity leads to antibiotic treatment failure and deciphering the underlying molecular mechanisms should be the next challenge in the field with the perspective to develop novel antibiotic therapies that target intracellular pathogenic reservoirs. Membrane-active molecules are promising in this context, but issues related to their pharmacokinetics and toxicity need to be addressed.

Expert opinion

The intracellular persistence of *S. aureus* presents a significant challenge for clinical microbiologists in terms of diagnosis, and for clinicians, in terms of treatment. These intracellular niches remain undetectable unless biopsy samples can be collected and examined using appropriate microscopic techniques.

At this stage, it remains challenging to establish a direct link between persistence or recurrence of an infection and the presence of intracellular bacteria. We lack in-vivo models of persistent or recurrent infections in which an intracellular reservoir can be easily monitored over time. Molecular mechanisms remain therefore mostly elusive. In addition, classical bulk-averaging methods such as OD measurements, CFU/mL counting, RNAseq, etc. miss out on phenotypic heterogeneity that is known to underly crucial phenotypes such as SCVs and bacterial persisters. Novel single-cell methods based on microscopy and flow cytometry should therefore be developed and used in conjunction to intracellular models to interrogate these phenotypic variants and identify the molecular mechanisms underlying prolonged antibiotic survival inside host cells. This will help identify potential Achille's heels that could be exploited to develop alternative therapies effective against the intracellular pathogenic reservoir of *S. aureus*.

From a therapeutic perspective, persisters or intracellular bacteria are currently not taken into consideration when selecting antibiotics for the treatment of staphylococcal infections. An exception to this is rifampicin, which is often used to treat complicated bone infections due to its proven ability to effectively penetrate bone tissue. In cases of therapeutic failure, clinicians commonly switch to another antibiotic or opt for drug combinations. However, it is worth noting that persisters can exhibit cross-tolerance to multiple classes of antibiotics.

Among the molecules discovered so far, peptides have shown promise in their ability to kill persister cells. However, there are significant hurdles to overcome before they can be routinely used in a clinical context. Pharmacokinetic challenges, including issues related to the route of delivery, stability, and cell penetration, need to be addressed. Additionally, there is a need to enhance their specificity against bacterial membranes.

Directions for future research are therefore multiple. In clinical research, we need to establish routine methods to evaluate the persister character or intracellular tropism of clinical isolates and to determine whether these characteristics could be used to predict the risk of recurrence of the infection. In fundamental research, we need to better understand the role of phenotypic heterogeneity for disease progression and antibiotic survival, which could inspire multi-target therapeutic approaches to eradicate intracellular niches. In pharmacological research, we need to search for small molecules active on persisters to overcome the difficulties posed by the use of peptides as drugs. In drug development research, companies should be urged to include activity testing against intracellular *S. aureus* or persisters early in the selection for best candidates for clinical development.

The good news however is that the intracellular character of *S. aureus* has been widely acknowledged. This increased awareness should drive further research dedicated to better understanding its intracellular survival and, potentially, lead to novel therapeutic approaches. In this context, the growing capabilities of single-cell analysis techniques and -omics approaches will play a pivotal role. Additionally, virtual drug design can assist in screening chemical diversity, enabling the concentration of synthesis efforts on the most promising scaffolds.

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Figure 1. Challenges faced by *S. aureus* when thriving inside professional phagocytes and mechanisms to overcome them: (1) To cope with the acidic medium of the phagolysosomes, *S. aureus* upregulates *agrA,*a response regulator that induces transcription of RNAIII to promote the production of virulence factors. **(2)** To resist to oxidative burst, *S. aureus* produces the antioxidant staphyloxanthin (STX), the superoxide dismutases (SOD) SodA and SodM, the KatA catalase and AhpC alkyl hydroperoxide reductase conferring resistance to H_2O_2, and the peroxidase inhibitor (SPIN) that binds to myeloperoxidase (MPO). **(3)** To grow in the presence of nitric oxide (produced, together with citrulline (Cit), from arginine (Arg) and O_2 by iNOS), *S. aureus* produces the Hmp flavohaemoglobin and switches to homolactic fermentation by overexpressing the LDH1 lactate deshydrogenase. **(4)** To survive to cationic antimicrobial peptides, *S. aureus* neutralizes its cell surface by upregulating the MrpF multiple peptide resistance factor (synthesizing and translocating Lysyl-PG) or enzymes encoded by the *dlt* operon (modifying teichoic acids). It overproduces the OatA O-acetyltransferase to acetylate the muramic acid residues of peptidoglycan and resist to lysozyme (LYS). It also produces peptidases to degrade the AMP.

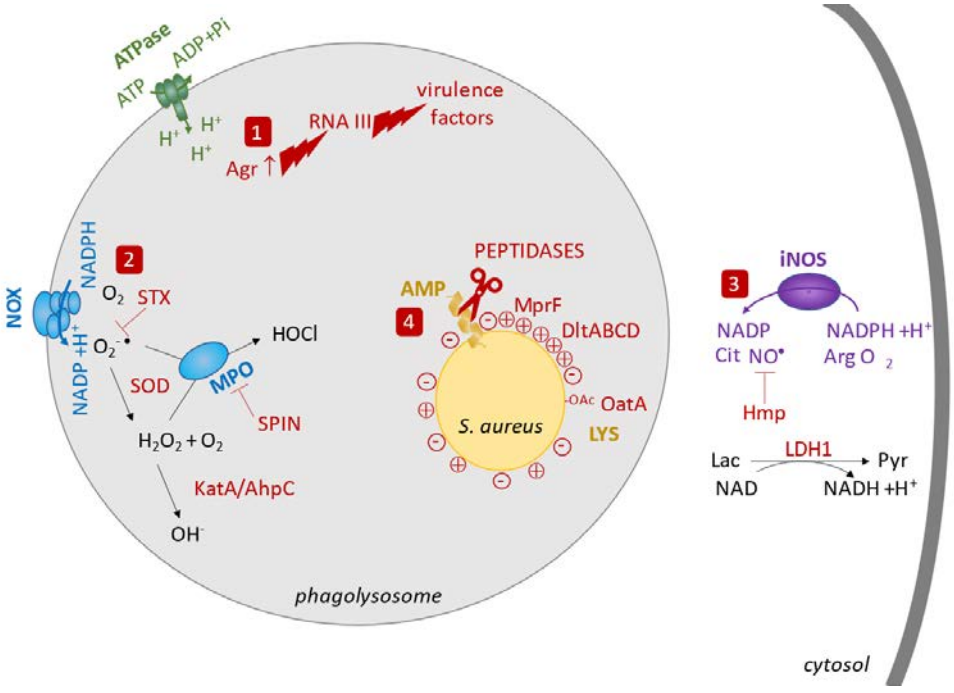

Figure 2: main factors affecting antibiotic activity against intracellular *S. aureus.*

Drug-related factors (left) include the cellular pharmacokinetic properties of the antibiotic, notably **(1)** its capacity to accumulate in the infected compartment, which can be defeated by active efflux and **(2)** its intracellular bioavailability, which can be reduced by binding to cell proteins or lipids, and **(3)** alteration of the ionization status of he drug in mild acidic vacuolar compartment, which may affect its intrinsic activity (modulation of its uptake in the bacteria or of its interaction with its target). Bacteria-related factors (right) are related to tolerance (poor responsiveness) to drug action, associated with a switch to **(4)** persister phenotype or **(5)** small colony variant (SCV) . Persisters and SCVs share some properties shown in the inset, like their capacity to survive in a non-replicating or slow-replicating state in the presence of antibiotics and revert to a replicating state when the antibiotic pressure is relieved, via a series of responses (in red) to stress (in yellow) which are not always well characterized.

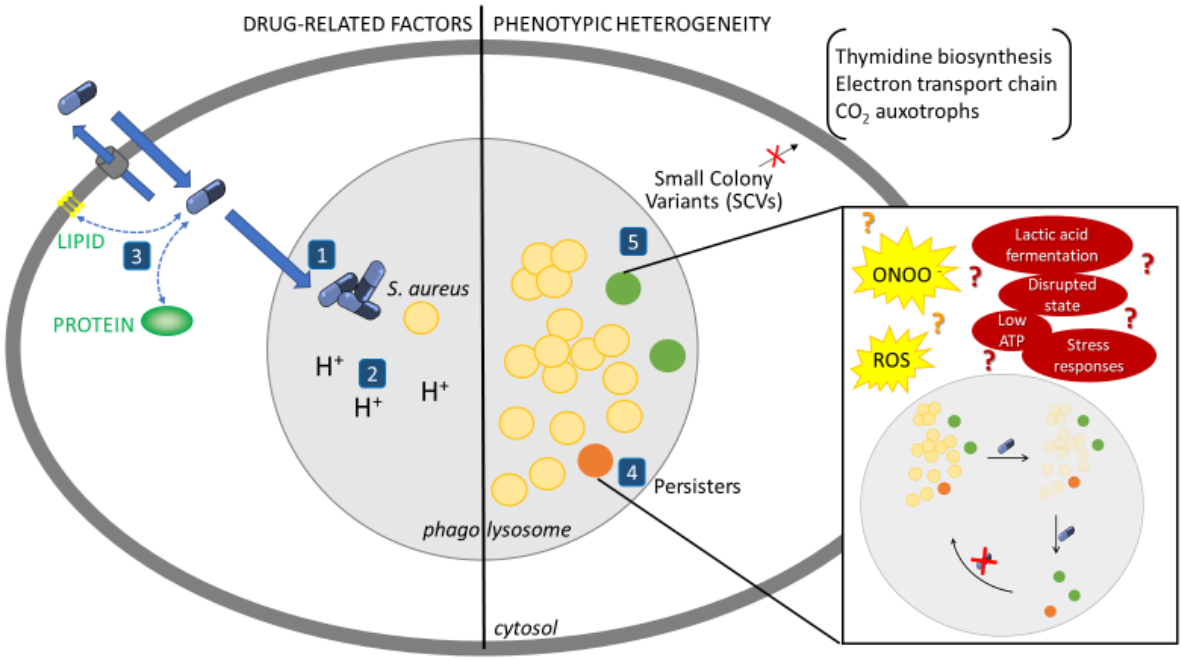

Figure 3: Stress responses induced by exposing intracellular *S. aureus* to oxidative stress or to antibiotics.

Among the oxidative stresses faced by intracellular *S. aureus,* peroxinitrite (ONOO-) resulting from the combination of ROS and NOS, is suggested to play the major role in inducing ATP depletion and reduced respiration, as well as tolerance to rifampicin. On the other hand, stress induced by exposure of intracellular *S. aureus* to the antibiotic oxacillin caused an early induction of the stringent response, as well as an induction of the cell wall stress stimulon, the SOS response, and the heat shock response, together with a decrease in oxidative phosphorylation, a shift of central metabolism to the use of lactose rather than glucose, and a reduction in the translation rate. Altogether, these changes could lead to multidrug tolerance.