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CELLULAR AND MOLECULAR PHARMACOLOGY

INTRACELLULAR PHARMACODYNAMICS OF ANTIBIOTICS AGAINST SMALL-COLONY VARIANTS OF *STAPHYLOCOCCUS AUREUS* IN *IN VITRO* MODELS OF PROFESSIONAL PHAGOCYTIC CELLS

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Promoteur : Professeur Françoise VAN BAMBEKE

« Reste devant la porte si tu veux que l'on t'ouvre.
Ne quitte pas la voie si tu veux que l'on te guide.
Rien n'est fermé jamais,
Sinon à tes propres yeux. »

FARID AL-DIN ATTAR
Extrait Du Langage des Oiseaux

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ABBREVIATION LIST

ABC	**A**TP-**B**inding **C**assette
CF	**C**ystic **F**ibrosis
CFTR	**C**ystic **F**ibrosis **T**ransmembrane conductance **R**egulator
CFU	**C**olony **F**orming **U**nit
CLSI	**C**linical and **L**aboratory **S**tandards **I**nstitute
Fn	**F**ibro**n**ectin
FnBPs	**F**ibro**n**ectin **B**inding **P**roteins
MICs	**M**inimal **I**nhibitory **C**oncentrations
MRSA	**M**ethicillin-**R**esistant ***S****tahylococcus* ***a****ureus*
MRPs	**M**ultidrug **R**esistance **P**roteins
MSSA	**M**ethicillin-**S**usceptible ***S****tahylococcus* ***a****ureus*
MSCRAMMs	**M**icrobial **S**urface **C**omponents **R**ecognizing **A**dhesive **M**atrix **M**olecules
PBPs	**P**enicillin **B**inding **P**roteins
ROS	**R**eactive **O**xygen **S**pecies
SCVs	**S**mall **C**olony **V**ariants
TLR	**T**oll-**L**ike **R**eceptor

FOREWORD

This work is part of the effort of the laboratory to decipher the parameters governing the activity of antibiotics against the intracellular forms of *Staphylococcus aureus*. In this context, a previous work examined the intracellular activities of a series of antibiotics against a thymidine-dependent Small-Colony Variant of *Staphylococcus aureus* and its isogenic normal-phenotype counterpart, in human THP-1 monocytes (186).

As Small-Colony Variants are frequently reported in Cystic Fibrosis patients (292), our initial goal was to study antibiotic activity against these particular forms in a more relevant model, namely bronchial epithelial cells displaying different CFTR genotype (wild-type, F508del or no CFTR), cultivated on a polarized manner to correlate the activity of antibiotics with their pharmacokinetic profile in these cells. Yet, obtaining the cell lines and cultivating them proved much more fastidious than anticipated.

While these models were being developed, we decided in the meantime to examine in parallel, in the previously described model of THP-1 cells, other types of Small Colony Variants, characterized by defects in the electron transport chain, namely menadione- and hemin-dependent Small-Colony Variants, for which data were still lacking. This model allowed us to make a series of original observations, which at the end, leaded us to reorient the aim of the thesis in this new direction.

The introduction will present (i) the existing state of knowledge of *Staphylococcus aureus,* its Methicillin-Resistant forms and the current therapeutic approaches available against these resistant strains, (ii) the Small-Colony Variant phenotype and its associated disease states, and (iii) the *in vitro* intracellular infection model used in this project, the THP-1 cells.

INTRODUCTION

1. *Staphylococcus aureus*

Sir Alexander Ogston introduced the term "*Staphylococcus*" (from the Greek *staphylé*) to describe grape-like clusters of bacteria responsible for inflammation and suppuration (193). Staphylococci are Gram-positive cocci (0.5 to 1.5 µM in diameter), non-motile, non-spore-forming, usually catalase positive and oxidase negative (138; 137). Staphylococci are also tolerant to high concentrations of sodium chloride (301) and show resistance to high temperature (138).

The genus *Staphylococcus* contains 32 species, 16 of which are found in humans. Only a few of them are pathogenic in the absence of predisposing host conditions such as immunosuppression or the presence of a foreign body. In humans and animals, the most virulent one is *Staphylococcus aureus* (295; 218).

S. aureus is endowed with many features (e.g. the ability to promote asymptomatic nasal colonization of a large fraction of the world's population, resistance to lysozyme and innate immune defense strategies, toxin production and multiple drug resistance to name a few) that help define it as a formidable human pathogen causing relatively mild skin infections to life-threatening, invasive disease (Table 1 [155]).

Table 1. Clinical manifestations associated with *Staphylococcus aureus* infections

Type of infections	
Skin & Soft Tissue Infections	Impetigo, folliculitis, furuncles, carbuncles, mastitis, cellulitis, fasciitis, abscesses, etc.
Blood Stream Infections	Bacteremia, sepsis Metastatic infections: Endocarditis, meningitis, pericarditis, pneumonia, osteomyelitis, septic arthritis, etc.
Toxin-Related Diseases	Food poisoning Scalded Skin Syndrome Toxic Shock Syndrome with multiple organ failure

1.1. Main Characteristics

Staphylococcus aureus genome is composed of a single chromosome of around 2.8 Mb, which is predicated to encode approximately 2600 genes. About 75% of the genome comprises the core component, which is generally conserved and composed of genes present in all strains. The core genome contains genes required for growth and survival, and also contains some genes associated with pathogenesis. Although the core genome is conserved between strains, many genes encode

hypothetical proteins of unknown function. In addition, some parts of the "core" genome are not as stable as the term suggests, and the core genome has therefore been subdivided into "stable core" and "core variable genome" (74). The latter tends to encode virulence factors (ranging from cell wall components, such as the capsular polysaccharide and peptidoglycan, to secreted proteins, such as exotoxins), has a higher rate of nucleotide variability, and a higher number of variable number tandem repeats.

1.1.1. Cell Surface Constituents

The cell wall of *S. aureus* is a strong protective coat, which is relatively amorphous in appearance, about 20-40 nm thick (252). Underneath the cell wall is the cytoplasm that is limited by the cytoplasmic membrane. Peptidoglycan is the basic component of the cell wall, and makes up 50% of the cell wall mass (295). It is integral in the formation of the tight multi-layered cell wall network, capable of withstanding the high internal osmotic pressure of staphylococci (301). Another cell wall constituent is a group of phosphate-containing polymers called teichoic acids, which contribute for about 40% of cell wall mass (139). There are two types of teichoic acids, cell wall teichoic acid and cell membrane associated lipoteichoic acid (which induces the secretion of the cytokines interleukine IL-1β and IL-6 [19] and the chemokine IL-8 by monocytes and stimulates nitric oxide production by macrophages [113; 134]); bound covalently to the peptidoglycan or inserted in the lipid membrane of the bacteria. Teichoic acids confer a negative charge to the staphylococcal cell surface and play a role in the activity of autolytic enzymes (301). Peptidoglycan and teichoic acid together account for about 90% of the weight of the cell wall, the rest is composed of surface proteins, exoproteins and peptidoglycan hydrolases (autolysins). Some of these components are involved in attaching the bacteria to surfaces and are virulence determinants. Finally, over 90% of *S. aureus* clinical strains have been shown to possess capsular polysaccharides (131; 272). Capsule production is reported to decrease phagocytosis *in vitro*, and to enhance *S. aureus* virulence in a mouse bacteremia model (302; 272), therefore acting as a form of biofilm.

1.1.2. Pathogenesis

S. aureus has genes that encode several virulence factors (Table 2), the expression of which is controlled by a complex regulatory network including the quorum-sensing *agr* system, transcriptional regulators of the Sar family, the two-component regulatory systems ArlRS and SaeRS, and the alternative sigma factor SigB (191). During *in vitro* cultivation, bacterial surface-associated virulence factors are preferentially expressed in the logarithmic growth phase, whereas secreted virulence

factors are released in the post-logarithmic phase. It is assumed that this biphasic expression of virulence factors orchestrates the infection process. Initially, surface-bound adhesins recognize host surface structures facilitating colonization, which is then followed by further growth of the microbes and secretion of toxins and enzymes, such as hemolytic toxins (α-, β- and γ-toxin), leucotoxins (e.g. Panton-Valentine leukocidin and LukFS), staphylococcal enterotoxins (SEA to SEJ), toxic shock syndrome toxine-1 (TSST-1), several proteases (e.g. metalloprotease aureolysin [Aur], serine proteases [SspA] and cysteine protease [SspB]), and lipases (e.g. Geh). Although in most cases the infection remains localized, the bacteria can also spread into deeper tissues and, importantly, are able to cause chronic diseases (155; 215; 127).

A characteristic feature of pathogenic *S. aureus* is the presence of adhesins that bind host extracellular-matrix proteins and serum components. These surface proteins either remain associated with the surface of the bacteria or are released into the culture supernatant. These have been collectively termed "MSCRAMMs" (Microbial Surface Components Recognizing Adhesive Matrix Molecules [e.g. collagen-binding protein {205}, fibronectin-binding proteins {78,123}, clumping factors {168; 189}, elastin-binding protein {201}]) and are involved in colonizing host tissues and in the evasion of host immune response (204; 82).

Table 2. Virulence factors of *Staphylococcus aureus* (adapted from 253)

Pathogenic action	Virulence factors	Functions
Colonization of host tissues	Surface proteins	Adhesins, fibronectin and fibrinogen-binding protein
Lysis of eukaryotic cell membranes and bacterial spread	Membrane-damaging toxins, invasins	Hemolysins, hyaluronidase, leukocidin, leukotoxin, lipases, nucleases
Inhibition of phagocytic engulfment	Surface factors	Capsule, protein A
Survival in phagocytes	Biochemical compounds	Carotenoids, catalase production
Immunological disguise and modulation	Surface proteins	Clumping factor, coagulase, protein A
Contribution to symptoms of septic shock	Exotoxins	Enterotoxins SEA to SEG, exfoliative toxin, TSST

1.2. Anti-Staphylococcal Agents

Choosing an appropriate anti-staphylococcal drug is a daunting task given the ever-changing resistance pattern of this organism. Five bacterial targets have been exploited in the development of antimicrobial drugs: cell wall synthesis, protein synthesis, ribonucleic acid synthesis, deoxyribonucleic acid synthesis and intermediary metabolism.

Currently, glycopeptides remain the gold-standard treatment option for resistant *S. aureus* infections, either alone or in combination with other agents (e.g. rifampin, fusidic acid, gentamicin [Table 3]).

Table 3. Antibiotics used for the treatment of *Staphylococcus aureus* (153; 177).

Pathogen	**Antibiotic**
MSSA	β-lactams: Penicillins: nafcillin, oxacillin, dicloxacillin, amoxicillin/clavulanate, ampicillin/sulbactam, piperacillin/tazobactam Cephalosporins: cephalexin, cephazolin Macrolides*: azithromycin, clarithromycin
MSSA & MRSA	Lincosamide: clindamycin Trimethoprim & sulfonamide: trimethoprim/sulfamethoxazole --- Used in conjunction to another agent: Ansamycin: rifampin Aminoglycoside: gentamicin Fusidane: fusidic acid
MRSA	Tetracyclines: doxycycline, minocycline Oxazolidinone: linezolid Glycopeptides: vancomycin, telavancin Lipopeptide: daptomycin

1.2.1. Inhibition of Cell-Wall Synthesis

β-lactams and glycopeptides are among the classes of antibiotics that interfere with specific steps in homeostatic cell wall biosynthesis. Successful treatment with a cell wall synthesis inhibitor can result in changes to cell shape and size, induce cellular stress responses and lead to cell lysis (275).

On the other hand, lipopeptides do not impede any of the enzymatic steps in cell wall biosynthesis, but inhibit peptidoglycan biosynthesis by inducing membrane depolarization (3; 255).

1.2.1.1. β-Lactams

While the discovery was made in 1928 by Alexander Fleming, the use of penicillin as a therapeutic agent to treat infections did not happen until the 1940s. The β-lactam ring proved to be the key in penicillin synthesis and modification, and opened up the floodgate where novel β-lactam agents could be produced by adding unusual side chains. Thereafter, semi-synthetic β-lactam

* In countries with low and stable rates of macrolides resistance (86)

compounds have been developed continuously and systematically (e.g. methicillin, ampicillin). Although the penicillins generation presented a great opportunity, natural sources continued to be explored. Many related naturally occurring compounds with important antibiotic activity were isolated from bacteria; these include cephalosporins (e.g. cefuroxime, cefepime), carbapenems (e.g. imipenem, meropenem, doripenem) and monobactams (e.g. aztreonam).

β-lactams block the cross-linking of peptidoglycan units by inhibiting the peptide bond formation reaction catalyzed by transpeptidases (Figure 1), which are also known as Penicillin-Binding Proteins (PBP [305; 274]). This inhibition is achieved by penicilloylation of a PBP's transpeptidase active site - the β-lactam drug molecule is an analog of the terminal D-alanyl-D-alanine dipeptide of peptidoglycan and acts as a substrate for the enzyme during the acylation phase of cross-link formation - which disables the enzyme due to its inability to hydrolyze the bond created with the now ring-opened drug (297; 124). Interestingly, β-lactam sub-types have distinct affinities for certain PBPs, which is correlated with the ability of these drugs to stimulate autolysin activity and induce lysis (264; 136).

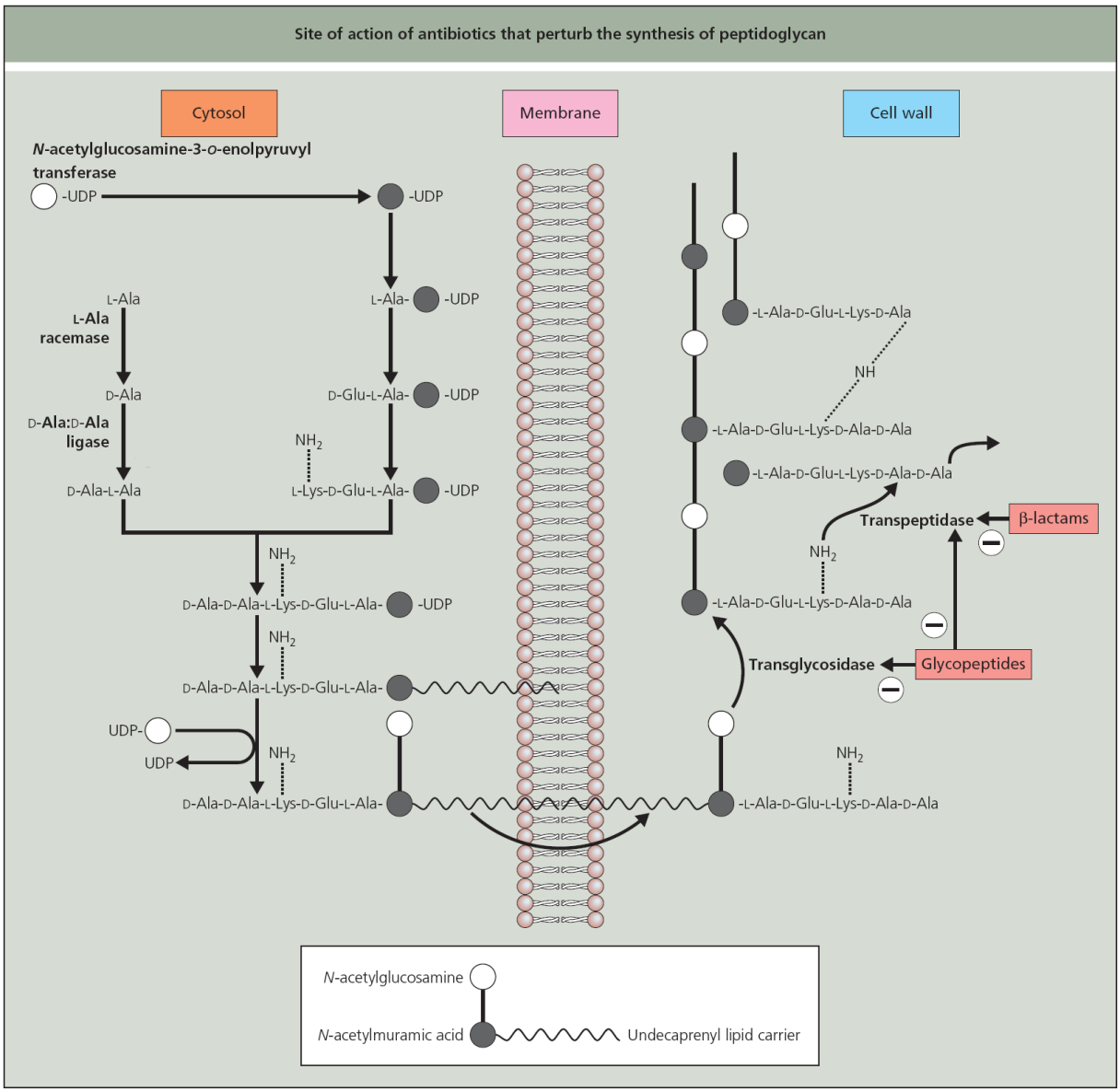


Figure 1. Site of action of antibiotics that perturb the synthesis of peptidoglycan (adapted from 282). The peptidoglycan unit is formed in the cytosol of bacteria by binding to uridine diphosphate (UDP) *N*-acetylmuramic acid of a short peptide (the nature of which differs between bacteria). This precursor is then attached to a lipid carrier and added to *N*-acetylglucosamine before crossing the bacterial membrane. At the cell surface peptidoglycan units are reticulated by the action of transglycosylases (catalyzing the polymerization between sugars) and of transpeptidases (catalyzing the polymerization between peptidic chains). The antibiotics act as follows: β-lactams are analogs of D-Ala-D-Ala and suicide substrates for transpeptidases; and vancomycin binds to D-Ala-D-Ala termini and thus inhibits the action of transglycosylases and transpeptidases.

1.2.1.2. Glycopeptides

Since their discovery, glycopeptide antibiotics (e.g. vancomycin, teicoplanin) have been used worldwide as a last-resort antibiotic to treat infections by Gram-positive bacterial pathogens (167;

110; 161). Over the last 10 years, active research for new glycopeptides has led to the production of a series of semisynthetic derivatives (e.g. telavancin, oritavancin).

Glycopeptide antibiotics inhibit peptidoglycan synthesis through binding with peptidoglycan units (at the D-alanyl-D-alanine dipeptide) and by blocking transglycosylase and transpeptidase activity (128). Thus, glycopeptides (whether free in the periplasm like vancomycin or membrane-anchored like teicoplanin [49]) generally act as steric inhibitors of peptidoglycan maturation and reduce cellular mechanical strength, although some chemically-modified glycopeptides have been shown to directly interact with the transglycosylase enzyme (91).

It should be noted that the new glycopeptide, oritavancin, involves an additional mechanism of action: the cell membrane interaction/disruption. The presence of the hydrophobic 4'-chlorobiphenylmethyl group allows for interaction and disruption of the cell membrane, resulting in depolarization, permeabilization and concentration-dependent, rapid cell death (Figure 2 [14; 15; 4; 135; 62]).

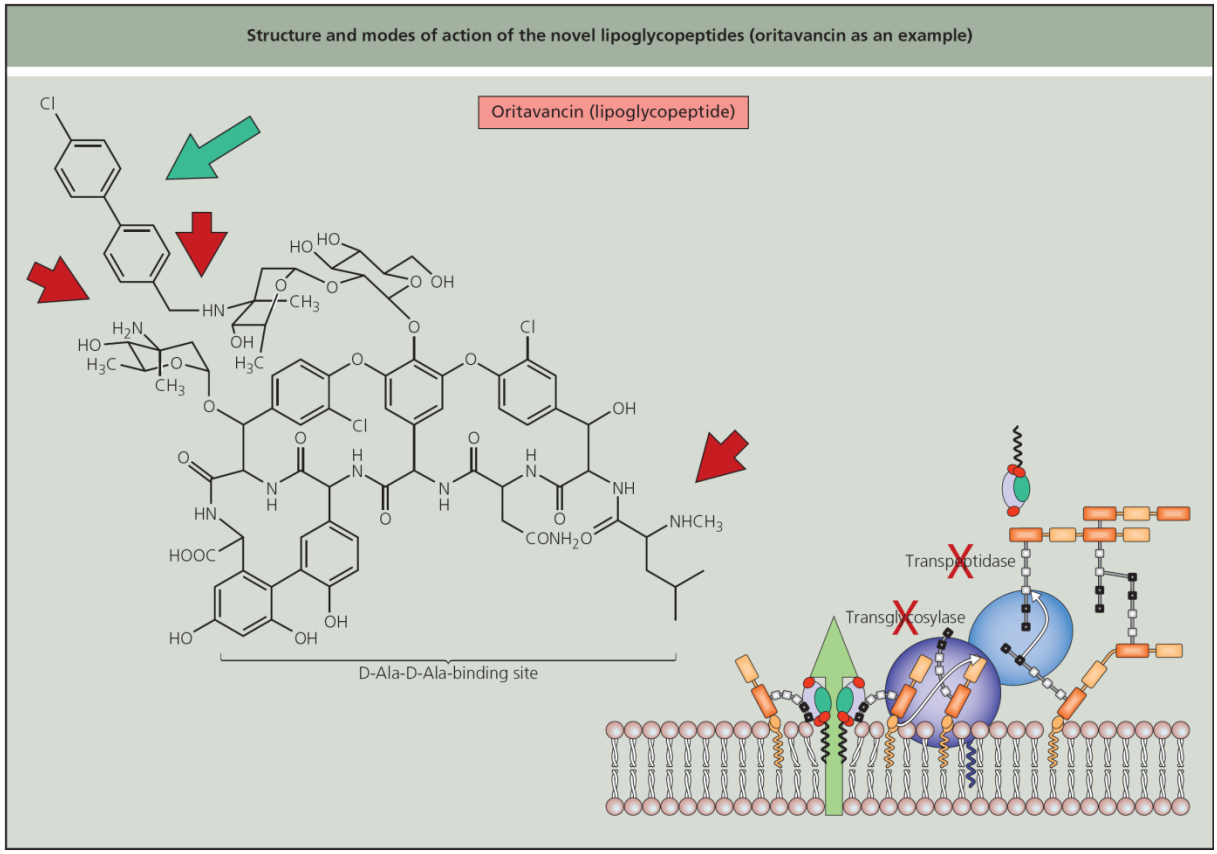


Figure 2. Structure and proposed modes of action of oritavancin (282). Inset: the drug is characterized by the presence of a bulky hydrophobic moiety (green arrow) and either additional amino groups (red arrows), resulting in a global amphiphilic character for the whole molecule. Note that the D-Ala-D-Ala binding site of the molecule remains unchanged. Main figure: the molecule interacts with the D-Ala-D-Ala termini of the pentapeptide (closed squares) as vancomycin, inhibiting both the

transpeptidase and the transglycosidase activities, but also inserts itself into the membrane through its lipophilic side chain (this is favored, in the case of oritavancin, by its strong capacity to dimerize). As a result, this lipoglycopeptide induces membrane leakage and thereby display a marked, concentration-dependent bactericidal effect. This second mode of action is exerted even if bacteria display a D-Ala-D-Lac or D-Ala-D-Ser in place of the D-Ala-D-Ala moiety, in as in vancomycin-resistant organisms. Specificity towards bacterial membranes probably stems from the fact that membrane anchoring is enhanced by the presence of phospatidylglycerol, an acidic phospholipid abundant in bacterial but not in eukaryotic cell membranes.

1.2.1.3. Lipopeptides

Daptomycin is a branched cyclic anionic lipopeptide antibiotic that was discovered in the early 1980's. Despite nearly 25 years of study and 10 years of clinical use, aspects of the mechanism of action of daptomycin remain poorly understood. However, a recent study support a mechanism for daptomycin with a primary effect on cell membranes that in turn redirects the localization of proteins involved in cell division and cell wall synthesis, causing dramatic cell wall and membrane defects, which may ultimately lead to a breach in the cell membrane and cell death (255; 211).

Insertion of daptomycin into bacterial membranes requires the presence of phosphatidylglycerol (125). Following insertion, daptomycin aggregates in the membrane (181). As daptomycin has a large head-group volume and a short small volume tail, the molecule is viewed as a lipid. Aggregation of such lipids will produce local alterations in membrane curvature (125; 169; 267). The localized region of membrane distortion and altered curvature is recognized by DivIVA, a protein playing an important role in cell division in diverse Gram-positive bacteria (152; 220), leading the cell to incorrectly identify the location as a site of potential cell division. Recruitment of DivIVA leads to local changes in peptidoglycan biogenesis, including localized inhibition of lateral cell wall biosynthesis. Because no corresponding change is made around the cell wall, lateral extension continues on the opposite side of the cell, leading to asymmetric extension.

When multiple sites of local membrane curvature are induced, discontinuities in the membrane at the site of daptomycin insertion lead to slow leakage of ions and loss of membrane potential. These changes may also result in local deregulation of the cell division or cell wall biosynthetic machinery, including activation of autolysins (122; 266). There may be other integral membrane proteins whose function is dependent on either proper localization or normal bilayer architecture, resulting in a highly pleiotropic effect of daptomycin on cell physiology.

It is important to note that laboratory-based studies using *in vitro* and animal models advocate that daptomycin should not be used in pulmonary infections, due to its inactivation by lung surfactant (254).

1.2.2. Inhibition of DNA Replication

Modulation of chromosomal supercoiling through topoisomerase-catalyzed strand breakage and rejoining reactions is required for DNA synthesis, mRNA transcription and cell division (72; 65; 94). These reactions are exploited by the synthetic quinolone class of antimicrobials, including the clinically-relevant fluoroquinolones, which target DNA-topoisomerase complexes (64; 270; 93).

Quinolones are derivatives of nalidixic acid, which was discovered as a byproduct of chloroquine (quinine) synthesis and introduced in the 1960s to treat urinary tract infections (117). Nalidixic acid and other first generation quinolones (e.g. oxolinic acid) are rarely used today owing to their toxicity (229). Second (e.g. ciprofloxacin), third (e.g. levofloxacin) and fourth (e.g. moxifloxacin, gemifloxacin) generation quinolone antibiotics can be classified based on their chemical structure along with qualitative differences in how these drugs kill bacteria (117; 157).

The quinolone class of antimicrobials interferes with the maintenance of chromosomal topology by targeting DNA gyrase (topoisomerase II) and topoisomerase IV (topoIV), trapping these enzymes at the DNA cleavage stage and preventing strand rejoining (64; 43; 66). As a result of quinolone-topoisomerase-DNA complex formation (Figure 3), DNA replication machinery becomes arrested at blocked replication forks, leading to inhibition of DNA synthesis, which immediately leads to cell death (64).

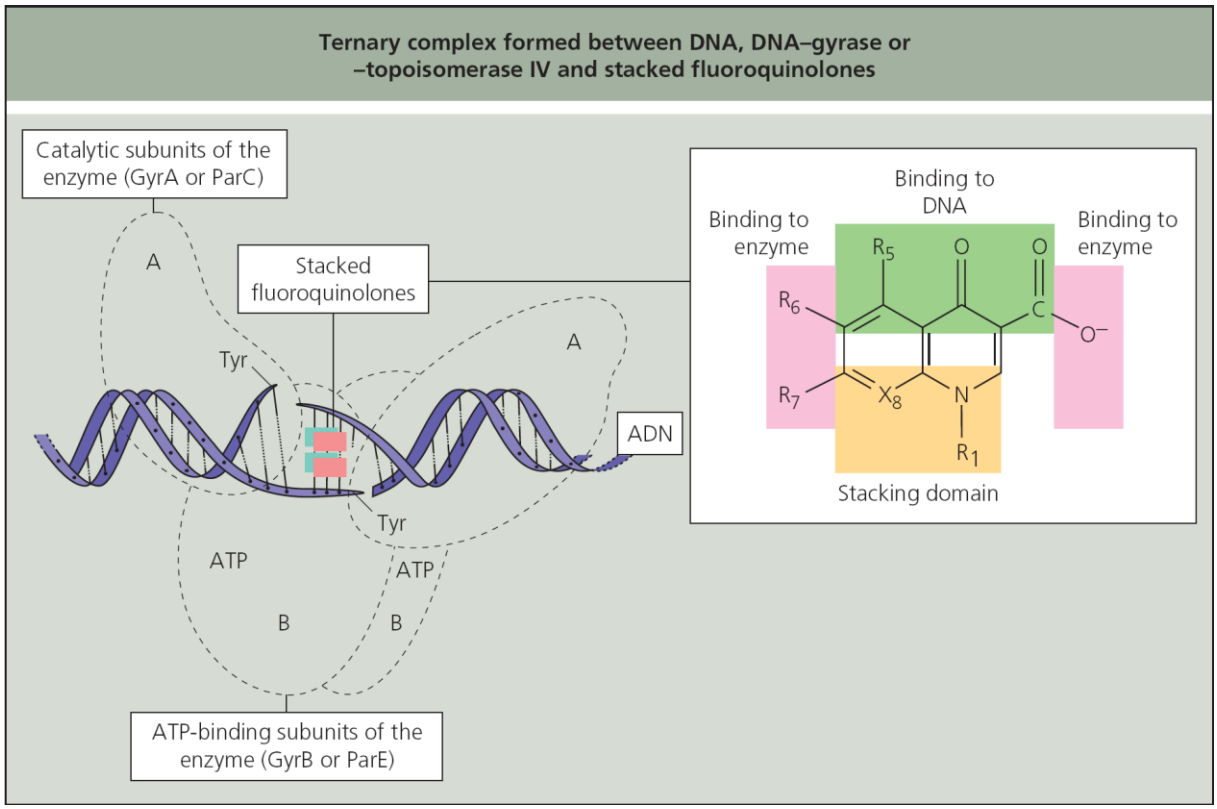


Figure 3. Ternary complex formed between DNA, DNA–gyrase or –topoisomerase IV and stacked fluoroquinolones (282). Subunits A form covalent bonds via Tyr122 with the 5' end of the DNA chain. The binding site for fluoroquinolones is located in the bubble formed during the local opening of the DNA molecule. The right panel shows the parts of the antibiotic molecules interacting with DNA, with the enzyme, or favoring the stacking of the fluoroquinolone molecules.

1.2.3. Inhibition of RNA Synthesis

The inhibition of RNA synthesis by the ansamycin class of semi-synthetic bactericidal antibiotics, much like the inhibition of DNA replication by quinolones, has a catastrophic effect on prokaryotic nucleic acid metabolism and is a potent means for inducing bacterial cell death (79).

Ansamycins were first discovered in the 1950's (245) and over time, more potent ansamycin forms were isolated and characterized (e.g. rifamycin SV, rifampin [244]). These antibiotics are often used in combinatorial therapies owing to the rapid nature of resistance development (298; 32).

Ansamycin drugs inhibit DNA-dependent transcription by stable binding, with high affinity, to the subunit of a DNA-bound and actively-transcribing RNA polymerase enzyme (111; 33; 185); the subunit is located within the channel formed by the polymerase-DNA complex, from which the newly synthesized RNA strand emerges (39). A unique mechanistic requirement of ansamycins is that RNA

synthesis has not progressed beyond the addition of two ribonucleotides, which is attributed to the ability of the drug molecule to sterically inhibit nascent RNA strand initialization (166).

1.2.4. Inhibition of Protein Synthesis

The process of mRNA translation occurs over three sequential phases (initiation, elongation and termination) involving the ribosome and a host of cytoplasmic accessory factors (88). The ribosome organelle is composed of two ribonucleoprotein subunits; the 50S and 30S. Drugs that inhibit protein synthesis are among the broadest classes of antibiotics and can be divided into two subclasses: the 50S inhibitors and 30S inhibitors.

50S ribosome inhibitors include the macrolide (e.g. erythromycin), lincosamide (e.g. clindamycin), streptogramin (e.g. dalfopristin/quinupristin), amphenicol (e.g. chloramphenicol) and oxazolidinone (e.g. linezolid) classes of antibiotics (133; 179). In general terms, 50S ribosome inhibitors work by physically blocking either initiation of protein translation (as it is the case for oxazolidinones [203]), or translocation of peptidyl-tRNAs, which serves to inhibit the peptidyltransferase reaction that elongates the nacent peptide chain. Studies of macrolide, lincosamide and streptogramin drugs have provided for a mode of action model that involves blocking the access of peptidyl-tRNAs to the ribosome (to varying degrees), subsequent blockage of the peptidyl-transferase elongation reaction by steric inhibition, and eventually triggering dissociation of the peptidyl-tRNA (286; 172).

30S ribosome inhibitors include the tetracycline, glycylcycline and aminoglycoside families of antibiotics. On the one hand, tetracyclines (e.g. minocycline, doxycycline) and glycylcyclines (e.g. tigecycline) work by blocking the access of aminoacyl-tRNAs to the ribosome (47; 194; 207; 228). Even if glycylcyclines exhibit antibacterial activities typical of earlier tetracyclines, they are more active against resistant pathogens (25). On the other hand, the aminoglycoside family of antibiotics (e.g. streptomycin, kanamycin, gentamicin) binds the 16S rRNA component of the 30S ribosome subunit. Spectinomycin, interferes with the stability of peptidyl-tRNA binding to the ribosome by inhibiting elongation factor-catalyzed translocation, but does not cause protein mistranslation (58; 108). By contrast, the interaction between aminoglycosides and the 16S rRNA can induce an alteration in the conformation of the complex formed between an mRNA codon and its cognate charged aminoacyl-tRNA at the ribosome, promoting tRNA mismatching which can result in protein mistranslation (57; 132; 83; 200).

By locking elongation factor G (EF-G) to the ribosome, fusidic acid also blocks bacterial protein synthesis (23). EF-G is a translational GTPase catalysing two different steps of protein synthesis (235). First, EF-G is needed for translocation of tRNAs and mRNA with respect to the ribosomal 30S subunit to make a new mRNA codon available for decoding. Second, EF-G acts together with ribosome recycling factor (RRF) in splitting of the ribosomal post-termination complex. In both of these steps, GTP hydrolysis by EF-G is used as an energy source, and in both cases fusidic acid prevents the release of EF-G from the ribosome after GTP hydrolysis (23; 115).

1.2.5. Inhibition of Folic Acid Synthesis

By inhibiting folic acid metabolism and more especially the biosynthesis of tetrahydrofolate, the synthetic antibacterial agents, trimethoprim and sulfonamide drugs, induce thymine starvation leading to cell death.

Sulfonamides inhibit dihydropteroate synthase, which blocks folate biosynthesis (44). This, in turn, leads to defective thymidine biosynthesis. Sulfonamides are bacteriostatic against *S. aureus* (159; 262; 263). Some strains of *S. aureus* overproduce para-aminobenzoic acid (PABA), causing resistance to sulfonamides (262; 263; 178). However, resistance to sulfonamides is more frequently caused by alterations in dihydropteroate synthase (53; 106; 118).

Trimethoprim is a tetrahydrofolate reductase inhibitor that, when added to sulfamethoxazole, provides a second step block in the folate biosynthetic pathway (118). Trimethoprim/sulfamethoxazole (TMP-SMZ) proved to be bactericidal (63; 104; 163; 273; 129). Blocking folate metabolism at two sites decreased the emergence of resistance (53; 106; 118). Nevertheless, resistance to TMP-SMZ has occurred because of amino acid substitutions in both enzymes. Plasmids carry the altered genes, which facilitate the spread of TMP-SMX resistance (16). Exogenous thymidine will render TMP-SMX inactive, because it bypasses the double biosynthetic blockade.

1.3. Strategies for recurrent and persistent infections

Decades of research have illuminated how *S. aureus* has evolved ingenious mechanisms to counteract immune response and to elaborate numerous virulence factors that contribute to the diversity and severity of staphylococcal diseases (81; 191; 227). This organism has the ability to transiently colonize the skin and mucous membranes of most humans with unnoticeable or mild

clinical features, and it is estimated that 20% of the world's population are persistent carriers. It is the most common cause of skin and soft tissue infections, and once it penetrates the subcutaneous tissues and reaches the blood, it can infect almost any organ, most notably bone tissue and cardiac valves (155). The existence of highly virulent methicillin-resistant *S. aureus* (MRSA) strains is a serious and alarming worldwide public health menace, and different epidemic strains have developed both in the community and in hospitals (98).

1.3.1. Methicillin-Resistant Staphylococcus aureus

MRSA is now recognized as a public health problem worldwide because of increasing rates of infection in many settings (156; 109). The terminology used for cases of colonisation or infection due to MRSA is varied. The Centers for Disease Control and Prevention (CDC) defines a case of MRSA infection as community-acquired (CA-) when it is diagnosed in outpatients or within 48 hours of hospitalization if the patient lacks the traditional risk factors for MRSA (56). The CDC defines hospital-acquired (HA-) MRSA infections as those in patients who have had frequent or recent contact with hospitals or healthcare facilities within the previous year or who have recently undergone an invasive medical procedure.

From a microbiological point of view, MRSA can be resistant either just to methicillin and other β-lactam antibiotics or also to other classes of antibiotics such as tetracyclines, trimethoprim/sulfamethoxazole, rifampin, clindamycin, quinolones and macrolides or even to multiple classes of antibiotics (156). Most HA-MRSA is resistant to several classes of antibiotics, whereas most CA-MRSA strains are susceptible to tetracyclines, trimethoprim/sulfamethoxazole and clindamycin (80; 40; 232). However, the level of resistance is increasing and geographically variable (Figure 4 [213]).

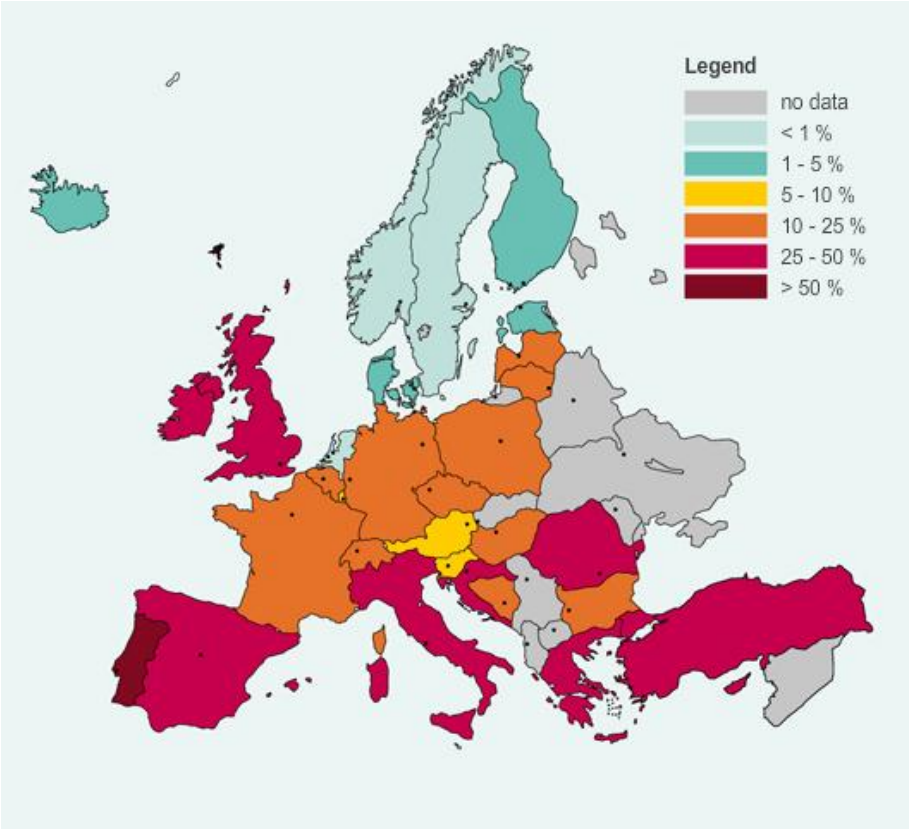


Figure 4. Prevalence of invasive isolates of MRSA in 2008 (European Centre for Disease Prevention and Control. Prevalence of MRSA in Europe 2008).

On a genetic level, the correct differentiation between CA-MRSA and HA-MRSA necessitates a specific molecular genetic approach that is available only in specialized laboratories. The molecular basis on which CA-MRSA and HA-MRSA are distinguished is the difference in the mobile genetic element SCC*mec* integrated into the MRSA chromosome. The resistance to penicillin and β-lactams is due to the production of altered penicillin-binding proteins (PBPs) with reduced affinity for methicillin and less toxic β-lactams. This genetic element carries the gene responsible for resistance to methicillin, the *mec*A gene, which encodes for a penicillin-binding protein (PBP-2) responsible for a decreased binding affinity for β-lactam antibiotics (Figure 5). SCC*mec* elements have two essential components: the *ccr* gene complex (*ccr*) and the *mec* gene complex (*mec*). Several *mec* and *ccr* allotypes have been found among SCC*mec* elements, which has led to the classification of SCC*mec* types I, II, III, IV and V (46). HA-MRSA strains belong to SCC*mec* types I, II and III, and SCC*mec* types IV and V to CA-MRSA. In SCC*mec* type I no antibiotic resistance determinants, except for those carried by *mec*A (β-lactams), are found. By contrast, types II and III contain multiple determinants for non-β-lactam antibiotic resistance and provide a molecular explanation for the multiple drug resistance often documented in MRSA isolates circulating in healthcare environments. The large size of SCC*mec* types I and II probably limits their horizontal transfer (120). The SCC*mec* types IV and V are smaller in

size than types I - III and, like SCC*mec* type I, lack resistance determinants other than *mec*A. SCC*mec* type IV has been found in most CA-MRSA isolates, which explains why CA-MRSA isolates are most often resistant only to β-lactam antibiotics.

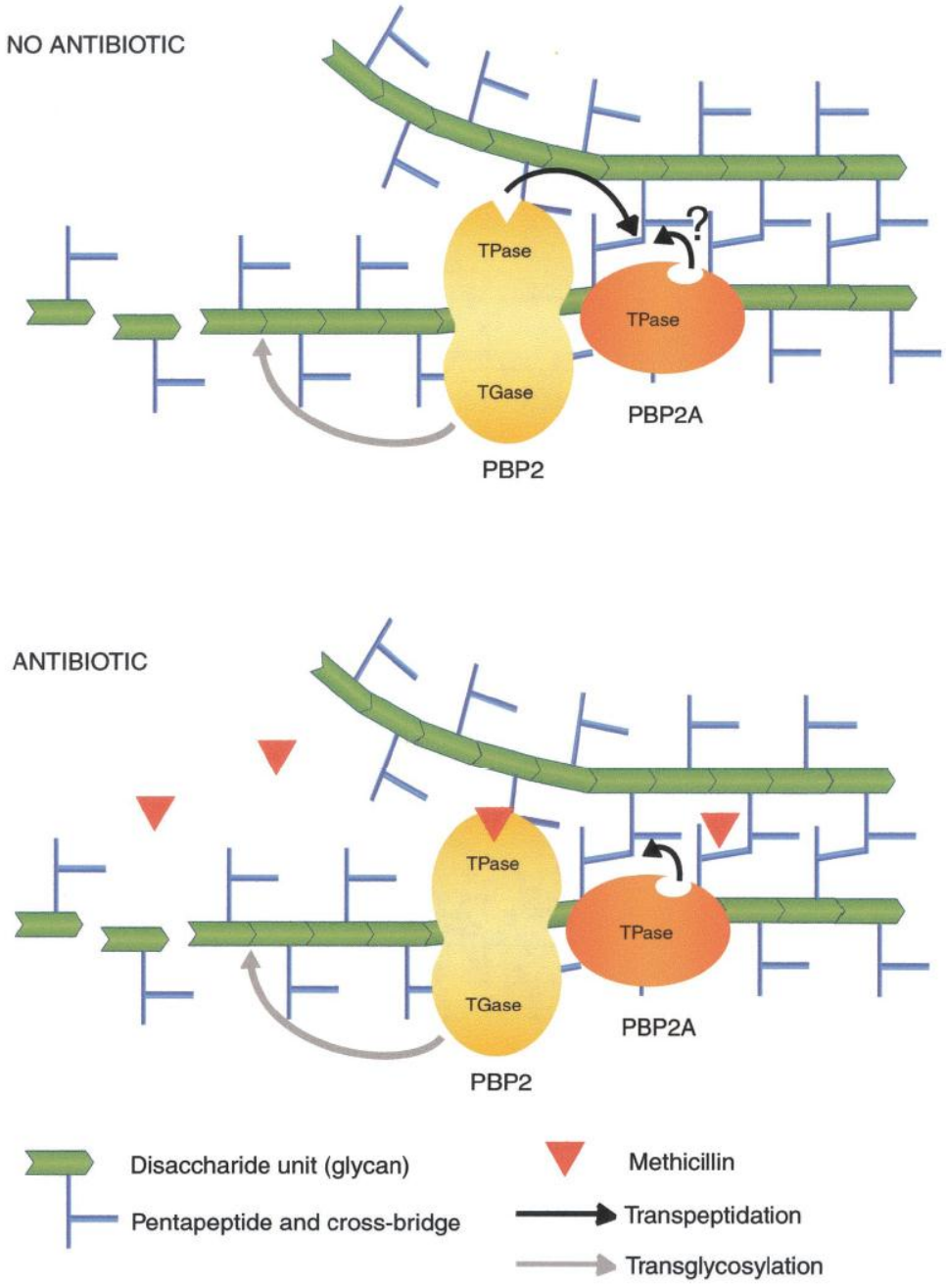


Figure 5. Model for the cooperative functioning of the TGase domain of PBP2 and the TPase activity of PBP2A in methicillin-resistant Staphylococcus aureus (210). (Upper) In the absence of antibiotic it is assumed that both the TPase and TGase domains of PBP2 participate in the biosynthesis of staphylococcal peptidoglycan. Whether or not the TPase activity of PBP2A (present in methicillin-resistant strains) also functions in the crosslinking of the peptidoglycan in the absence of antibiotic in the medium is not clear at the present time. (Lower) When antibiotic (▼) is added to the medium, the TPase domain of PBP2 is acylated and is no longer capable of performing its peptide crosslinking

activity. However, the observations described in this paper demonstrate that the penicillin-insensitive TGase domain of PBP2 remains functional and cooperates with the TPase activity of the acquired PBP2A and is actually essential for cell-wall synthesis and bacterial growth in the presence of β-lactam antibiotics in the surrounding medium.

Furthermore, CA-MRSA strains ususally carry the two genes encoding for the pore-forming toxin named Panton-Valentine leukocidin (PVL). These strains are responsible for tissue necrosis, are cytolytic to macrophages, monocytes and polymorphonuclear granulocytes and have been linked epidemiologically to cutaneous and more severe infections (285; 268).

1.3.2. Intracellular persistence

Gaining an intracellular niche, even briefly, might afford a window of opportunity for *S. aureus* to survive and promote disease.

1.3.2.1. Intracellular *Staphylococcus aureus*

Professional phagocytic cells such as macrophages are designed to engulf microorganisms and clear debris. Nearly all cells discussed in the scientific literature as non-professional phagocytic cells possess mechanisms that nevertheless permit endocytic uptake of microorganisms (24). A variety of human pathogens (e.g. *Listeria monocytogenes*, *Shigella flexneri* and *Legionella pneumophila*) are classified as intracellular pathogens (50). *S. aureus* has not been traditionally considered among these bona fide intracellular pathogens, although a substantial mass of evidence from cell culture studies now convincingly argues that this microorganism can invade and persist for varying lengths of time in many cell types in laboratory cell culture models.

The list of host cells used in *S. aureus* internalization and intracellular survival assays includes endothelial cells, epithelial cells, fibroblasts, osteoblasts and keratinocytes. Recent reports even document bacterial survival within professional phagocytes such as human monocyte-derived macrophages, and some studies have examined *S. aureus* survival in neutrophils (103; 147; 294).

The intracellular persistence is a bacterial strategy to subvert immunological defense mechanisms, as well as extracellular bactericidal concentrations of antibiotics. As a consequence of cellular invasion and survival, an intracellular niche might then serve as a reservoir for chronic or relapsing staphylococcal infections and/or contribute to chronic carriage.

1.3.2.2. Internalization

In cell culture models, host-cell invasion in non-professional phagocytic cells by *S. aureus* depends on the presence of fibronectin-binding proteins (FnBPs) on the bacterial surface, fibronectin (Fn) and host-cell integrins (257). Bacterial entry proceeds by an F-actin-dependent zipper-type mechanism that resembles professional phagocytosis. FnBPs contain multiple modules, each of which can bind to a stretch of type I (F1) repeats at the N terminus of Fn (165; 239). Presumably, FnBPs are thereby capable of associating with several Fn molecules at once (239). Furthermore, the FnBP binding site is located at some distance from the central integrin binding RGD motif in Fn. Therefore, Fn can form a bridge between FnBPs and integrins (256; 85). Apparently through these features, FnBPs are capable of inducing integrin clustering and signaling in cells. This leads to invasion of staphylococci via src tyrosine kinase activation and reorganization of the actin cytoskeleton (1; 84).

Physiological clustering of integrins by extracellular cues causes formation of adhesion structures that differ by morphology, molecular composition, and spatiotemporal dynamics. Focal adhesions are tight clusters of cytoskeletal and signaling proteins, including focal adhesion kinase (FAK), vinculin, paxillin, talin, and tensin. These proteins cooperatively organize the association of actin stress fibers with the cytoplasmic domains of integrins (307; 28; 59). It has been proposed that fibrillar adhesions develop by translocation of Fn-ligated α5β1 integrins out of focal adhesions. Fibrillar adhesions have an elongated or beaded morphology and contain tensin, but they are devoid of most other focal adhesion components. The current notion is that fibrillar adhesions stretch extracellular Fn molecules and thereby perpetuate Fn fibrillogenesis (199; 307; 54).

Together, these investigations support the view that Fn-coated staphylococci induce fibrillar adhesion-like contact sites, which are regulated by protein tyrosine kinase signaling. The recruitment of this protein complex to cell-attached microbes indirectly links the bacteria-occupied integrins with the intracellular actin cytoskeleton, and mediates the uptake of the pathogen (Figure 6).

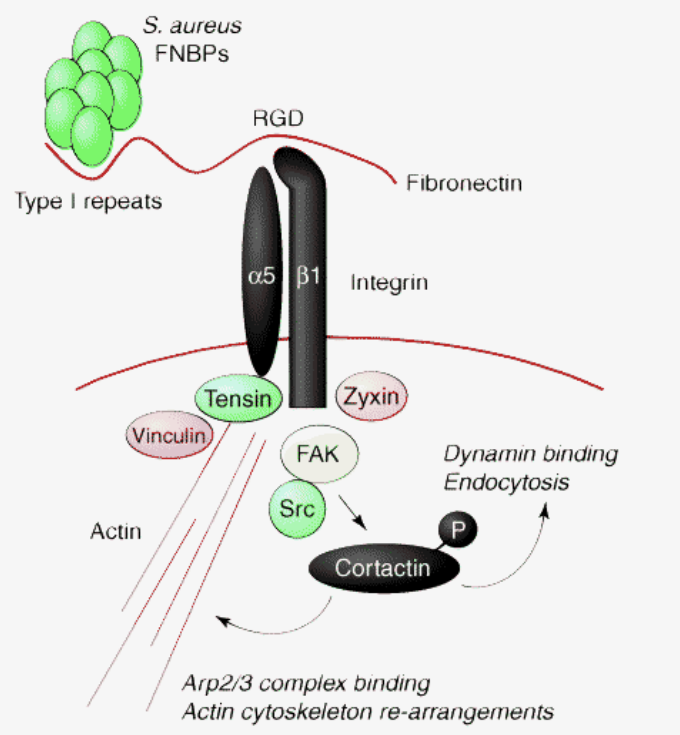


Figure 6. Schematic summary of some of the signaling events involved in invasion of non-professional phagocytic host cells by Staphylococcus aureus (114). *S. aureus* associates through FnBP with the type I repeats of host-derived Fn. Fn deposited on the pathogen surface is recognized by the cellular Fn receptor, integrin $\alpha_5\beta_1$, that binds to the RGD motif contained within this matrix protein. Bacteria-induced clustering of integrins leads to the local recruitment of structural proteins such as tensin, vinculin and zyxin, as well as signaling enzymes such as Src family PTKs and FAK, to the site of bacterial attachment. The combined activity of FAK and Src results in tyrosine phosphorylation (P) of multiple downstream effectors including cortactin. Cortactin is functionally involved in bacterial internalization most likely by its influence on cytoskeleton rearrangements by the Arp2/3 complex or the regulation of endocytosis by dynamin.

Studies with MRSA strains showed that internalization was also dependent on SCCmec and spa type, implying that in some circumstances, uptake is multifactorial (300).

1.3.2.3. Post-Internalization Adaptation

The environmental change encountered by invading *S. aureus* during passage from an extracellular to intracellular milieu probably requires altered gene expression to promote survival. Garzoni and colleagues provide a first glimpse of the complexities associated with intracellular adaptation of this organism and a basis of comparison with other intracellular organisms (89). In the first two hours post-internalization, extensive alteration in gene expression occurred. Considerable transcriptional shutdown was measured for genes involved in bacterial metabolic properties, nutrient transport and cell wall synthesis. By contrast the expression of numerous genes involved in virulence, neutralization of free radicals and iron acquisition increased. Six hours post-infection,

bacteria restarted transcription of genes involved in metabolic activity consistent with minimal bacterial replication.

Another important issue is the production of toxins by internalized bacteria. Available data indicate that toxin-forming ability does not necessarily lead to or correlate with predictable host-cell cytotoxicity or intracellular survival under all circumstances. For example, the accessory gene regulator *agr* has been shown to control the expression of a large number of staphylococcal toxins, including α-toxin. The mechanism of release of bacteria from the phagosomes into the cytoplasm of the host cell is thought in some *S. aureus*-host-cell models to coincide with the induction of *agr*. *S. aureus* mutants lacking *agr*, however, were reported to be unable to escape from the endocytic vesicle, evade lysosomal fusion events in fibroblasts (236) or induce cell death (112). Interestingly, when autophagy is induced in fibroblasts by external stimuli, intracellular *agr*-deficient *S. aureus* are converted to a fully cytotoxic state and bacteria replicate within the phagosomes compartment, then escape into the cytosol and eventually kill their host cells (236). The determination of subcellular localization in various models mentioned above is not standardized and it is prudent to examine most findings with caution.

1.3.2.4. Clearance or Persistence

Once inside cells, the fate of internalized *S. aureus* can vary from rapid clearance to persistence from several days-to-weeks (Figure 7). A survey of results from numerous *in vitro* models of intracellular *S. aureus* indicates several scenarios: (i) clearance by lysosomal degradation or innate immune effectors such as antimicrobial peptides, autophagy or apoptosis, (ii) persistence of viable bacteria within vacuoles or (iii) endosomal escape and persistence within the cytosol. There is no clear means at this time to predict confidently whether acute cellular cytotoxic effects (apoptosis or necrosis), or bacterial survival with minimal or absent cytotoxicity occurs with any given combination of host cell and *S. aureus* strain.

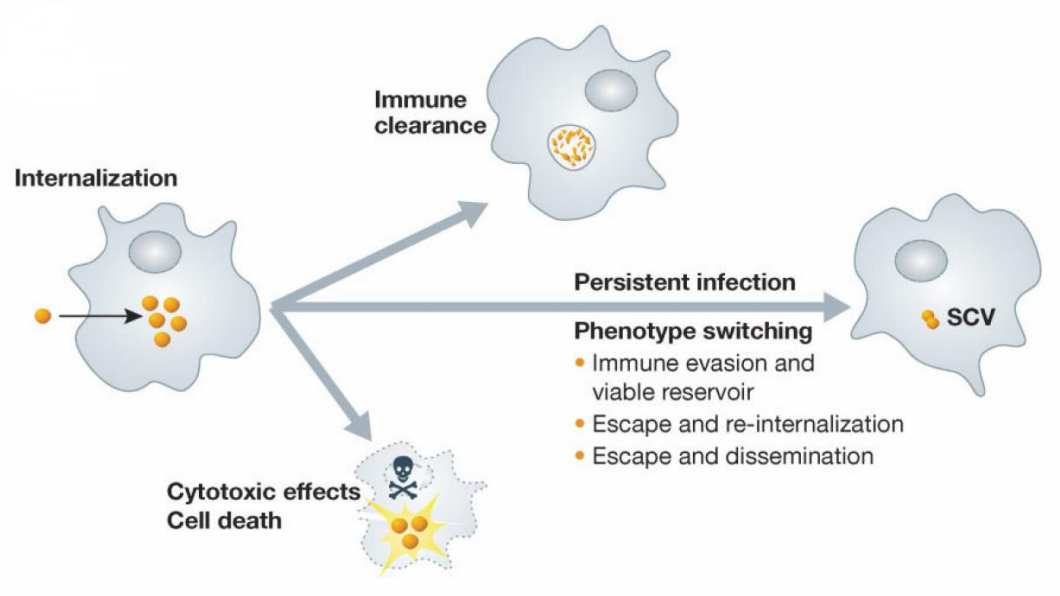


Figure 7. Possible outcomes after intracellular Staphylococcus aureus infection (89). After internalization into the host cell, not all bacteria undergo phenotype switching. *S. aureus* can be cleared, it can cause cell death or it can undergo a phenotype switch and cause a persistent infection. The phenotype switch to SCVs allows *S. aureus* to evade the immune response and leads to the formation of a viable reservoir of bacteria from which the bacteria can escape and disseminate or re-infect the host.

1.3.3. Small-Colony Variants

A related *S. aureus* survival strategy was elegantly illustrated: the induction of reversible phenotype switching that can occur following bacterial internalization and facilitates intracellular survival. Instead of classic persisters induced by antibiotic exposure, a type of metabolically dormant bacteria, termed Small-Colony Variants (SCVs), can arise during prolonged residence inside host cells (Table 4). Although some recovered SCVs harbor heritable auxotrophism for biosynthetic defects in electron transport chain co-factors (i.e. menadione or hemin) or thymidine biosynthesis, a large proportion of recovered SCVs are unstable and can revert to the wild-type phenotype. The capacity to interconvert by this mode of phenotype switching permits *S. aureus* to alter its virulence potential rapidly. Importantly, intracellular survival and phenotypic switching defines a powerful immune escape mechanism and an alternative route to explain chronic infection.

Table 4. Typical features of *Staphylococcus aureus* SCVs (adapted from 291)

Characteristics	Normal phenotype	SCV phenotype
Colony morphology	Yellow-orange colonies with a size of ca. 4-8 mm in diameter	Gray-white, pinpoint colonies (ca. 1 mm)
Growth rate on solid media	12-18 hours	Delayed (48-72 hours incubation)
Auxotrophism	None	Thymidine, Menadione and/or Hemin
Toxins activity (α- and β-hemolysins)	Normal	Reduced
Coagulase activity	Normal	3-4 fold delayed
Biochemical reactions	Normal	Delayed and/or changed
Resistance to antibiotics	Varying	Reduced susceptibility to gentamicin and TMP-SMX

SCVs are broadly defined as slow growing colonies with diameters roughly 1/10 the parental strain when cultivated on agar plates (Figure 8). In *S. aureus*, SCVs have been shown to have enhanced surface expression of adhesins, particularly fibronectin binding proteins (FnBPs) which aid internalization and decreased expression of exotoxins and thus diminishes cytotoxicity (243). Their decreased susceptibility to antibiotics and the absence of routine testing in clinical samples to detect their presence also provide a definite advantage. These characteristics of SCVs, coupled with their capacity to revert to more rapidly growing forms, renders them ideal candidates to provoke persistent human infections.

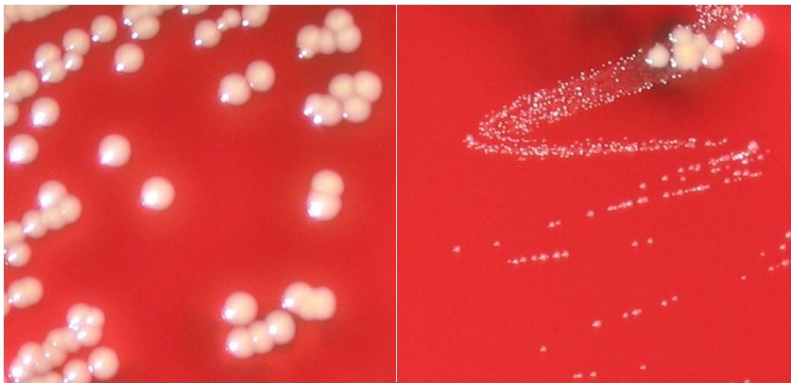


Figure 8. Morphological appearance of Staphylococcus aureus colonies on Columbia blood agar after incubation for 24 h at 37°C. Shown are normal phenotype and SCV and spontaneous revertant strains. Differences in colony size, shape, and hemolytic activity are evident (186).

1.3.3.1. Hemin- and Menadione-Dependent Mutants

Electron transport defective SCVs are characterized by being hemin- or menadione-auxotrophs (Figure 9). Hemin and menadione are essential in the formation of cytochromes and menaquinone,

respectively. Disruption of the electron transport chain causes reduced production of ATP, important for many metabolic processes in bacteria including cell wall biosynthesis, amino acid transport and protein synthesis. Loss of electron transport also reduces the transmembrane potential, which is required for the effect of many cationic antimicrobial compounds like aminoglycosides, lantibiotics and antimicrobial peptides, resulting in decreased susceptibility to these agents (170; 144; 231; 11; 12).

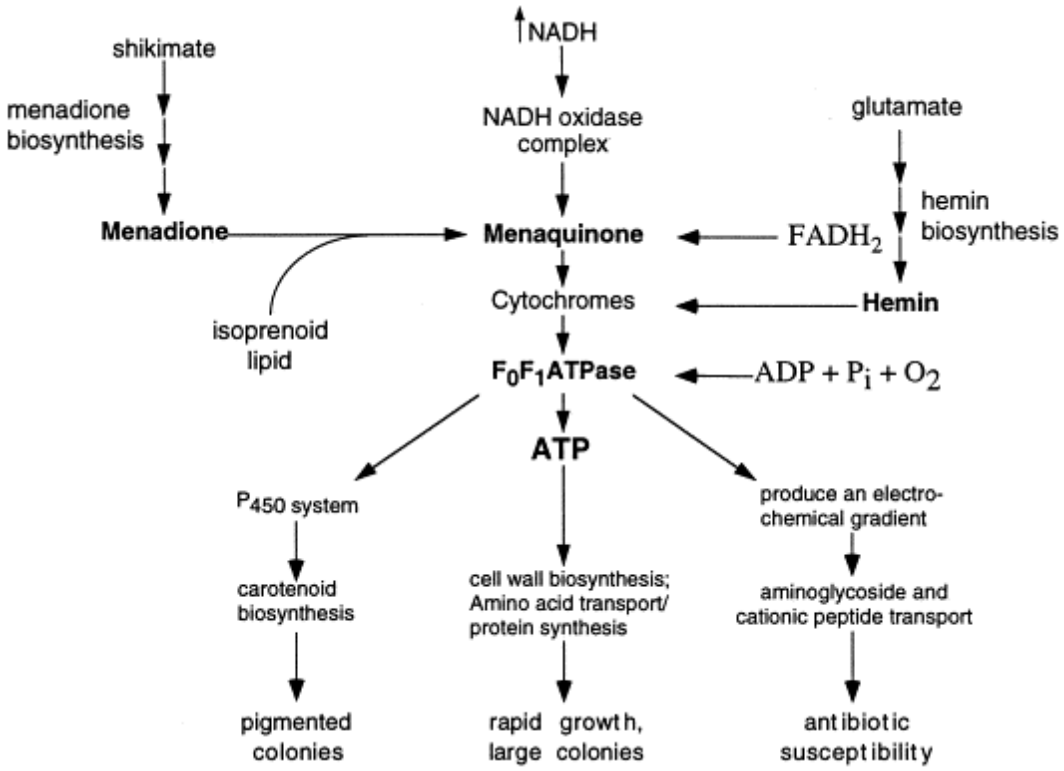


Figure 9. Relationship between electron transport and menadione- or hemin-dependent SCV phenotype in Staphylococcus aureus (170).

1.3.3.2. Thymidine-Dependent Mutants

It has been recently shown that random mutations in the thymidylate synthase - encoding *thyA* gene which lead to an intracellular lack of dTMP - are responsible for the formation of the thymidine-dependent SCV phenotype (17; 42; 308) and that hypermutability due to a defect DNA mismatch repair system favors the acquisition of these mutations (18). As thymidine-dependent SCVs are apparently able to use thymidine and/or the metabolite dTMP from the environment, these variants are able to bypass the antibiotic effect of folic acid antagonists and consequently are resistant to trimethoprim/sulfamethoxazole (96; 214; 308). A schematic model of metabolic pathways associated with the thymidine- dependent SCV phenotype is shown in Figure 10.

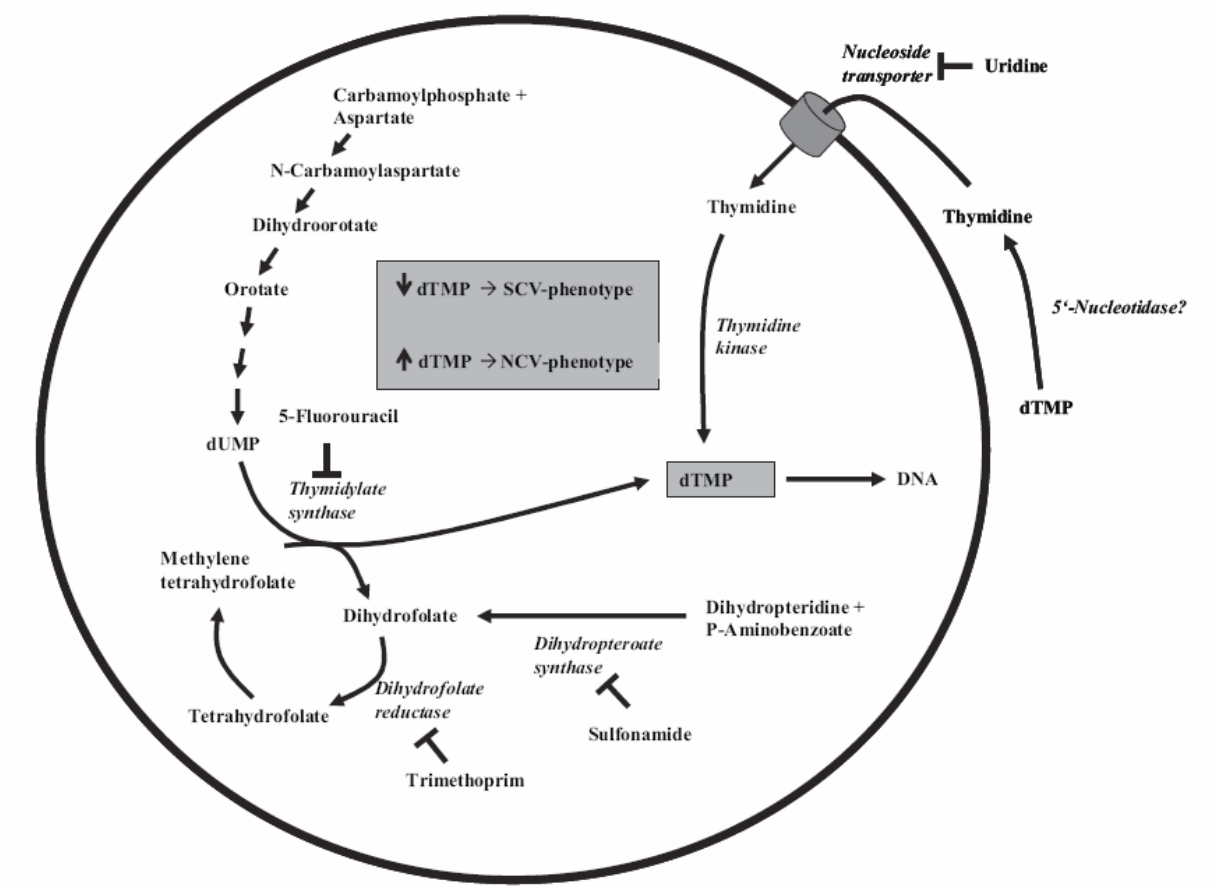


Figure 10. Metabolic pathways associated with thymidine-dependent S. aureus SCVs (308). Intracellular dTMP can be generated either by thymidylate synthase, leading to a larger amount of intracellular dTMP and consequently to a normal phenotype, or by thymidine kinase, which is dependent on nucleoside transporter integrity and an extracellular supply of thymidine.

1.4. Staphylococcal Diseases Associated with Small-Colony Variants

That there is a connection between SCVs and persistent, recurrent infections has only been appreciated since the mid-1990s (241; 290; 127; 216; 261). While SCVs dependent on hemin or menadione can be isolated from patients with osteomyelitis, persistent soft tissue infection, and device-related infections (216; 260; 289; 293), thymidine-dependent SCVs are well-known in the context of cystic fibrosis lung disease, especially after use of trimethoprim/sulfamethoxazole (17; 96; 127).

1.4.1. Device-Related Infections

Staphylococci are the most common cause of infections associated with medical devices. Cases of persistent pacemaker-related bloodstream and ventriculo-peritoneal-shunt infections caused by

S. aureus SCVs have been reported (293; 242; 260). Device-related infections with SCVs respond poorly to antibiotic therapy and can be misidentified by automated diagnostic systems (293; 260). The reported cases also illustrate that complete removal of any foreign-body material is essential for the complete cure of prosthetic intra vascular-device-related infections caused by staphylococcal SCVs, and this idea is consistent with previous *in vitro* models in which *S. aureus* SCVs were found to be almost completely resistant to antibiotics (48).

1.4.2. Osteomyelitis

S. aureus is the major etiological agent of human osteomyelitis (71). Several investigators have shown that SCVs can often be recovered from cultures of normal *S. aureus* strains that have been exposed to gentamicin or other aminoglycosides (149; 216; 8; 12; 176; 144; 217; 41; 183; 304). Beads containing gentamicin are used as an adjunct to systemic antibiotic therapy and debridement to treat patients with osteomyelitis. The beads release the aminoglycoside slowly (over weeks to months), providing a sustained local concentration of the antimicrobial agent. Although the patient milieu is clearly complex, the results of a case-control study provide evidence that gentamicin-containing bead placement might be an efficient way to select *in vivo* for *S. aureus* SCVs (290).

1.4.3. Pulmonary Infections Associated with Cystic Fibrosis

Patients with cystic fibrosis (CF) are highly susceptible to *S. aureus* lung infections. In these patients, the pathogen evokes an intense host immune response in the lungs which is characterized above all by polymorphonuclear leukocytes (230). *S. aureus* multiplies and persists in the airways of CF patients for months or even years despite appropriate anti-staphylococcal therapy (29; 127, 126). During chronic infection, pathogens will experience changing selection pressures as they encounter new habitats and different co-infecting species and as they respond to medical intervention. In the short term, regulatory mechanisms allow the pathogen to quickly change its phenotype in response to the microenvironment. In the long term, mutation or recombination together with purging selection enforced by the changing environment leads to inheritable shifts in the bacterial population. Adaptive strategies of *S. aureus* are illustrated by the formation of biofilms, the switch to Small-Colony Variants, the occurrence of hypermutable strains, the down-regulation of virulence genes and the manifestation of a heterogeneous bacterial population. Some of these strategies lead to a resistant phenotype of *S. aureus*, making appropriate antibiotic therapy a challenge. Additionally, classical antibiotic-resistant bacteria (i.e. MRSA) may be selected due to the high antibiotics consumption in these patients.

2. Intracellular infection models

In the present work, we attempted to get a better understanding of the intracellular fate of *à* and its Small-Colony Variants forms in professional phagocytic cells.

Monocytes and macrophages constitute one of the first lines of defense of the innate immune response against infectious agents. These cells are dedicated to the elimination of infectious microbes by phagocytosis. Newly formed phagosomes containing bacteria will mature along the endocytic pathway, which forms highly degradative phagolysosomes through fusion with lysosomes (76).

2.1. THP-1 Monocytes

The THP-1 human myelomonocytic cell line displaying macrophage-like activity was originally isolated from the blood of a one-year-old boy with acute monoctytic leukemia (278). THP-1 monocytes display poor intrinsic defenses against intracellular infection (36). This feature not only permitted the intracellular multiplication of bacteria but also offered valuable information concerning the specific intra- and extracellular capacities of the drug by excluding other parameters that could affect the antimicrobial activity. In addition, THP-1 monocytes could be differentiated into active macrophages with increased bactericidal activity by incubation with phorbol-12-myristate-13-acetate (PMA [283]).

2.2. THP-1 Macrophages

Macrophage heterogeneity is influenced by differentiation state, with marked differences between monocytes and macrophages (99; 92). PMA treatment, which activates protein kinase C (PKC), enhances a greater degree of differentiation in THP-1 cells as reflected by increased adherence and expression of surface markers associated with macrophage differentiation (240). It is also associated with an increased number of mitochondria and lysosomes, resistance to apoptosis stimuli and the potency of Toll-Like Receptor 2 (TLR2) responses as important discriminators of the level of macrophage differentiation for transformed cells (52).

2.3. Oxidative Burst against Intracellular Pathogens

2.3.1. Reactive Oxygen Species

During the process of infection, *S. aureus* is engulfed by phagocytic cells and is exposed to the microbicidal activity of these cells. One of the most powerful antimicrobial mechanisms is based on the production of reactive oxygen species (ROS) by phagocyte NADPH oxidase (107). ROS are also generated during incomplete electron transfer in the bacterial respiratory chain (119).

ROS include the superoxide anion ($O2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$) and the hydrogen peroxide (H_2O_2) (38). All these free radicals can interact with numerous targets, including lipids, DNA, and proteins leading to the destruction of the internalized microorganisms (306).

2.3.2. Impact on Antibiotic Action

A novel oxidative damage cell death pathway, which involves ROS generation and a breakdown in iron regulatory dynamics following norfloxacin-induced DNA damage induction, was uncovered. More specifically, norfloxacin treatment was found to promote superoxide generation soon after gyrase poisoning, and was ultimately shown to result in the generation of highly-destructive hydroxyl radicals through the Fenton reaction (119). Under these conditions, the Fenton reaction was found to be fueled by superoxide-mediated destabilization of iron-sulfur cluster catalytic sites, repair of these damaged iron-sulfur clusters, and related changes in iron-related gene expression (69).

It has been shown that all major classes of bactericidal antibiotics (β-lactams, aminoglycosides, quinolones), despite the stark differences in their primary drug-target interactions, promote the generation of lethal hydroxyl radicals in both Gram-negative and Gram-positive bacteria (142). Stress response network analysis methods employed in this study suggested that antibiotic-induced hydroxyl radical formation is the end product of a common mechanism, wherein alterations in central metabolism related to NADH consumption (increased tricarboxylic acid [TCA] cycle and respiratory activity) are critical to superoxide-mediated iron-sulfur cluster destabilization and stimulation of the Fenton reaction. These predictions were validated by the results of additional phenotypic experiments, biochemical assays and gene expression measurements, confirming that lethal levels of bactericidal antibacterials trigger a common oxidative damage cellular death pathway, which contributes to killing by these drugs (Figure 11).

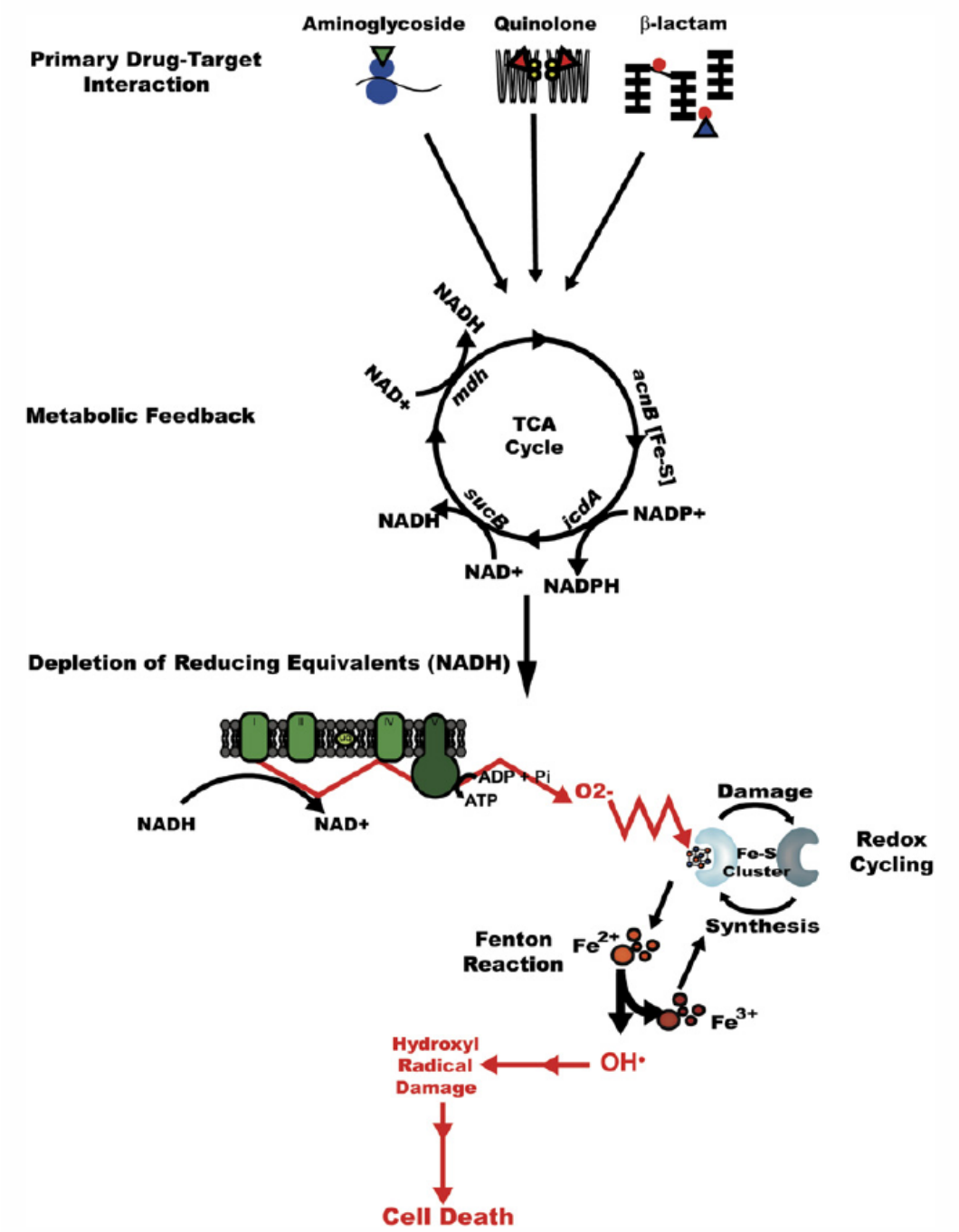


Figure 11. Proposed model for common mechanism of killing by bactericidal antibiotic (141). The primary drug-target interactions (aminoglycoside with the ribosome, quinolone with DNA gyrase, and β-lactam with penicillin-binding proteins) stimulate oxidation of NADH via the electron transport chain that is dependent upon the TCA cycle. Hyper-activation of the electron transport chain stimulates superoxide formation. Superoxide damages iron-sulfur clusters, making ferrous iron available for oxidation by the Fenton reaction. The Fenton reaction leads to hydroxyl radical formation, and the hydroxyl radicals damage DNA, proteins, and lipids, which results in cell death.

Most recently, studies of antibiotic-induced stress response networks have been conducted to determine exactly how the primary effect of a given bactericidal drug can trigger cell death via a mechanism common to all bactericidal drugs. For example, a comparative analysis of stress response

networks, reconstructed using gene expression data from aminocyclitol-treated (spectinomycin, gentamicin and kanamycin) *E. coli*, was used to identify the incorporation of mistranslated proteins into the cell membrane as the trigger for aminoglycoside-induced oxidative stress (143). Interestingly, mistranslated membrane proteins were shown to stimulate radical formation by activating the envelope stress (Cpx) and redox-responsive (Arc) two-component systems, ultimately altering TCA cycle metabolism; the TCA cycle had previously been implicated in aminoglycoside susceptibility (142; 238).

The discovery of the common oxidative damage cellular death pathway has important implications for the development of more effective antibacterial therapies. Specifically, it indicates that all major classes of bactericidal drugs can be potentiated by inhibition of the DNA stress response network, which plays a key part in the remediation of hydroxyl radical-induced DNA damage.

AIMS

Staphylococcus aureus, especially Methicillin-Resistant *S. aureus* (MRSA), is a major human pathogen causing a wide spectrum of nosocomial and community-associated infections with high morbidity and mortality. The presence of a bacterial subpopulation constituted of Small-Colony Variants (SCVs) is believed to account in part for the increased persistence of the pathogen during chronic and antibiotic-refractory infections. SCVs have fastidious growth requirements and therefore present a challenge to clinical microbiologists. Moreover, their reduced production of virulence factors allows their persistence within the intracellular compartment, which protects them from both the host defense system and antimicrobial agents.

The aim of this project is to characterize the ability of electron transport defective SCVs to invade and persist within phagocytic cells and to examine the activity of antibiotics against these intracellular forms following a pharmacodynamic approach that allows the establishment of antibiotic relative potency and maximal efficacy.

To this effect, we used the well-characterized COL MRSA and its genetically stable menadione- and hemin-dependent mutants, for which very few data are currently available. Three main areas were investigated:

SCVs & Monocytes: Intracellular Infections by Itself?

In the context of a pharmacological study, a first and important step was to assess antibiotic activity without interference by cell defenses. We selected for this purpose the THP-1 monocytic cell line.

SCVs & Macrophage: Influence of the Cell Defense Mechanisms?

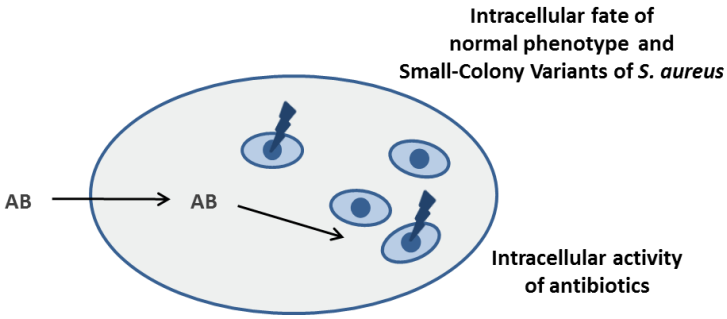
As reactive oxidant species (ROS) may be critical in antibiotic-dependent bacterial killing, we also used THP-1 macrophages differentiated from monocytes by the phorbol ester, phorbol-12-myristate-13-acetate (PMA).

SCVs & β-lactams: Intracellular Restoration of the Susceptibility?

It has been previously demonstrated in our laboratory that phagocytized MRSA are susceptible to β-Lactams because the acid pH of phagolysosomes induces a conformational change of PBP2a allowing its acylation by these antibiotics. We have finally investigated here whether this mechanism also applies to SCV-MRSA strains. Moreover, we have examined the influence of host cell defenses on this restoration of susceptibility.

RESULTS

1. SCVs & Antibiotics in Monocytes



THP-1 monocytic cell line has been previously used in our laboratory to assess quantitatively the intracellular pharmacodynamics of antibiotics. This model displays poor intrinsic defenses and therefore offers the possibility of examining specifically the intra- and extracellular activities of a drug, excluding other parameters that could affect the antimicrobial activity.

In this study, we have thus used this model to compare the internalization and the intracellular growth of genetically stable menadione- and hemin-dependent SCVs of the COL parental strain. We have then studied the pharmacodynamic profile of different antibiotics against extracellular and intracellular bacteria. Finally, we have investigated the influence of medium supplementation with menadione or hemin on both bacterial growth and antibiotic activity.

AUTHOR'S CONTRIBUTION:

1. Conceived and designed experiments: LGG, SL, PMT, FVB;
2. Performed experiments: LGG;
3. Analyzed data: LGG, SL, BCK, KB, RAP, PMT, FVB;
4. Contributed to reagents, strains and financial support: BCK, KB, RAP, OD, FVB;
5. Wrote the paper: LGG, SL, PMT, FVB.

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Pharmacodynamic Evaluation of the Activity of Antibiotics against Hemin- and Menadione-Dependent Small-Colony Variants of *Staphylococcus aureus* in Models of Extracellular (Broth) and Intracellular (THP-1 Monocytes) Infections

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Staphylococcus aureus **small-colony variants (SCVs) persist intracellularly, which may contribute to persistence/recurrence of infections and antibiotic failure. We have studied the intracellular fate of *menD* and *hemB* mutants (corresponding to menadione- and hemin-dependent SCVs, respectively) of the COL methicillin-resistant *S. aureus* (MRSA) strain and the antibiotic pharmacodynamic profile against extracellular (broth) and intracellular (human THP-1 monocytes) bacteria. Compared to the parental strain, SCVs showed slower extracellular growth (restored upon medium supplementation with menadione or hemin), reduced phagocytosis, and, for the *menD* SCV, lower intracellular counts at 24 h postinfection. Against extracellular bacteria, daptomycin, gentamicin, rifampin, moxifloxacin, and oritavancin showed similar profiles of activity against all strains, with a static effect obtained at concentrations close to their MICs and complete eradication as maximal effect. In contrast, vancomycin was not bactericidal against SCVs. Against intracellular bacteria, concentration-effect curves fitted sigmoidal regressions for vancomycin, daptomycin, gentamicin, and rifampin (with maximal effects lower than a 2-log decrease in CFU) but biphasic regressions (with a maximal effect greater than a 3-log decrease in CFU) for moxifloxacin and oritavancin, suggesting a dual mode of action against intracellular bacteria. For all antibiotics, these curves were indistinguishable between the strains investigated, except for the *menD* mutant, which systematically showed a lower amplitude of the concentration-effect response, with markedly reduced minimal efficacy (due to slower growth) but no change in maximal efficacy. The data therefore show that the maximal efficacies of antibiotics are similar against normal-phenotype and menadione- and hemin-dependent strains despite their different intracellular fates, with oritavancin, and to some extent moxifloxacin, being the most effective.**

Small-colony variants (SCVs) are a naturally occurring subpopulation of bacteria with distinctive phenotypic features, among which the most characteristic is the formation of colonies having about 1/10 of the normal size. They have been described in many bacterial species and recovered in clinical samples from patients presenting persistent or recurrent infections (42). In *Staphylococcus aureus*, SCVs are mostly nonpigmented and nonhemolytic; their slow growth is due to auxotrophism for distinct growth factors such as menadione, hemin, and/or thymidine. Menadione- and hemin-dependent strains are defective in electron transport (35). Mutations in the menadione biosynthetic enzymes cause depletion in menaquinone, which normally forms a complex with cytochromes involved in electron transport, while failure to produce hemin blocks the synthesis of these cytochromes. Thymidine-dependent strains are deficient for dTMP synthesis and therefore unable to synthesize DNA (42). These SCVs often escape detection in routine laboratory testing because their auxotrophic character requires specific nutritional supplementation or prolonged culture (64). However, in epidemiological surveys looking specifically for their presence, SCVs of *S. aureus* are easily detected in a number of situations of chronic and relapsing infections. For instance, SCVs are observed in the sputum of 70% of patients suffering from cystic fibrosis (22), with about two-thirds of these strains harboring a thymidine-dependent phenotype (related to the chronic administration of trimethoprim-sulfamethoxazole in these patients) and one-third showing dependence on menadione or hemin or even double auxotrophism (12, 18, 21). Hemin- or menadione-dependent mutants are most frequent in osteomyelitis or device-associated infections, especially in patients treated with aminoglycosides (41, 42).

Small-colony variants show an enhanced ability to invade and persist in both phagocytic and nonphagocytic cells (2, 50, 56, 63, 65), which protects them from the immune system, makes them less accessible to antibiotics, and contributes to their survival. Therefore, the treatment of these intracellular forms requires the use of antibiotics not only displaying activity against SCVs but also presenting cellular pharmacokinetic and pharmacodynamic properties that enable them to act intracellularly (59).

In a previous study, we compared the intracellular activities of a series of antibiotics against a thymidine-dependent SCV isolated from a cystic fibrosis patient and its isogenic normal-phenotype

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counterpart (38). The present study focuses on hemin- and menadione-dependent SCVs, for which data are still lacking. We used in parallel genetically stable hemin- and menadione-dependent mutants of the well-characterized COL methicillin-resistant *S. aureus* (MRSA) strain (9, 66). This allowed us to compare the intracellular fates of these organisms, their extracellular and intracellular susceptibilities to antibiotics, and the influences of supplementation on these parameters. The studies were performed in a model of THP-1-infected monocytes that had been specifically developed for a quantitative assessment of the intracellular pharmacodynamics of antibiotics (7). These cells are indeed considered permissive to many intracellular bacteria (55), such as *S. aureus* (7), *Listeria monocytogenes* (48), *Legionella pneumophila* (53), *Chlamydia* spp. (68), *Coxiella burnetii* (17), *Brucella* spp. (26), *Francisella tularensis* (69), *Yersinia pestis* (67), and *Mycobacterium tuberculosis* (52). This cellular model therefore allows analysis of the true effect of antibiotics (in a pharmacological context) with minimal interference from cell defense mechanisms.

MATERIALS AND METHODS

Antibiotics and main reagents. The following antibiotics were obtained as microbiological standards from their respective manufacturers: daptomycin from Novartis Pharma AG (Basel, Switzerland), moxifloxacin from Bayer HealthCare (Leverkusen, Germany), and oritavancin from The Medicines Company (Parsippany, NJ). The other antibiotics were obtained as the branded products commercialized in Belgium for human use (gentamicin and vancomycin as Geomycin and Vancocin [distributed in Belgium by GlaxoSmithKline s.a./n.v., Genval, Belgium] and rifampin as Rifadine [Merrell Dow Pharmaceuticals Inc., Strasbourg, France]). Human serum was obtained from healthy volunteers (Lonza, Basel, Switzerland) and stored at −80°C as pooled samples until use. Cell culture media and sera were from Invitrogen Corp. (Carlsbad, CA), and other reagents were from Sigma-Aldrich (St. Louis, MO) or Merck KGaA (Darmstadt, Germany).

Bacterial strains, susceptibility testing, and 24-h dose-response studies in broth. We used four isogenic strains throughout this study, namely, *S. aureus* strain COL (wild-type [WT] hospital-acquired [HA] MRSA strain [14, 44]), its *menD* and *hemB* SCV mutants, and the *hemB* genetically complemented strain. The *hemB* and *menD* mutants were constructed by allelic replacement with an *ermB* cassette-inactivated *hemB* gene and an *ermC* cassette-inactivated *menD* gene, respectively (9, 66). MICs were determined by microdilution according to CLSI recommendations (13), and 24-h concentration-response studies in the acellular medium (broth) were performed in Mueller-Hinton broth as described previously (7). For experiments with oritavancin, 0.002% polysorbate (Tween) 80 was added to the medium according to CLSI recommendations to prevent drug binding to plastic surfaces (1). For studies with daptomycin, media were supplemented with 50 mg $CaCl_2$/liter (16). Readings or colony counts were made after 24 or 48 h. The maintenance of the SCV character of the strains was checked at the end of all experiments by observation of the size of the colonies growing on agar plates. No reversion was observed.

Cells, cell infection, and morphological studies. All experiments were conducted with human THP-1 cells (ATCC TIB-202 [American Tissue Culture Collection, Manassas, VA]), a myelomonocytic cell line that behaves like monocytes, showing moderate phagocytic activity and low cell defense mechanisms (47, 55). These cells were maintained in our laboratory as described previously (7). Cell infection, assessment of antibiotic activities, and morphological studies were performed exactly as described previously (7, 38), except that the concentration of gentamicin added to the culture medium of the controls (to prevent extracellular growth and the subsequent acidification of the medium) was reduced to 0.25× MIC to minimize its influence on *S. aureus* intracellular growth while still effectively preventing extracellular contamination (verified to be <5% of the total number of bacteria in samples under these conditions). Antibiotic activity was assessed by CFU counting (the large dilution of samples before spreading on agar plates for CFU counting ensured the absence of carryover effect [39]). The SCV character of the strains was maintained based on the observation of the size of the colonies. Electron microscopy was performed on samples prepared as previously described, with infection carried out at a bacterium/macrophage ratio of approximately 20 to allow visualization of a sufficiently large number of intracellular bacteria (7).

Curve fitting and statistical analyses. Concentration-response studies were performed against both extracellular and intracellular bacteria, as described previously (7). Curve-fitting analyses were made using GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA). Data were used to fit monophasic or biphasic sigmoidal functions (Hill equations with slope factors set to 1). The monophasic equation is

$$Y = E_{max} + \left[\left(E_{min} - E_{max}\right)/1 + 10^{\log_{10} EC_{50} - X}\right] \quad (1)$$

and the biphasic equation is

$$Y = E_{max} + \left(E_{min} \times \left\{\text{fract} + \left[1 + 10^{\log_{10} EC_{50\_1} - X}\right]\right\} + E_{min} \times \left\{\text{fract} + \left[1 + 10^{\log_{10} EC_{50\_2} - X}\right]\right\}\right) \quad (2)$$

where (i) Y is the $\log_{10}$ of the change in CFU and X is the $\log_{10}$ of the antibiotic concentration (in multiples of the MIC in broth) in both functions, (ii) EC_{50} is the concentration at which the change in $\log_{10}$ CFU is halfway between E_{min} and E_{max} function in the monophasic function, and (iii) EC_{50_1} and EC_{50_2} are the concentrations at which the change in $\log_{10}$ CFU is halfway between E_{min} and the first plateau and between this first plateau and E_{max}, respectively, and fract is the fraction of the total response associated with the first phase in the biphasic function. This allowed us to obtain, for each condition, numeric values of (i) relative minimal efficacy (E_{min}; CFU increase in $\log_{10}$ units at 24 h compared to the original inoculum, as extrapolated for an infinitely low concentration of antibiotic), (ii) relative maximal efficacy (E_{max}; CFU decrease in $\log_{10}$ units at 24 h compared to the original inoculum, as extrapolated for an infinitely large antibiotic concentrations), and (iii) drug static concentration (C_s, concentration of antibiotic resulting in no apparent bacterial growth compared to the original inoculum as determined by graphical interpolation and, in the case of a biphasic response, the proportion of the total response that could be ascribed to the first and the second wave of CFU decrease). Statistical analyses were performed with GraphPad Instat version 3.06 (GraphPad Software).

RESULTS

Extracellular growth and effect of menadione or hemin supplementation. In the first series of experiments, we compared the growth rate of SCVs with that of the parental isogenic strain in broth, by following over time the optical density at 620 nm (OD_{620}) of the bacterial suspension (Fig. 1). Compared to the wild-type isogenic strain, both *menD* and *hemB* mutants were characterized by a reduced apparent extracellular growth, reaching only 20 to 30% of the optical density of the parental strain at 24 h. Bacterial counts were recorded in parallel and confirmed this slower growth (CFUs for SCVs were ~60% of the value measured for the wild-type strain). The genetically complemented *hemB* strain showed a growth similar to that of the parental strain.

Addition of hemin (0.5 to 4 mg/liter; data are shown for 2 mg/liter) fully restored the growth of the *hemB* mutant without affecting that of the parental strain. In contrast, the addition of 2 mg/liter menadione sodium bisulfite (MSB) (or menadione; data not shown) only partially increased the growth rate of the *menD* mutant without effect on the parental strain. Higher concentrations of MSB were tested but appeared toxic, as they caused a

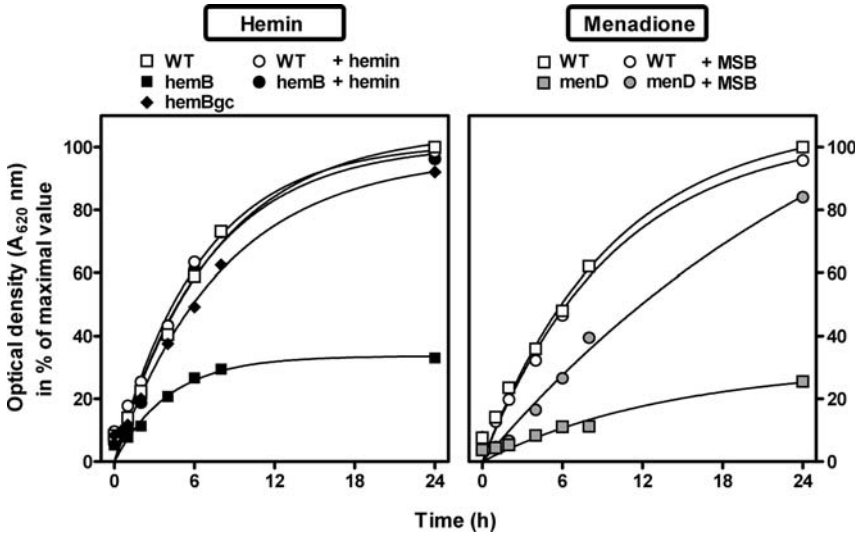

FIG 1 Extracellular growth (evaluated by optical density at 620 nm) of the strains under study under control conditions or in cation-adjusted Mueller-Hinton broth supplemented with hemin (2 mg/liter; left panels) or menadione sodium bisulfite (MSB; 2 mg/liter; right panels). WT, wild-type, normal-phenotype parental COL strain; hemB, *hemB* mutant; menD, *menD* mutant; hemBgc, genetically complemented *hemB* mutant; menDs, *menD* mutant in the presence of MSB.

concentration-dependent decrease in the culture OD_{620} (data not shown).

Phagocytosis and intracellular growth. In a subsequent step, we compared the capacities of the parental strain, its two SCV derivatives, and the genetically complemented *hemB* strain to infect THP-1 monocytes (Fig. 2). Considering phagocytosis first (left panel), both SCV strains were less efficiently internalized by monocytes than were the normal-phenotype or *hemB*-complemented strains, which showed similar rates of uptake. To assess the intracellular growth (right panel), viable counts were measured after 24 h of incubation (with data obtained in broth presented in parallel for comparison). The growth rates of all strains were much lower intracellularly than in broth. Thus, the parental strain, the *hemB* mutant, and the genetically complemented strain gained 1 to 1.5 log CFU versus 2.3 to 3.1 log CFU in broth. Interestingly, the *menD* mutant did not show significant intracellular growth, with only a 0.2-log increase in CFU over the 24-h period. Yet, MSB supplementation brought the intracellular counts at 24 h of the *menD* mutant back to the value observed for the parental strain, while hemin supplementation had no effect on the *hemB* mutant.

Intracellular localization of bacteria. Electron microscopy showed that all strains were present in membrane-delineated vacuoles, some of which contained several bacteria (Fig. 3).

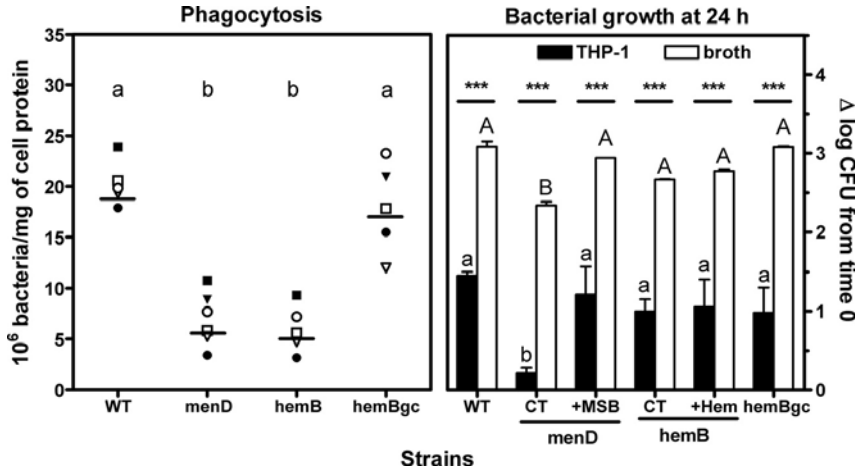

FIG 2 Comparative phagocytosis and intracellular versus extracellular survival of SCVs (*menD* and *hemB*) in THP-1 monocytes, compared to the isogenic *S. aureus* normal-phenotype strain (WT) and the *hemB* genetically complemented strain (hemBgc). (Left) Enumeration of cell-associated CFU after 1 h of phagocytosis by human monocytes. Each data set corresponds to the actual counts from 6 independent experiments performed in triplicate. The horizontal line indicates the corresponding mean value. (Right) Comparative extracellular (Mueller-Hinton broth, pH 7.4) and intraphagocytic (THP-1 monocytes) growth of strains after 24 h of incubation. For SCVs, media were supplemented with 2 mg/liter of MSB or hemin for comparison with control conditions (CT). Values are expressed as the change in CFU (in log scale) per ml of medium (broth) or per mg of cell protein compared to the initial inoculum. Results are means ± standard deviations of three independent determinations. For statistical analysis (by analysis of variance, with Tukey *post hoc* test), data with different letters indicate significant differences between strains; asterisks indicate significant differences between extracellular and intracellular values ($P < 0.05$).

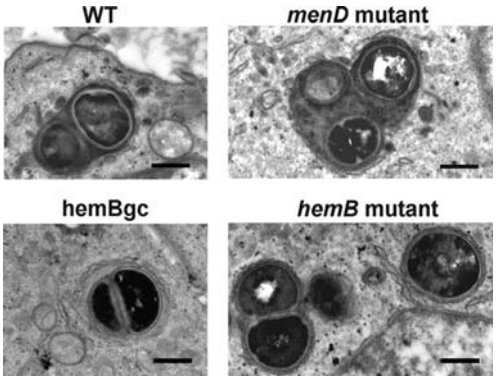

FIG 3 Morphological appearance of infected cells after 24 h of incubation. Both normal and SCV phenotypes were seen in membrane-bound structures with, in some cases, the appearance of several bacteria in a single vacuole, suggestive of intracellular multiplication. Bar, 500 nm.

Susceptibility testing. The MICs of a large series of antibiotics were tested against the COL strain and its derivatives. It appeared susceptible to most of them except β-lactams, tetracycline, and trimethoprim-sulfamethoxazole (see Table S1 in the supplemental material). Six antibiotics were selected for this study based on their known bactericidal character in broth against *S. aureus* (β-lactams were not included because of the MRSA phenotype of the COL parental strain). MICs were measured in broth at pH 7.4 and 5.5, to mimic the pHs of the extracellular milieu and of the lysosomal environment, respectively (Table 1). Among these antibiotics, only gentamicin and moxifloxacin showed a significant (2-$\log_2$-dilution) increase in MIC against SCVs, which was reversed by hemin supplementation for the *hemB* strain but not by MSB for the *menD* strain. Oritavancin was slightly more active against the *menD* mutant than against the parental strain. When measured at acidic pH, MICs were globally similar, except for gentamicin, which showed higher MICs against the two SCVs. Again, susceptibility was recovered upon addition of hemin in tests involving the *hemB* strain but not upon addition of MSB in tests involving the *menD* strain.

Activities of antibiotics against extracellular and intracellular *S. aureus*. The activities of antibiotics were then examined in parallel against extracellular (in broth) and intracellular (infected THP-1 cells) bacteria after 24 h of incubation. We compared the parental wild-type strain with its *menD* and *hemB* mutants, the *menD* mutant in medium supplemented with 2 mg/liter MSB, and the genetically complemented *hemB* strain. Antibiotics were added to the medium over a wide range of concentrations, which allowed us to obtain the pertinent pharmacological descriptors of activity (relative minimal efficacy [E_{min}], relative maximal efficacy [E_{max}], and apparent static concentration [C_s]; see reference 7 and Materials and Methods for a description of the models used and of the corresponding parameters). A graphical representation of the data is presented in Fig. 4 and 5, with the numerical values for pharmacological descriptors shown in Table 2. All data are presented as a function of the extracellular concentration (expressed in multiples of the MIC measured at pH 7.4 for extracellular bacteria and at pH 5.5 for intracellular bacteria).

Figure 4 shows the concentration-response profiles of the 3 antibiotics with moderate activity against SCVs (MICs, ≥0.25 mg/liter), and Fig. 5 shows the concentration-response profiles for those displaying lower MICs and which previously proved the most active against a thymidine-dependent SCV of *S. aureus* (38). Numerical data are presented overall in Table 2, and data for individual strains and antibiotics are presented in Tables S2 and S3 in the supplemental material.

Considering extracellular bacteria (left panels, Fig. 4 and 5), a monophasic sigmoidal regression was found to fit the data for each antibiotic and for each strain. For all strains, E_{min} (bacterial growth in the absence of antibiotic) values were similar (all comprised between approximately a 2- and a 3-$\log_{10}$-CFU increase over the initial inoculum), and the static concentrations (C_s; in

TABLE 1 MICs of antibiotics against bacterial strains and influence of medium supplementation in MSB or in hemin (24/48 h)[a]

		MIC (mg/liter)					
			menD mutant		*hemB* mutant		
Antibiotic	pH	Wild type	Control	With MSB at 2 mg/liter	Control	With hemin at 2 mg/liter	*hemB* genetically complemented strain
Vancomycin	7.4	1/2	1	1	1/2	1	1
	5.5	1/2	1	1	1	1	1
Daptomycin	7.4	0.5/2	0.25/0.5	0.25/0.5	0.5	0.5	1
	5.5	0.5	0.5	0.5	0.5	1	2
Gentamicin	7.4	0.25	1	1	0.5	0.125/1	0.25/0.5
	5.5	1	32/64	32/64	32	0.5	1
Rifampin	7.4	0.016	0.016/0.03	0.016/0.06	0.016/0.03	0.016/0.06	0.016
	5.5	0.002	0.002	0.002	0.002	0.002	0.002
Moxifloxacin	7.4	0.03/0.06	0.125	0.125	0.125/0.25	0.06/0.125	0.03/0.06
	5.5	0.125/0.25	0.25/0.5	0.5	0.25/0.5	0.125	0.125/0.5
Oritavancin	7.4	0.25/1	0.03/0.06	0.03/0.06	0.125	2/4	0.25
	5.5	0.25/1	0.125	0.06/0.125	0.125/0.25	0.25/0.5	0.25/1

[a] Only values that were different at 48 h and at 24 h are indicated.

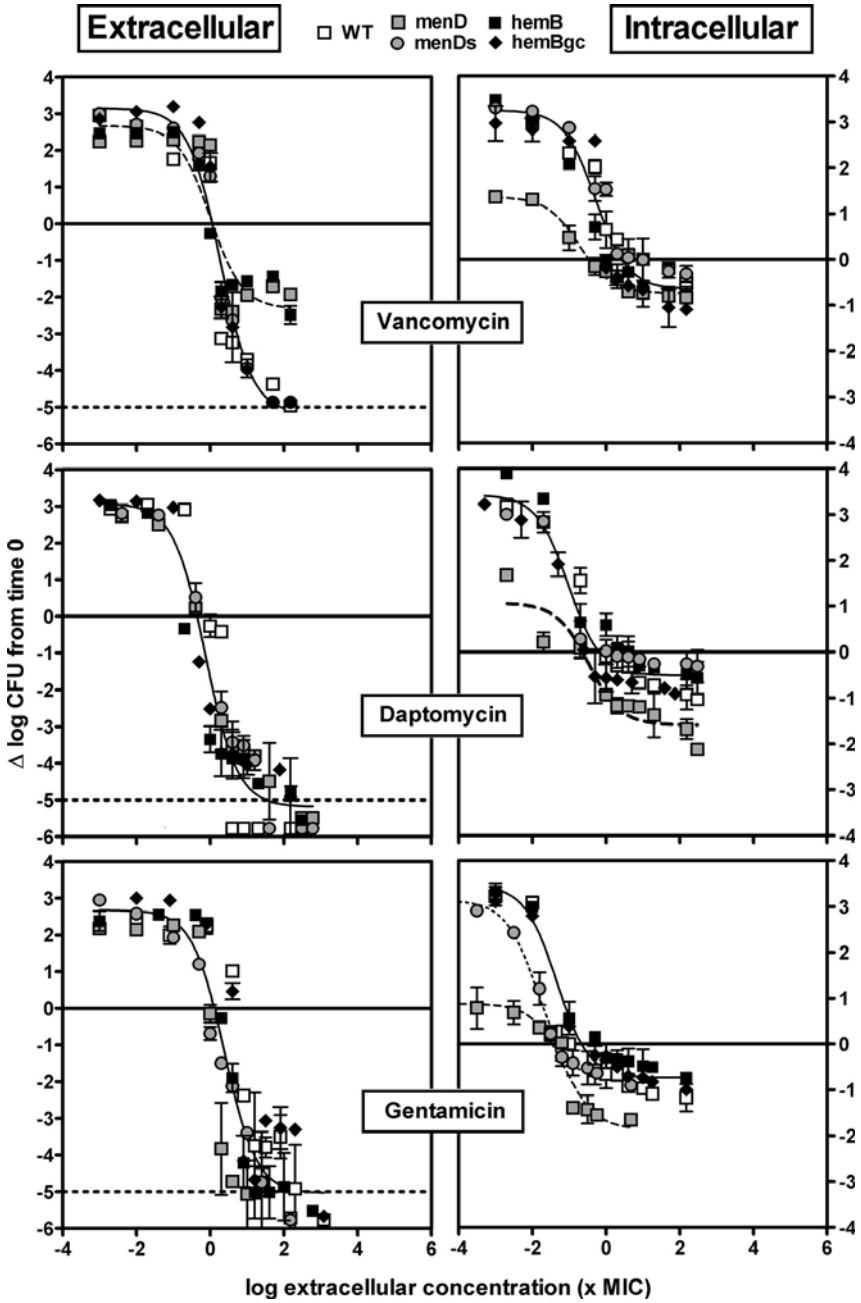

FIG 4 Concentration-response curves of vancomycin, daptomycin, and gentamicin against the extracellular (broth, left panels) and intracellular (THP-1, right panels) forms of the *S. aureus* parental strain with the wild-type phenotype (WT), its *menD* mutant under control conditions (menD) or in medium supplemented with 2 mg/liter MSB (menDs), its *hemB* mutant (hemB), and the *hemB* genetically complemented mutant (hemBgc). Bacteria and infected cells were incubated in the presence of increasing concentrations of antibiotics (total drug) for 24 h. The ordinate shows the change in the number of CFU (log scale) per ml of broth (extracellular bacteria) or per mg of cell protein (intracellular bacteria). The solid horizontal line corresponds to an apparent static effect, and the dashed line corresponds to the limit of detection. The abscissa shows the drug concentration in broth (extracellular) or in the culture medium (intracellular) expressed in multiples of the MIC measured at pH 7.4 (extracellular), pH 5.5 (intracellular, except *hemB* strain), or pH 5.5 in the presence of hemin (*hemB* strain, based on data in Fig. 2 suggesting the availability of hemin-like compounds in the cellular medium). All values are means ± standard deviations (SD) of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Experiments have been reproduced 3 times with similar results.

multiples of the MIC in broth) were similar for all antibiotics (all at a value close to the corresponding MIC). For all antibiotics except vancomycin, E_{max} (maximal relative efficacy) was close to the lower limit of detection (about a 5-$\log_{10}$-CFU decrease compared to the original inoculum; this was even observed for gentamicin, indicating that this antibiotic remained highly bactericidal against SCVs in spite of its increased MIC). In contrast, vancomycin was clearly less effective against the two SCV strains (*menD* and *hemB*) with E_{max} values at ~2.5-$\log_{10}$ CFU only ($P < 0.05$ by analysis of variance [ANOVA] compared with the other strains)

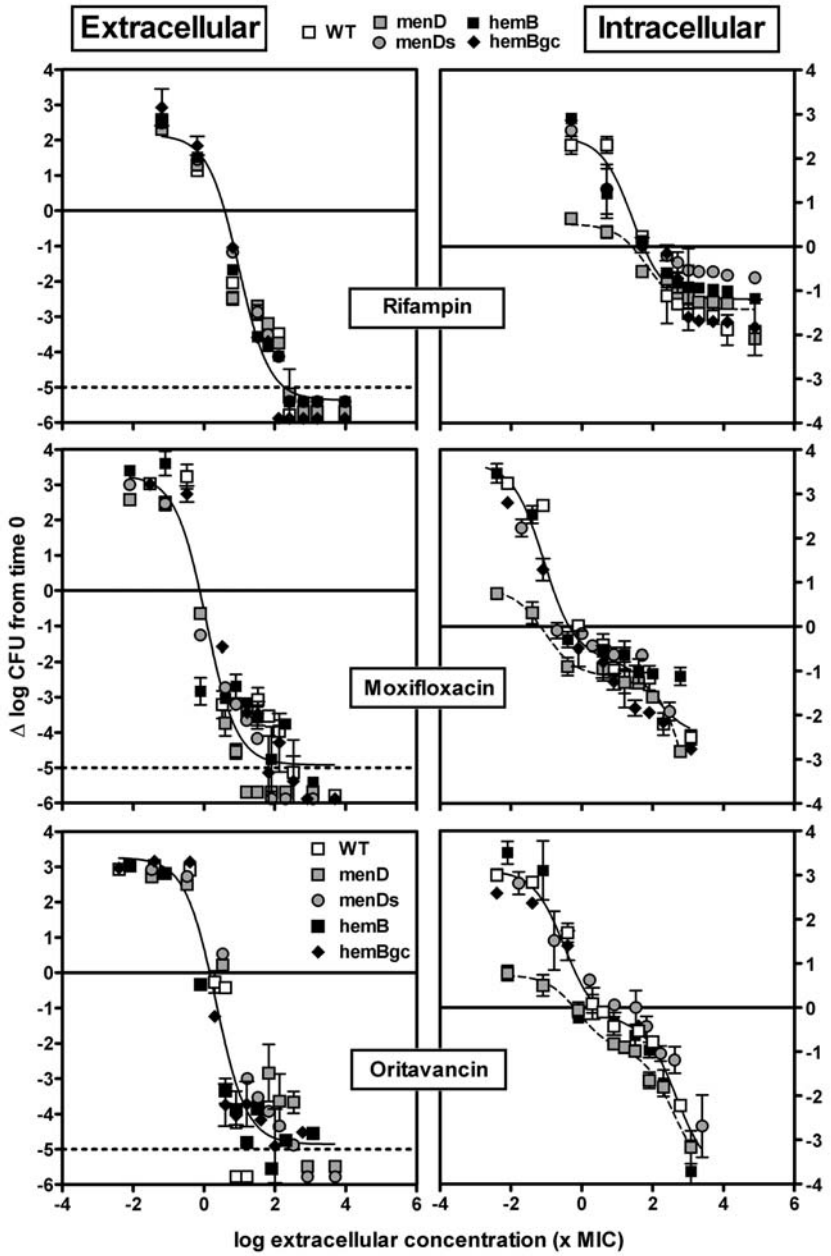

FIG 5 Concentration-response curves of rifampin, moxifloxacin, and oritavancin against the extracellular (broth, left panels) and intracellular (THP-1, right panels) forms of the *S. aureus* parental strain with wild-type phenotype (WT), its *menD* mutant under control conditions (menD) or in medium supplemented with 2 mg/liter MSB (menDs), its *hemB* mutant (hemB), and the *hemB* genetically complemented mutant (hemBgc). Bacteria and infected cells were incubated in the presence of increasing concentrations of antibiotics (total drug) for 24 h. The ordinate shows the change in the number of CFU (log scale) per ml of broth (extracellular bacteria) or per mg of cell protein (intracellular bacteria). The plain horizontal line corresponds to an apparent static effect, and the dotted line corresponds to the limit of detection. The abscissa shows the drug concentration in broth (extracellular) or in the culture medium (intracellular) expressed in multiples of the MIC measured at pH 7.4 (extracellular), pH 5.5 (intracellular, except the *hemB* strain), or pH 5.5 in the presence of hemin (*hemB* strain, based on data in Fig. 2 suggesting the availability of hemin-like compounds in the cellular medium). All values are means ± standard deviations (SD) of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Experiments have been reproduced 3 times with similar results.

but it showed a maximal relative efficacy similar to that of the other antibiotics when tested against the wild-type (WT) strain, the *hemB*-complemented strain (hemBgc), or the *menD* strain in the presence of MSB (menDs).

Moving now to intracellular bacteria, the *menD* mutant (i) grew considerably more slowly than all the other strains (E_{min} ranging between 0.5- and 1.5-$\log_{10}$-CFU increase versus approximately 3 $\log_{10}$ CFU increase for the other strains or for the same strain in the presence of MSB [menDs]) and (ii) showed a C_s somewhat lower (3- to 16-fold) than those for the other strains. A second key observation is that for all strains and for all antibiotics except moxifloxacin and oritavancin, E_{max} never reached more

TABLE 2 Pertinent regression parameters[a] (with confidence intervals) and statistical analysis[f] of the dose-response curves illustrated in Fig. 4 and 5

	Extracellular activity					Intracellular activity					
Antibiotic	Strain(s)	E_{min}[b]	E_{max}[c]	C_s[d]	R^2	Strain(s)	E_{min}[b]	E_{max}[c]	C_s[d]	Fract[e]	R^2
Vancomycin	WT, menDs strain, hemBgc strain	3.15 (2.61 to 3.68); a, A	<−5; a, A	1.16	0.948	WT, menDs strain, *hemB* strain, hemBgc strain	3.26 (2.94 to 3.57); a, A	−0.64 (−0.91 to −0.37); a, B	2.68	NA	0.916
	menD strain, *hemB* strain	2.67 (1.84 to 3.50); a, A	−2.32 (−3.14 to −1.49); b, A	1.16	0.847	*menD*	1.36 (1.14 to 1.58); b, B	−0.74 (−0.88 to −0.60); a, B	0.34	NA	0.984
Daptomycin	All strains	3.08 (2.52 to 3.63); a, A	<−5; a, A	0.44	0.934	WT, menDs strain, *hemB* strain, hemBgc strain	3.43 (3.09 to 3.76); a, A	−0.51 (−0.67 to −0.34); a, B	0.65	NA	0.940
						menD strain	1.07 (0.27 to 1.86); b, B	−1.59 (−2.09 to −1.09); b, B	0.23	NA	0.881
Gentamicin	All strains	2.68 (2.02 to 3.34); a, A	<−5; a, A	1.29	0.879	WT, *hemB* strain, hemBgc strain	3.46 (3.15 to 3.77); a, A	−0.74 (−0.87 to −0.60); a, B	0.22	NA	0.969
						menD strain	0.88 (0.50 to 1.26); b, B	−1.86 (−2.38 to −1.33); b, B	0.03	NA	0.948
						menDs strain	3.13 (2.89 to 3.37); a, A	−0.82 (−1.02 to −0.62); a, B	0.05	NA	0.993
Rifampin	All strains	2.16 (1.68 to 2.64); b, A	<−5; a, A	3.90	0.948	WT, menDs strain, *hemB* strain, hemBgc strain	2.49 (2.04 to 2.93); a, A	−1.19 (−1.39 to −0.99); b, B	56.38	NA	0.891
						menD strain	0.51 (−0.07 to 1.10); b, B	−1.42 (−1.78 to −1.07); b, B	25.32	NA	0.882
Moxifloxacin	All strains	3.27 (2.51 to 4.03); a, A	<−5; a, A	0.76	0.908	WT, menDs strain, *hemB* strain, hemBgc strain	3.69 (3.13 to 4.25); a, A	<−2; c, B	0.45	0.80	0.937
						menD strain	0.85 (0.52 to 1.18); b, B	<−2; c, B	0.07	0.72	0.990
Oritavancin	All strains	3.27 (2.56 to 3.97); a, A	<−5; a, A	1.42	0.907	WT, menDs strain, *hemB* strain, hemBgc strain	3.10 (2.72 to 3.49); a, A	<−2; c, B	3.0	0.50	0.951
						menD strain	0.77 (0.41 to 1.06); b, B	<−2; c, B	0.63	0.36	0.991

[a] Calculated based on sigmoidal regressions with a Hill coefficient of 1 for extracellular data and for intracellular data with vancomycin, daptomycin, gentamicin, and rifampin and based on biphasic sigmoidal regressions with Hill coefficients of 1 for intracellular data with moxifloxacin and oritavancin.

[b] Increase in CFU (in $\log_{10}$ units) from the corresponding original inoculum as extrapolated for an infinitely low concentration of antibiotic (mean with 95% confidence interval).

[c] Decrease in CFU (in $\log_{10}$ units) from the corresponding original inoculum as extrapolated for an infinitely large concentration of antibiotic (mean with 95% confidence interval). For moxifloxacin and oritavancin, the plateau value was not reached intracellularly, preventing us from calculating accurate E_{max} values. It was therefore estimated as being greater than a 5-log (extracellular) or a 2-log (intracellular) decrease (<-5 or <-2 in the table).

[d] Concentration (multiple of the MIC) resulting in no apparent bacterial growth as determined by graphical interpolation. The MICs used were values at pH 7.4 for extracellular activity, values at pH 5.5 for intracellular activity for all strains from the *hemB* mutant, and the value at pH 5.5 in the presence of hemin for the *hemB* mutant based on the data from Fig. 2 suggesting the availability of hemin-like compounds in the cellular medium.

[e] Proportion of the total response that could be ascribed to the first wave of CFU decrease in the biphasic curve. NA, not applicable.

[f] For statistical analysis per column (one-way ANOVA with Tukey test for multiple comparisons between each parameter for all drugs), values with different lowercase letters are significantly different from each other ($P < 0.05$); for statistical analysis per row (unpaired, two-tailed *t* test between corresponding parameters of extracellular and intracellular activities), values with different uppercase letters are significantly different from each other ($P < 0.05$).

TABLE 3 Antibiotic efficacy at clinically relevant concentrations (free trough value [fC_{min}] and free peak value [fC_{max}]) observed in the serum of patient receiving conventional dosages

		Pharmacokinetic parameter[a]			Extracellular activity[b]			Intracellular activity[b]		
Antibiotic	Daily dose	Free fraction (%)	C_{min} (total/free)	C_{max} (total/free)	Strains	*E* at fC_{min}	*E* at fC_{max}	Strain(s)	*E* at fC_{min}	*E* at fC_{max}
Vancomycin	15 mg/kg of body wt BID[d]	45	8/3.6	50/22.5	WT, menDs strain, hemBgc strain	−2.33	−4.53	WT, menDs strain, *hemB* strain, hemBgc strain	−0.15	−0.55
					menD strain, *hemB* strain	−1.24	−2.10	*menD* strain	−0.63	−0.72
Daptomycin	6 mg/kg/day	10	7/0.7	94/9.4	All strains	−2.38	−4.96	WT, menDs strain, *hemB* strain, hemBgc strain	−0.13	−0.50
								menD strain	−1.08	−1.54
Gentamicin	6 mg/kg/day	>70	2/1.4	18/13	All strains	−2.09	−4.75	WT, *hemB* strain, hemBgc strain	−0.56	−0.74
								menD strain	−0.16	−1.44
								menDs strain	0.11	−0.69
Rifampin	600 mg BID	20	1.2/2.4	18/1.6	All strains	−4.93	−4.73	WT, menDs strain, *hemB* strain, hemBgc strain	−1.11	−1.07
								menD strain	−1.31	−1.26
Moxifloxacin	400 mg/day	70	1/0.7	4/2.8	All strains	−4.04	−4.69	WT, menDs strain, *hemB* strain, hemBgc strain	−0.77	−0.94
								menD strain	−1.06	−1.16
Oritavancin	1,200-mg single dose	15	NA[c]	129/20	All strains	NA	<−5	WT, menDs strain, *hemB* strain, hemBgc strain	NA	−0.81
								menD strain		−1.80

[a] Values collected in the summary of product characteristics of the corresponding drugs; for oritavancin, data are from reference 10.
[b] Intrapolated from the equations of the concentration-response curves shown in Fig. 4 and 5 (with concentrations converted into mg/liter).
[c] NA, not applicable. Trough concentrations are not relevant since oritavancin will be administered as a single, 1,200-mg dose for treatment of skin and soft tissue infections thanks to its long terminal half-life in plasma ($t_{1/2}$ = 393 h [43]).
[d] BID, twice a day.

than a 2-log_{10}-CFU decrease compared to the original inoculum, which is considerably less than that in broth (E_{max} values of the *menD* mutant strain were, however, slightly more negative than those of the other strains for daptomycin and gentamicin). Moreover, with gentamicin, both the *hemB* mutant and the genetically complemented *hemB* mutant differed from the *menD* mutant in medium supplemented with MSB (the latter shows a C_s as low as that of the *menD* strain, which can be ascribed to the fact that MSB supplementation does not decrease its MIC [$P < 0.001$ by ANOVA]). In all those cases, a monophasic Hill equation could be fitted to the data. In contrast, the shape of the responses to moxifloxacin and oritavancin for all strains (including the *menD* mutant) was clearly biphasic, as previously described for oritavancin with a thymidine-dependent SCV (38). A first plateau could be identified at ~1-log_{10} CFU decrease (compared to the original inoculum) for an extracellular concentration of about 10× the MIC (and accounting for about half of the total response for oritavancin and about three-fourths for moxifloxacin) followed by a second response that reached 2.5 to 3.5 log_{10} decrease from the initial inoculum at the maximal concentration tested. Lastly, C_s values were close to those observed extracellularly, except for rifampin (probably in relation to its very low MIC at acidic pH).

Table 3 illustrates for each antibiotic the efficacy that could be reached when extracellular or intracellular bacteria are exposed to a range of concentrations mimicking those than can be achieved in human serum upon treatment with conventional dosages. All drugs were bactericidal against extracellular bacteria, except vancomycin against the *menD* mutant. In contrast, only oritavancin, moxifloxacin, and rifampin were able to reduce the intracellular inoculum of more than 0.8 log_{10} CFU for all strains over a range of concentrations mimicking free trough and peak in human serum.

DISCUSSION

The present study is the first to systematically evaluate the activity of antibiotics against the intracellular forms of *hemB* and *menD* mutants mimicking the SCV phenotype using a pharmacodynamic approach. A series of novel observations has been made, with respect to both the intracellular infection of monocytes by these strains and antibiotic activity.

Considering initial intracellular infection by itself, we found

that the internalization rate was lower for both SCVs than for their parental strain with normal phenotype. A similar behavior was reported for a *menB* SCV mutant in the same model of THP-1 cells (46), as well as for a series of SCVs collected from cystic fibrosis patients, which were less phagocytized by polymorphonuclear leukocytes (PMN) than were their normal-phenotype counterparts (45). The effect is probably not related to differences in the abilities of SCVs and parental strains to adhere to the macrophage cell surface, because the COL strain used here does not express a series of surface proteins playing a role in adhesion (62). This is not specific to COL, which was initially a clinical isolate, since a huge variability in adhesion properties has also been documented for other clinical isolates of *S. aureus* (3, 23, 49, 58). Our data, therefore, may suggest that the differences in intracellular inocula observed are probably due to a less avid phagocytosis of SCVs. We know, for example, that the genes involved in capsule synthesis are upregulated in the *menD* mutant (24), which may contribute to reducing its internalization. Interestingly, also, we found that the intracellular survival of the *hemB* mutant was similar to that of the parental strain while that of the *menD* mutant was much lower. Because the growth of the latter was reversed upon menadione bisulfite supplementation, we may suggest that the cellular medium contains the nutrients needed for counterbalancing the slow growth of the hemin-dependent strain but not that of the menadione-dependent strain. These nutrients may possibly come from the culture medium, which contains transferrin that may act as an alternative hemic iron transporter. Other reports suggest that supplementation of the medium by hemin or menadione actually reduces the capacity of the SCVs to persist within the cells by increasing toxin production, thereby favoring its capacity to escape from phagolysosomes (5, 21, 66). While these studies have all been performed in nonprofessional phagocytes where internalized *S. aureus* cells seem to reach the cytosol, our experiments were made with macrophages where we show here that SCVs remain localized in vacuoles of phagocytes after 24 h of infection. This suggests that the nature of the host may be critical.

Considering antibiotic activity against extracellular bacteria, we show, as anticipated (40), that gentamicin is less active against SCVs than against the normal-phenotype strain, the difference being particularly impressive at acidic pHs. This can be explained by a decrease of susceptibility to aminoglycosides in SCVs, which is known to result from a low electrical potential across the plasma membrane ($\Delta\Psi$) within the bacteria (36), the latter being further decreased in acidic medium (15, 34). We also noted a slight increase in moxifloxacin MIC against SCVs compared to wild-type bacteria, but this may not be generalized as it is not observed in surveys of clinical isolates of SCVs (19). More conspicuously, these changes were reversible upon supplementation by hemin but not by menadione. This contrasts with the fact that genetically complemented menadione-dependent clinical isolates with mutations in the *menB* gene have been shown to fully recover their susceptibility to gentamicin (25). Yet, previous studies showed that supplementation does not necessarily restore gentamicin activity (36), suggesting either that mutations in *menD* are less easily compensated for by menadione supplementation than are mutations in *menB* (MenD is involved in an earlier step of menadione synthesis than is MenB [20]) or that other defects affecting gentamicin transport do exist in the mutant that we used. We cannot exclude, however, the possibility that the bisulfite form of menadione is less active *in vitro* than *in vivo* due to a limited metabolism. Concentration-effect studies single out vancomycin as the only antibiotic which loses its bactericidal character against SCVs while its MIC remains unchanged (thus turning vancomycin into a bacteriostatic drug). This effect could result from the fact that cell wall synthesis is reduced in SCVs (40). In contrast, daptomycin, aminoglycosides, fluoroquinolones, oritavancin, and, to a lesser extent, rifampin have been shown to remain bactericidal against extracellular slow-growing bacteria (11, 33, 37, 54).

Considering intracellular activities, we demonstrate overall a reduced maximal efficacy for all tested antibiotics, compared to what can be obtained in broth. This is in line with what we have repeatedly described for *S. aureus* of different origins and resistance phenotypes and for most antibiotics tested in this *in vitro* model of THP-1 monocytes as well as in other models of phagocytic and nonphagocytic cells (7, 27, 29, 31, 38). The present data, however, expand those observations in two main directions.

First, and most strikingly, we see that the impairment of intracellular growth exhibited by the *menD* mutant is not accompanied by a decrease of its susceptibility to antibiotics when examined in concentration-dependent experiments. Thus, even though the amplitude of the response (as defined by the E_{min}-to-E_{max} span) is reduced, this is entirely due to the decrease in E_{min}. This is consistent with the fact that the bactericidal activity of all antibiotics tested (except for vancomycin) was maintained against this strain when grown in broth in spite of its lower growth rate and is in sharp contrast to what has been originally shown for *Escherichia coli* exposed to β-lactams, for which killing rates are reduced in inverse proportion to their generation time (57). We have not studied β-lactams here because of the resistance phenotype of our strains, but the lower response of vancomycin in broth may point to effects specific to cell wall synthesis inhibitors. Yet, our present data question the general hypothesis raised to explain the decreased susceptibility of intracellular *S. aureus* toward most antibiotics, namely, that their growth rate is impaired in phagolysosomes compared to broth and other extracellular milieus (4, 8, 51, 59). Further studies will need to establish whether our observations are specific to the *menD* strain and/or can be extended to other slow-growing strains. They also will need to differentiate among the other potential causes, as the overall susceptibility may be dependent on a combination of several environmental as well as bacterial factors (see reference 59 for a review).

A second and perhaps even more striking observation is the shape of the response exhibited by all strains to moxifloxacin and oritavancin. While the modeling performed here may be of limited value because of the restricted number of independent data points, it nevertheless suggests that these two drugs might be able to eradicate intracellular pathogens much more effectively than other antibiotics if their extracellular concentration is further increased. While possibly clinically irrelevant (because this feature could involve drug concentrations that cannot reasonably be obtained in the extracellular milieus in patients), it raises interesting perspectives in terms of a dual mode of action for these drugs. A biphasic shape of the bacterial response to an increase in concentration has already been seen with telavancin (a lipoglycopeptide with structural similarities with oritavancin [61]) for methicillin-susceptible *S. aureus* (MSSA) and MRSA (6) and with oritavancin for a thymidine-dependent SCV (38). In both cases, this has been ascribed to the known dual mode of action of these drugs that probably involves an impairment of the building-up of the pepti-

doglycan at low concentrations and membrane-destabilizing effects at higher concentrations (see reference 60 for a review). Conversely, we can offer no explanation for moxifloxacin at this stage, as this will require more extensive studies and detailed comparisons with other fluoroquinolones and other strains. Notably, however, a biphasic shape was also observed when studying the intracellular activity of delafloxacin, a very potent investigational fluoroquinolone (30), against *S. aureus*. In this case, as in the present study, a biphasic response was observed when extending the range of concentrations to high multiples of the MIC (typically 1,000×, which was possible for delafloxacin in view of its very low MICs, but explains why it escaped our attention in previous studies with other fluoroquinolones).

Concentrating on the SCVs, the present data, combined with those from our previous study using a thymidine-dependent strain (38), suggest that the intracellular behaviors and susceptibilities of thymidine-, hemin-, and menadione-dependent SCVs to antibiotics are rather different. Our data reveal that antibiotics show the same profile of intracellular activity against the hemin-dependent SCV as against a normal-phenotype strain. They also display a similar intracellular maximal efficacy (as defined by the decrease in CFU from the initial inoculum at high concentration) against a wild-type and a menadione-dependent SCV but a much lower E_{min}, which is reversed upon medium supplementation in menadione bisulfite and therefore attributable to its slower growth. This contrasts with our previous observations using a thymidine-dependent SCV, against which antibiotic activity (measured at a fixed concentration mimicking the human maximum concentration in serum [C_{max}]) remained lower than against a normal-phenotype strain, even when its intracellular growth was restored by thymidine supplementation. These differences in drug profiles of activity further show that there is no direct correlation between growth rate and susceptibility to antibiotics intracellularly.

As a conclusion, our work has thus shown that the intracellular fates of the two types of mutants are highly dissimilar, with specific consequences for antibiotic activity. Thus, the *hemB* mutant that grows intracellularly like the parental strain also responds similarly to antibiotics; the *menD* mutant, the growth of which is impaired in cells, sees its intracellular growth controlled by lower antibiotic concentrations (higher antibiotic potency) but with no change in maximal efficacy. This may be highly relevant when considering the effects that can be achieved at clinically relevant concentrations. Yet, a potential limitation of the present study is that it was performed with a single strain of each phenotype obtained by genetic engineering. Extending it to clinical isolates would be of clear interest, especially because SCVs are now recognized as a cause of persistence and recurrence in many infections, including osteomyelitis, device-associated infections, and pulmonary infections in CF patients (42). However, our model offers the advantage of comparing strains sharing the same genetic background, which is a first, necessary step when performing the type of systematic pharmacological comparison we undertook here. Taking this limitation into account, the present data suggest that oritavancin and, to a lesser extent, moxifloxacin (for strains that remain susceptible; intracellular breakpoint of susceptibility, MIC, ≤0.125 mg/liter [28]) may offer the most promising activity against these particular forms of intracellular infections, showing a high activity at clinically relevant concentrations. Notably, fluoroquinolones or lipoglycopeptides do not appear in the current recommendations of the Infectious Diseases Society of America for the management of MRSA infections (32). They would therefore deserve further investigations using clinical isolates and *in vivo* models.

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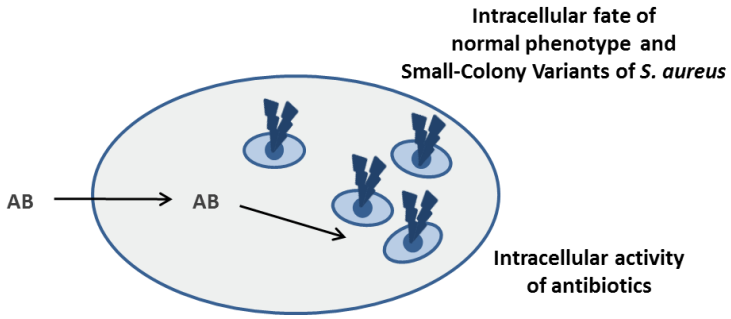
2. SCVs & Antibiotics in Macrophages



Non-adherent THP-1 monocytes can be easily differentiated into adherent macrophages by stimulation with phorbol esters such as phorbol-12-myristate-13-acetate (PMA). These macrophages are a well-established model that closely resembles native monocyte-derived macrophages. Although these differentiated cells lose their ability to replicate, their antibacterial properties are markedly enhanced, allowing them to participate in the inflammatory and immune responses.

In this study, we have examined the influence of PMA stimulation on the intracellular fate of menadione- and hemin-dependent mutants and on their intracellular susceptibilities to antibiotics. These experiments represent a first attempt to systematically compare the monocytic cell line THP-1 to its mature macrophage-like form with respect to antibiotic activity.

AUTHOR'S CONTRIBUTION:

1. Conceived and designed experiments: LGG, SL, PMT, FVB;
2. Performed experiments: LGG;
3. Analyzed data: LGG, SL, BCK, KB, RAP, PMT, FVB;
4. Contributed to reagents, strains and financial support: BCK, KB, RAP, FVB;
5. Wrote the paper: LGG, SL, PMT, FVB.

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Influence of the Protein Kinase C Activator Phorbol Myristate Acetate on the Intracellular Activity of Antibiotics against Hemin- and Menadione-Auxotrophic Small-Colony Variant Mutants of *Staphylococcus aureus* and Their Wild-Type Parental Strain in Human THP-1 Cells

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In a previous study (L. G. Garcia et al., Antimicrob. Agents Chemother. 56:3700–3711, 2012), we evaluated the intracellular fate of *menD* and *hemB* mutants (corresponding to menadione- and hemin-dependent small-colony variants, respectively) of the parental COL methicillin-resistant *Staphylococcus aureus* strain and the pharmacodynamic profile of the intracellular activity of a series of antibiotics in human THP-1 monocytes. We have now examined the phagocytosis and intracellular persistence of the same strains in THP-1 cells activated by phorbol 12-myristate 13-acetate (PMA) and measured the intracellular activity of gentamicin, moxifloxacin, and oritavancin in these cells. Postphagocytosis intracellular counts and intracellular survival were lower in PMA-activated cells, probably due to their higher killing capacities. Gentamicin and moxifloxacin showed a 5- to 7-fold higher potency (lower static concentrations) against the parental strain, its *hemB* mutant, and the genetically complemented strain in PMA-activated cells and against the *menD* strain in both activated and nonactivated cells. This effect was inhibited when cells were incubated with *N*-acetylcysteine (a scavenger of oxidant species). In parallel, we observed that the MICs of these drugs were markedly reduced if bacteria had been preexposed to H_2O_2. In contrast, the intracellular potency of oritavancin was not different in activated and nonactivated cells and was not decreased by the addition of *N*-acetylcysteine, regardless of the phenotype of the strains. The oritavancin MIC was also unaffected by preincubation of the bacteria with H_2O_2. Thus, activation of THP-1 cells by PMA may increase the intracellular potency of certain antibiotics (probably due to synergy with reactive oxygen species), but this effect cannot be generalized to all antibiotics.

Small-colony variants (SCVs) of *Staphylococcus aureus* have a propensity to survive within eukaryotic cells, and this propensity has been associated with the persistent and/or recurrent character of the infections that they cause (26, 40–42, 51), most conspicuously in cystic fibrosis patients (49, 54). Moreover, their slow growth and metabolic defects impair the activity of many antibiotics (8, 25).

In a previous paper (20), we compared the intracellular growth of isogenic menadione- and hemin-dependent SCVs with that of their parental strain in human THP-1 cells and examined the activity of antibiotics against the intracellular forms of these bacteria. These cells are indeed widely used for studying the intracellular activity of antibiotics against phagocytized bacteria (6, 27, 30, 33–35, 38, 46). We observed that the menadione-dependent mutant grew much more slowly in THP-1 cells than the hemin-dependent strain but remained as susceptible to antibiotics in terms of maximal reduction of the inoculum compared to the postphagocytosis values.

THP-1 cells were originally obtained from a patient suffering from acute monocytic leukemia. Although displaying many of the characteristics of macrophages (47), these cells maintain their monomyelocytic phenotype in culture and have notoriously poor antibacterial defense mechanisms. THP-1 cells, however, can be differentiated into macrophage-like cells by exposure to the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) (43). The most remarkable changes accompanying this differentiation are a capacity to adhere to surfaces, increased phagocytic activity toward latex beads, and stimulation of superoxide production (19, 43). The consequences of these changes concerning the uptake and intracellular survival of bacteria and how they modulate the intracellular activity of antibiotics remain, however, poorly understood. In the present study, we examined how differentiation of THP-1 cells by PMA affects the intracellular fate of an *S. aureus* strain with a normal phenotype and its menadione- and hemin-auxotrophic SCV mutants when exposed to antibiotics. Antibiotics were selected from among those having proved the most and least effective in nonactivated cells, namely, moxifloxacin and oritavancin (20), with the aim to examine whether cell activation could contribute to further improvement of their activity. Gentamicin was studied in parallel as a control drug showing less activity against intracellular bacteria but being highly efficient against extracellular bacteria.

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MATERIALS AND METHODS

Antibiotics and main reagents. Moxifloxacin and oritavancin were obtained as microbiological standards from Bayer HealthCare (Leverkusen, Germany) and from The Medicines Company (Parsippany, NJ), respectively. Gentamicin was obtained as the branded product commercialized in Belgium for human use (Geomycin [distributed by GlaxoSmithKline S.A.-N.V., Genval, Belgium]). Pooled human serum from healthy volunteers was purchased from Lonza Ltd. (Basel, Switzerland) and stored at −80°C until use. Cell culture media and sera were from Invitrogen Corp. (Carlsbad, CA), and other reagents were from Sigma-Aldrich (St. Louis, MO) or Merck KGaA (Darmstadt, Germany).

Bacterial strains and MIC determinations. Four isogenic strains were used through this study, namely, strain COL (wild-type, hospital-acquired methicillin-resistant *Staphylococcus aureus*), its *menD* and *hemB* SCV mutants (constructed by allelic replacement with an *ermB* cassette-inactivated *hemB* gene and an *ermC* cassette-inactivated *menD* gene, respectively [7, 52]), and a *hemB* genetically complemented (*hemB*gc) strain. SCVs were grown either in Mueller-Hinton broth (MHB) or in the same medium supplemented with 2 μg/ml menadione sodium bisulfite (MSB) or hemin (20). Unless stated otherwise, MICs were determined in MHB adjusted to pH 7.4 or pH 5.5 after 24 h and following the general recommendations of the Clinical and Laboratory Standards Institute (CLSI) (12, 13).

Cells and differentiation method. All experiments were conducted with human THP-1 monocytic cells (ATCC TIB-202 [American Type Culture Collection, Manassas, VA] [47]) differentiated to adherent macrophages by incubation with PMA (200 μg/liter; Sigma-Aldrich) for 48 h at 37°C (45).

Cell infection and intracellular activity of antibiotics. Cell infection and assessment of antibiotic activities were performed exactly as described previously (20).

Influence of H_2O_2 on antibiotic activity. Bacteria were preincubated for 30 min in the dark with 10 mM H_2O_2 in MHB. This concentration was selected as minimally affecting the growth of normal-phenotype strains in the absence of added antibiotic (37). Bacteria were then pelleted and resuspended in fresh broth for determination of the MICs as described above, but with readings made after both 24 and 48 h. Both values were recorded because significant differences were seen when dealing with bacteria preexposed to H_2O_2.

Curve-fitting and statistical analyses. Curve-fitting analyses and determination of the pertinent regression parameters of the concentration-response experiments were made with GraphPad Prism software (version 4.03; GraphPad Software, San Diego, CA). Data were used to fit monophasic or biphasic sigmoidal functions as previously described (20). Statistical analyses were performed with GraphPad Instat software (version 3.06; GraphPad Software).

RESULTS

Phagocytosis and intracellular growth. In a first series of experiments (Fig. 1, top), we examined the influence of cell activation by PMA on phagocytosis and intracellular growth of the wild-type strain, its *menD* and *hemB* SCVs, and the *hemB* genetically complemented strain. In nondifferentiated cells and as previously described, SCVs, detected by determination of the numbers of CFU (viable bacteria), were less avidly internalized than the parental or the genetically complemented *hemB*gc strains (20). In differentiated cells, the internalization of all strains was further drastically decreased (15- to 30-fold). After 24 h of incubation, all strains except for the *menD* mutant showed an approximately 1.5-log-CFU increase per mg of cell protein in nonactivated cells but only an approximately 0.5-log-CFU increase per mg cell protein in PMA-activated cells; the *menD* mutant did not grow in either cell type. The actual counts of viable bacteria associated with the cells were therefore 150- to 400-fold lower in PMA-activated cells than nonactivated cells at 24 h for all strains except the *menD* mutant, for which there was only a 50-fold difference between PMA-activated and nonactivated cells. Interestingly, because of their lower initial value, the supplemented *menD* mutant and the *hemB* mutant reached 2- to 10-fold lower CFU counts than the parental strain in each cell type. However, by normalizing all values to those of the postphagocytosis inoculum of each strain (Fig. 1, bottom), all strains (except the *menD* mutant) were found to have a similar growth rate when considering a given cell type, but this rate was about 2-fold lower in PMA-activated cells than nonactivated cells.

Intracellular activity of antibiotics. The activity of the three selected antibiotics in PMA-activated THP-1 cells at concentrations ranging from 0.001 to 150 mg/liter was then examined after 24 h of incubation. This time point was selected because such a length of time allows the reproducible growth of bacteria (except for the *menD* mutant, which grows more slowly) and comparison with data previously obtained with nonactivated cells (20). Results are presented in Fig. 2 for both activated and nonactivated cells. Sigmoidal functions could be fitted to all data, with the best fits obtained using a monophasic function for gentamicin and a biphasic function for moxifloxacin and oritavancin. There was no significant difference between the functions describing the individual behaviors of the parental strain, the *hemB* mutant, the genetically complemented strain (*hemB*gc), and the *menD* mutant grown in MSB-supplemented medium. All data from these strains were therefore used together to build a single function, as illustrated in Fig. 2. In contrast, the *menD* mutant showed a considerably lower minimal efficacy (E_{min}) value because of its slower intracellular growth. Table 1 compares the pharmacodynamic parameters of these curves with those observed in nonactivated cells. The more salient observation concerns the differences in antibiotic potencies (apparent static concentrations [C_ss]). Thus, the potency of gentamicin against the parental strain, the *hemB* mutant, and the genetically complemented *hemB* mutant was about 7-fold higher (7-fold lower C_s) in PMA-activated cells than nonactivated cells, whereas no significant difference was seen for the *menD* mutant, whether it was tested in the presence of MSB or not. The potency of moxifloxacin was about 5-fold higher (5-fold lower C_s) against all strains (except the *menD* mutant) when measured in PMA-activated cells than nonactivated cells. In sharp contrast, the apparent static concentration of oritavancin toward all strains was similar in both cell types. No systematic differences in maximal efficacy (E_{max}) could be observed within the limits of our experimental conditions (but true E_{max} values could not be determined with confidence for several antibiotic and strain combinations due to the biphasic character of several of the concentration-effect responses and the limitations to the highest antibiotic concentrations that could be used without toxic effects to the THP-1 cells), yet global analysis of the curves showed a significant effect of cell activation on the activity of gentamicin and moxifloxacin against all strains except the *menD* mutant; these differences clearly rely on improved potency (lower apparent static concentrations) in activated cells.

Influence of *N*-acetylcysteine on antibiotic intracellular activity. Differentiation of THP-1 cells by PMA is known to activate cell defense mechanisms, including the production of reactive oxygen species (ROS) (43). We therefore examined whether the general antioxidant *N*-acetylcysteine, a scavenger of oxidative species widely used to counteract the pro-oxidant effects of phorbol esters

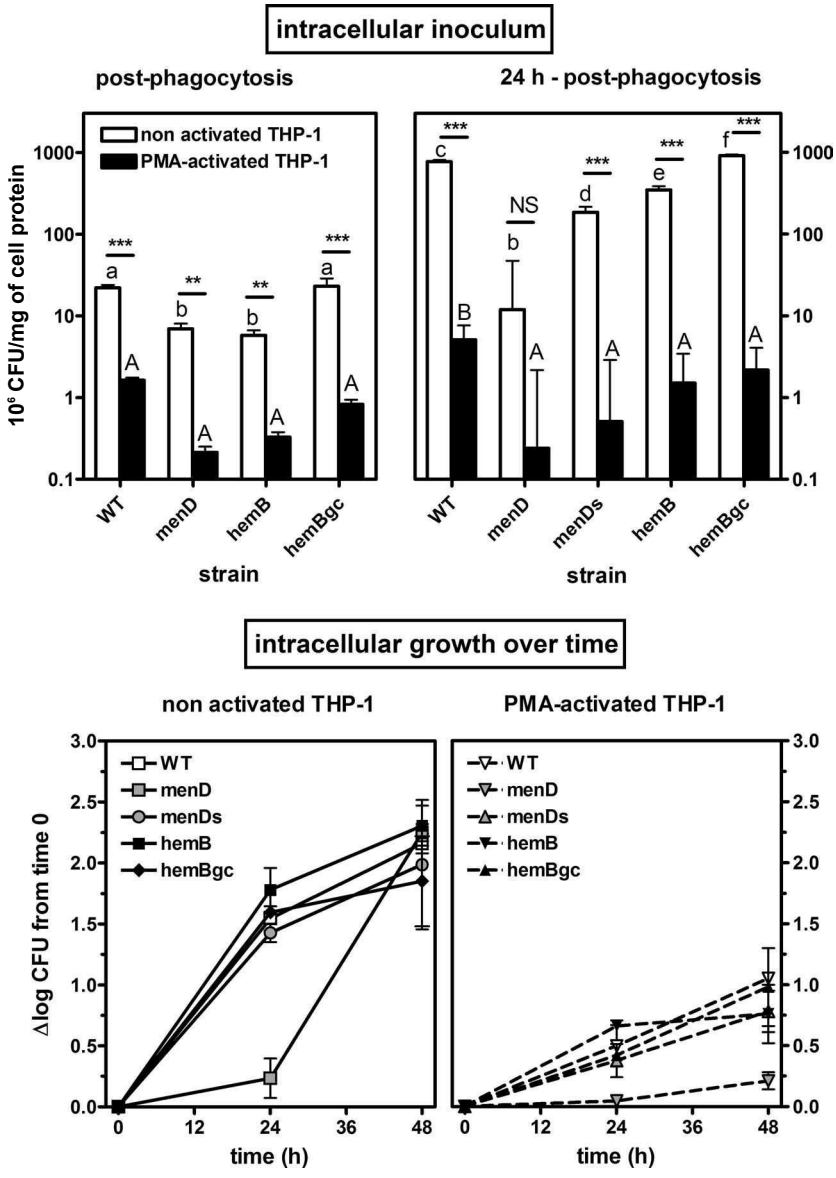

FIG 1 Comparative phagocytosis and intracellular survival of *S. aureus* of the normal phenotype (wild type [WT]), SCVs (*menD* [supplemented {menDs} or not {menD} with 2 μg/ml MSB for intracellular survival] and *hemB*), and the *hemB* genetically complemented strain (*hemB*gc) in nonactivated THP-1 cells (open bars) or in THP-1 cells activated by 48 h of incubation with 200 μg/liter PMA (solid bars). (Top) Enumeration of cell-associated CFU after 1 h of phagocytosis (left) and after 24 h of incubation postphagocytosis (right). (Bottom) Comparative growth of the strains over 48 h of incubation, with data expressed as changes from the initial inoculum. Data are means of 3 to 6 independent experiments performed in triplicate. Statistical analysis was by analysis of variance with the Tukey *post hoc* test) for differences between activated and nonactivated cells (***, $P < 0.001$; **, $P < 0.01$; NS, not significant) and differences between strains for a given type of cell (lowercase letters, comparison between strains in nonactivated cells; uppercase letters, comparison between strains in activated cells; different letters show significant differences with P values of <0.05).

(1, 11, 50), modulated the potency of antibiotics. For this purpose, infected cells were incubated for 24 h with each of the investigated antibiotics at its C_s determined from the results of the concentration-effect experiments [Table 1]) alone or in the presence of 25 mM *N*-acetylcysteine. Figure 3 shows that *N*-acetylcysteine markedly decreased the activity of gentamicin and moxifloxacin against all strains in both nonactivated and PMA-activated cells. Thus, rather than an apparent static effect, we observed substantial bacterial growth (about 1 to 3 log CFU) compared to what was observed for these antibiotics in the absence of *N*-acetylcysteine. The effect was more important in PMA-activated cells for gentamicin toward all strains except the parental strain and for moxifloxacin toward the parental and the *hemB*gc strains. The effect of *N*-acetylcysteine on oritavancin activity was less marked, with a gain of only 0.5 to 1.5 $\log_{10}$ CFU for the parental, *menD*, *hemB*, and *hemB*gc strains and a slight decrease in the numbers of CFU for the supplemented *menD* mutant (*menD*s).

Influence of H_2O_2 on antibiotic extracellular activity. To further investigate the potential cooperation between antibiotics and the oxidant species produced by phagocytes suggested by the previous experiment, we evaluated the influence of H_2O_2 on the activity of antibiotics. To determine this effect, MICs were measured

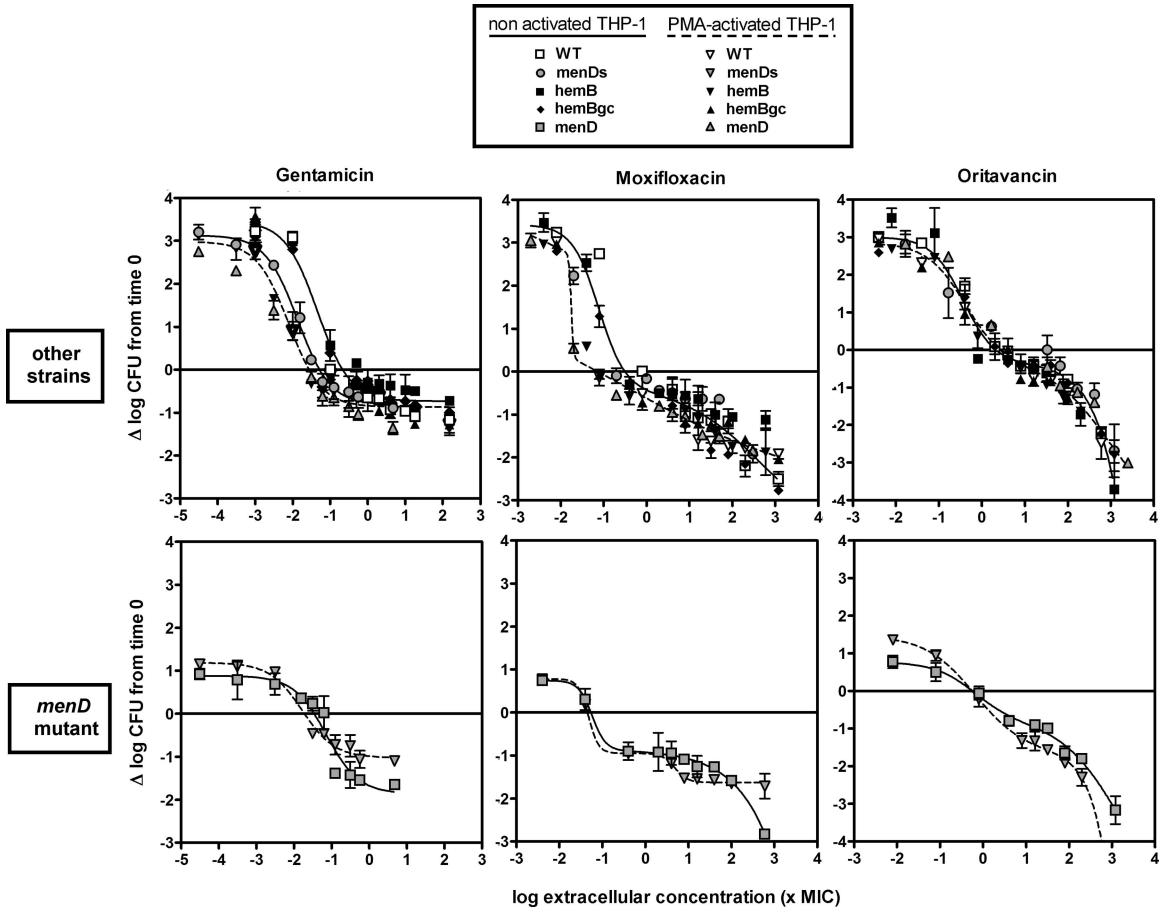

FIG 2 Concentration-response curves of gentamicin, moxifloxacin, and oritavancin in nonactivated THP-1 cells (solid line) or in cells activated by 48 h of incubation with 200 μg/liter PMA (dotted line) against the *S. aureus* parental strain with the wild-type phenotype, its *menD* mutant in medium supplemented by 2 μg/ml MSB (menDs), its *hemB* mutant (hemB) and the *hemB* genetically complemented mutant (hemBgc) (top), or its *menD* mutant under control conditions (menD) (bottom). Infected cells were incubated in the presence of increasing concentrations of antibiotics (total drug) for 24 h. The ordinate shows the change in the number of CFU (log scale) per mg of cell protein compared to the postphagocytosis inoculum. The solid horizontal line corresponds to an apparent static effect. The abscissa shows on the log scale the drug concentration in the culture medium expressed in multiples of the MIC measured at pH 5.5 (for all strains except the *hemB* strain) or pH 5.5 in the presence of hemin (for the *hemB* strain, based on data having demonstrated the availability of hemin-like compounds in the cellular medium [20]). All values are means ± standard deviations (SDs) of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Experiments were reproduced 3 times with similar results.

in broth at neutral as well as at acidic pH for bacteria preexposed to 10 mM H_2O_2 for 30 min and compared with those for unexposed bacteria. Table 2 shows that preexposing bacteria to H_2O_2 caused a drastic decrease of the MIC of gentamicin and moxifloxacin toward all strains when using readings made at 24 h. Values, however, were higher when readings were made after 48 h. Interestingly, preincubation with H_2O_2 restored the susceptibility of the *menD* mutant (supplemented or not with MSB) to gentamicin at acidic pH, and this effect partially persisted at 48 h. In contrast to the other two antibiotics, the MIC of oritavancin was essentially unaffected by preincubation of the bacteria with H_2O_2.

DISCUSSION

Although differentiation of monocytes in macrophages by PMA has been quite extensively studied, the influence of PMA on the phagocytosis of bacteria and their intracellular survival, as well as on the intracellular activity of antibiotics, has rarely been described. The present study is, therefore, one of the first to have examined these parameters in detail. We used THP-1 cells and *S. aureus* because this pair has been extensively studied by us and others using nondifferentiated cells (6, 21, 33, 35, 36, 39). To make the study as informative as possible with respect to the role played by the persistence of intracellular bacteria in the relapsing and recurrent character of staphylococcal infection, we examined wild-type *S. aureus* cells and also their SCV counterparts using a series of isogenic strains, the behavior of which in nonactivated cells has recently been characterized (20).

Considering the phagocytosis and intracellular survival of the bacteria first, we show that cell activation by PMA causes an apparent decrease in the number of viable bacteria associated with the cells after phagocytosis, as well as reduces intracellular growth upon further incubation. The reduction in the number of phagocytosed bacteria is *a priori* surprising since PMA is known to favor phagocytosis by THP-1 cells. However, this effect of PMA was studied only with latex beads (14, 43, 55), the uptake of which is nonspecific. In contrast, phagocytosis of *S. aureus* requires its attachment at the cell surface mainly via fibronectin-binding proteins FnBP-A and FnBP-B that connect to cellular integrins via

TABLE 1 Pertinent regression parameters[a] and statistical analysis of the dose-response curves illustrated in Fig. 2

	Nonactivated THP-1 cells							PMA-activated THP-1 cells							
				C_s							C_s				
Antibiotic	Strain	E_{min}[b]	E_{max}[c]	Multiple of MIC[d]	μg/ml[e]	Fract[f]	R^2	Strain	E_{min}	E_{max}	Multiple of MIC	μg/ml	Fract	R^2	ANOVA[g]
Gentamicin	WT, *hemB*, *hemB*gc	3.46 (3.15 to 3.77)a,A[h]	−0.74 (−0.87 to −0.60)a,A	0.22	0.11–0.22	NA[i]	0.97	WT, *menD*s, *hemB*, *hemB*gc	2.99 (2.67 to 3.30)a,A	−0.87 (−1.01 to −0.73)a,A	0.03	0.02–0.03	NA	0.95	<0.001
	menD	0.88 (0.50 to 1.26)b,A	−1.86 (−2.38 to −1.33)b,A	0.03	0.96	NA	0.95	*menD*	1.19 (0.95 to 1.42)b,A	−1.03 (−1.24 to −0.82)a,B	0.02	0.64	NA	0.98	>0.05
	*menD*s	3.13 (2.89 to 3.37)a,A	-0.82 (−1.02 to −0.62 a,A	0.05	1.06	NA	0.99								>0.05
Moxifloxacin	WT, *menD*s, *hemB*, *hemB*gc	3.43 (2.80 to 4.04)a,A	<−2 c	0.32	0.04–0.08	0.52	0.94	WT, *menD*s, *hemB*, *hemB*gc	4.44 (−4.39 to 13.27)c,A	<−2 b	0.07	0.01–0.02	0.33	0.99	<0.05
	menD	0.85 (0.52 to 1.18)b,A	<−2 c,A	0.07	0.02	0.72	0.99	*menD*	0.78 (−2.55 to 4.10)b,A	−1.63 (−1.86 to −1.39)c,B	0.05	0.01	0.72	1.00	>0.05
Oritavancin	WT *menD*s, *hemB*, *hemB*gc	3.10 (2.72 to 3.49)a,A	<−2 c	3.0	0.38–0.75	0.50	0.95	WT, *menD*s, *hemB*, *hemB*gc	2.85 (2.46 to 3.24)a,A	<−2 b	2.63	0.33–0.66	0.44	0.98	>0.05
	menD	0.77 (0.41 to 1.06)b,A	<−2 c	0.63	0.08	0.36	0.99	*menD*	1.48 (0.63 to 3.32)b,A	<−2 b	0.56	0.07	1.14	1.00	>0.05

[a] Calculated on the basis of the sigmoidal regressions with a Hill coefficient of 1 for extracellular data and for intracellular data with gentamicin and on biphasic sigmoidal regressions with Hill coefficients of 1 for intracellular data with moxifloxacin and oritavancin.

[b] E_{min}, increase in the number of CFU (in $\log_{10}$ units) from the corresponding original inoculum extrapolated for an infinitely low concentration of antibiotics (mean with the 95% confidence interval in parentheses).

[c] E_{max}, decrease in the number of CFU (in $\log_{10}$ units) from the corresponding original inoculum extrapolated for an infinitely large concentration of antibiotics (mean with the 95% confidence interval in parentheses). For moxifloxacin and oritavancin, the absence of clear plateaus in the change in the number of intracellular CFU at the highest antibiotic concentrations tested prevented us from calculating accurate E_{max} values. For some curves, the plateau was not reached at the maximal concentration tested; the maximal effect was therefore estimated as being greater than a 2-log-unit decrease in the inoculum (<−2 in the table).

[d] Concentration (multiple of the MIC) resulting in no apparent bacterial growth determined by graphical interpolation. MICs (readings made at 24 h) were used as follows: values at pH 7.4 were used for extracellular activity, values at pH 5.5 were used for intracellular activity for all strains by the *hemB* mutant, and values at pH 5.5 were used in the presence of hemin for the *hemB* mutant on the basis of previous data suggesting the availability of hemin-like compounds in the cellular medium (20).

[e] Concentration calculated on the basis of the MICs given in Table 2; a range is given when a single fit was applied to strains having different MICs.

[f] Fract, fraction (proportion) of the total response that could be ascribed to the first wave of decrease in the number of CFU in the biphasic curve.

[g] Statistical analysis comparing all data points from each condition between nonactivated and PMA-activated cells using the fitted Hill functions (one-way analysis of variance with the Tukey test for multiple comparisons).

[h] Statistical analysis comparing the E_{min} and E_{max} values of each data set: (i) analysis per column (one-way analysis of variance with the Tukey test for multiple comparisons between each parameter for all drugs; data with different lowercase letters are significantly different from each other [$P < 0.05$]); (ii) analysis per row (unpaired, two-tailed *t* test between nonactivated and PMA-activated cells; data with different uppercase letters are significantly different from each other [$P < 0.05$]).

[i] NA, not applicable.

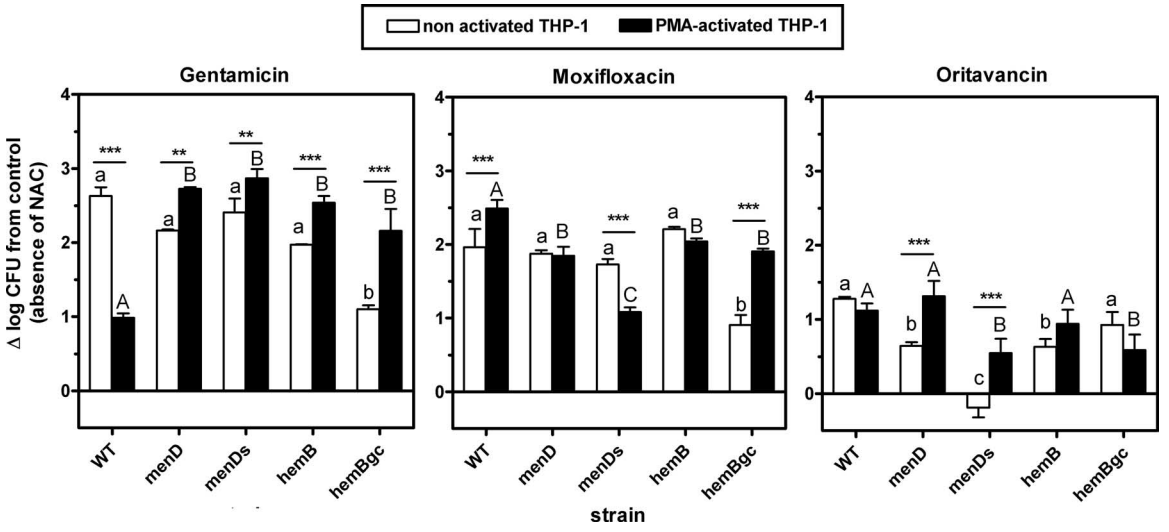

FIG 3 Influence of *N*-acetylcysteine on the intracellular activity of antibiotics in nonactivated THP-1 cells (open bars) or in cells that have been activated by incubation with 200 μg/liter PMA for 48 h (solid bars). Antibiotics were used at their C_s, i.e., the concentration at which no apparent change from the initial inoculum was observed, as interpolated from concentration-effects studies (Table 1), and the number of viable bacteria (CFU) per mg of cell protein was determined after 24 h of incubation in the presence of 25 mM *N*-acetylcysteine. Values are expressed as the difference in CFU from the values recorded in the absence of *N*-acetylcysteine (NAC; close to 0 in all cases). All values are means ± standard deviations (SD) of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Experiments have been reproduced 3 times with similar results. Statistical analysis was by analysis of variance with the Tukey *post hoc* test, and data with different letters indicate significant differences ($P < 0.05$) between strains (lowercase letters, in nonactivated cells; uppercase letters, in activated cells) and cell types (***, $P < 0.001$; **, $P < 0.01$).

soluble fibronectin and trigger internalization (23, 24, 44). The COL strain used in the present study, however, is described to be deficient in fibronectin-binding proteins (48), which may explain why internalization may not be favored in PMA-activated cells, despite their increased capacity to bind fibronectin (19). PMA also induces a marked stimulation of the production of oxidant species (19, 43). Oxidative burst is a major mechanism by which phagocytes defend themselves against bacterial invasion, including invasion by *S. aureus* (17, 22). Thus, we may reasonably hypothesize that phagocytized bacteria are more quickly killed in PMA-activated THP-1 cells than unstimulated cells. This would explain (i) the differences in recovery and the apparent slower growth of phagocytized wild-type bacteria since the endpoint (number of CFU) is the net result of the two opposite processes of bacterial growth and killing (as already observed by us in *Listeria monocytogenes*-infected THP-1 cells upon stimulation of the production of reactive oxygen species by exposure to gamma interferon [38]) and (ii) the still lower recovery of SCVs compared to that of the parent strain, because SCVs are hypersusceptible to oxidant agents *in vitro* (53).

TABLE 2 Influence of preincubation with H_2O_2 on MICs of antibiotics against the COL strain, its SCVs, and the genetically complemented *hemB* strain, with readings made at 24 h and 48 h[a]

			MIC (mg/liter)					
				menD mutant		*hemB* mutant		
Antibiotic	pH	H_2O_2	Wild type	Without MSB	With MSB	Without hemin	With hemin	*hemB*gc
Gentamicin	7.4	−	0.25/0.25	1/1	1/1	0.5/0.5	0.125/1	0.25/0.5
		+	0.03/1	0.125/1	0.125/1	0.125/1	0.125/0.5	0.03/1
	5.5	−	1/1	32/64	32/64	32/32	0.5/0.5	1/1
		+	0.125/2	0.125/2	0.125/4	0.125/2	0.06/2	0.125/2
Moxifloxacin	7.4	−	0.03/0.06	0.125/0.125	0.125/0.125	0.125/0.25	0.06/0.125	0.03/0.06
		+	0.008/0.125	0.008/0.125	0.008/0.125	0.002/0.06	0.008/0.06	0.008/0.125
	5.5	−	0.125/0.25	0.25/0.5	0.5/0.5	0.25/0.5	0.125/0.125	0.125/0.5
		+	0.002/0.125	0.002/0.125	0.002/0.125	0.001/0.125	0.002/0.125	0.002/0.125
Oritavancin	7.4	−	0.25/1	0.03/0.06	0.03/0.06	0.125/0.125	2/4	0.25/0.25
		+	0.25/1	0.03/0.06	0.03/0.06	0.125/0.125	2/4	0.25/0.25
	5.5	−	0.25/1	0.125/0.125	0.06/0.125	0.125/0.25	0.25/0.5	0.25/1
		+	0.25/1	0.125/0.125	0.06/0.125	0.125/0.25	0.25/0.5	0.25/1

[a] Preincubation was in medium supplemented or not in menadione sodium bisulfite (MSB; 2 μg/ml) or in hemin (2 μg/ml). Data are for readings made at 24 h/48 h. The slow growth of SCVs sometimes made readings difficult to obtain at 24 h. *hemB*gc, genetically complemented *hemB* strain.

Moving now to the analysis of the data concerning antibiotic activity, a first striking observation is that moxifloxacin and gentamicin share a similar response to cell activation by PMA, characterized by an increased potency (lower apparent static concentration) compared to that against the parental strain, the genetically complemented strain, and the *hemB* mutant which becomes indistinguishable from that measured against the *menD* strain in nonactivated cells. Two complementary pieces of evidence suggest that this effect results from cooperation between these antibiotics and the oxidant cell defense mechanisms that have been stimulated by PMA. First, their activity is impaired in cells coincubated with the general antioxidant *N*-acetylcysteine. Second, the MICs of both drugs markedly decreased when bacteria were preincubated with an oxidant species like H_2O_2. This is consistent with the fact that the bactericidal mode of action of these two drugs involves the formation of radical species within the bacteria (15, 28, 29). The fact that MICs were higher at 48 h is probably due to the short duration of the effect of H_2O_2 over time. Of note, however, the activity of the two drugs appeared to be unaffected by activation of cells when tested with the *menD* strain. A plausible explanation is that the deficiency in electron transport in this strain already makes it sensitive to the basal level of oxidant species produced by nonactivated cells, in relation to the fact that SCVs have been described to be hypersensitive to oxidants (53). The *hemB* mutant does not show this hypersusceptibility, but this strain behaves like the complemented strain when exposed to the intracellular medium (20), probably because this milieu contains heme-rich compounds capable of restoring its normal metabolic activity. Interestingly, an oxidant environment is also able to restore the activity of gentamicin against both types of SCVs, suggesting that it can compensate for the defect in electrostatic potential ($\Delta\Psi$) responsible for the intrinsic resistance of SCVs to aminoglycosides (8, 40).

A second and contrasting observation is that the concentration-response profile of oritavancin is not altered by cell activation by PMA, regardless of the strain examined. This suggests that, in contrast to aminoglycosides or fluoroquinolones, the bactericidal activity of oritavancin is less dependent on the formation of radical species. We show indeed that the oritavancin MIC is not affected upon preincubation of bacteria with H_2O_2 and its intracellular activity is less affected by *N*-acetylcysteine. While aminoglycosides or fluoroquinolones act upon intrabacterial targets, oritavancin bactericidal activity is assumed to result from inhibition of cell wall synthesis and anchoring to the bacterial membrane, causing depolarization and alteration of membrane permeability (9). Indeed, both *in vitro* and animal studies document that oritavancin and the two other classes of drugs interact very differently with cell host defenses. First, at clinically relevant concentrations oritavancin does not modify the production of reactive oxygen species by THP-1 cells (32) and neither increases nor decreases the capacity of phagocytic cells to kill pathogens that are out of its spectrum of activity, like *Acinetobacter baumannii* or *Candida albicans* (5). In contrast, moxifloxacin induces the release of reactive oxygen species by THP-1 cells (21). Second, oritavancin is highly effective in neutropenic animal models of infection (2), i.e., in the absence of oxidative defenses, whereas higher doses of fluoroquinolones are needed to reach a bacteriostatic effect when used in neutropenic versus immunocompetent animals (3). We also know that aminoglycosides show shorter postantibiotic effects in neutropenic animals (18).

Another and mutually nonexclusive explanation for the difference between oritavancin and the other drugs with respect to the influence of PMA on their potency could result from the delayed growth of bacteria in PMA-activated cells. Moxifloxacin suffers from an inoculum effect (31), which may explain why it is less active against intracellular bacteria when the amount of viable organisms is large. Gentamicin is more active against highly dividing bacteria (16), and we have seen that THP-1 differentiation slows the growth of intracellular *S. aureus*. In contrast, oritavancin remains bactericidal against both small and large inocula and remains bactericidal against slow-growing bacteria (4, 10). Yet, because of the pleiotropic effects exerted by PMA on THP-1 cells (43), we cannot exclude other concomitant mechanisms. We must also acknowledge that intracellular antibiotic activity was examined at a single time point; cooperation with cell defense mechanisms could have been different if shorter or longer incubation times had been used.

In conclusion, the data presented here show that activation of THP-1 cells by PMA affects the intracellular potency (apparent static concentration) of antibiotics to an extent that may reflect the dependence of their action on reactive oxygen species, without affecting their maximal efficacy. This may point to a useful process of cooperation between antibiotics and host defenses for certain agents, such as gentamicin and moxifloxacin. Conversely, agents for which cell activation has a minimal effect (like oritavancin) may remain as effective when host defense mechanisms are weakened.

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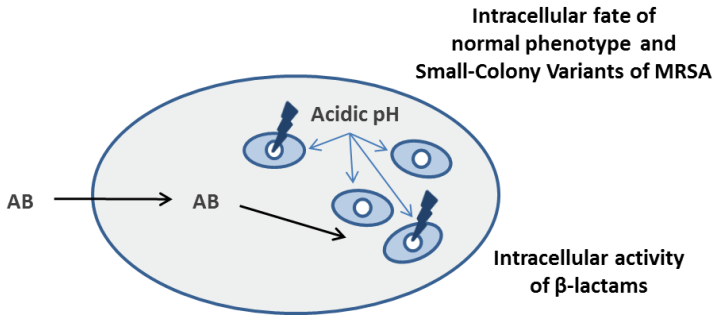

Our laboratory has previously demonstrated that acidic pH was associated with the restoration of the susceptibility of β-lactams against methicillin-resistant *S. aureus* (MRSA) due to an acidic pH-mediated conformational change of transpeptidase catalytic site of penicillin-binding protein (PBP) 2a from a closed to an open state.

In infected THP-1 monocytes, *S. aureus* survives and thrives in phagolysosomes, the pH of which is acidic, and becomes therefore susceptible to β-lactams.

The COL strain used in this project is an MRSA. We therefore wondered restoration of β-lactams activity in the intracellular environment would also take place for its SCV mutants. We have thus examined the intracellular activity of three β-lactams (cloxacillin, meropenem, doripenem) towards the intracellular forms of the COL strain and of its menadione- and hemin-dependent derivatives.

AUTHOR'S CONTRIBUTION:

1. Conceived and designed experiments: LGG, SL, PMT, FVB;
2. Performed experiments: LGG;
3. Analyzed data: LGG, SL, BCK, KB, RAP, PMT, FVB;
4. Contributed to reagents, strains and financial support: BCK, KB, RAP, FVB;
5. Wrote the paper: LGG, SL, PMT, FVB.

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Intracellular forms of menadione-dependent small-colony variants of methicillin-resistant *Staphylococcus aureus* are hypersusceptible to β-lactams in a THP-1 cell model due to cooperation between vacuolar acidic pH and oxidant species

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Objectives: Phagocytosed methicillin-resistant *Staphylococcus aureus* (MRSA) are susceptible to β-lactams because of an acid-induced conformational change of penicillin-binding protein (PBP) 2a within phagolysosomes. We have examined whether this mechanism applies to *menD* and *hemB* small-colony variants (SCVs) of the COL MRSA strain, using cloxacillin, meropenem, doripenem, and vancomycin as comparator.

Methods: Intracellularly, the change in cfu from post-phagocytosis inoculum was measured after 24 h of incubation with antibiotics combined or not with *N*-acetylcysteine (NAC; oxidant species scavenger); the relative potency (C_s) was calculated from the Hill equation of concentration–response curves. Extracellularly, the effect of a pre-incubation with H_2O_2 was determined on MICs and killing at pH 7.4 and 5.5.

Results: Intracellularly, the β-lactam C_s was similar for the COL strain and the *hemB* mutant and not modified or slightly decreased (2- to 16-fold) by NAC. In contrast, the C_s was 100- to 900-fold lower for the *menD* mutant, but similar to that for the COL strain when NAC was present. Extracellularly, β-lactam MICs were markedly reduced at pH 5.5 for the parental strain and the haemin-supplemented *hemB* mutant, with limited additional effect of pre-incubation with H_2O_2. In contrast, MICs remained elevated at pH 5.5 for the *menD* mutant (supplemented with menadione sodium bisulphite or not), but were 7–10 dilutions lower after pre-incubation with H_2O_2. Vancomycin MICs were unaltered in all conditions, with no marked effect of NAC on C_s.

Conclusions: Cooperation between acidic pH and oxidant species confers high potency to β-lactams against intracellular forms of *menD* SCVs of MRSA.

Keywords: cloxacillin, meropenem, doripenem, H_2O_2, haemin

Introduction

Small-colony variants (SCVs) of *Staphylococcus aureus* are associated with persistent infections.[1] They have a particular tropism for the intracellular environment, which is often assumed to protect them from the action of most antibiotics.[1] In a recent study,[2] we examined the intracellular susceptibility of menadione- and haemin-dependent SCV stable mutants obtained from the methicillin-resistant *S. aureus* (MRSA) COL strain[3,4] to antistaphylococcal antibiotics. We observed that, although showing a much slower intracellular growth than the other strains, the *menD* mutant was as susceptible as its isogenic wild-type parent (no change in maximal relative efficacy and slight increase in relative potency, as determined by concentration–effect experiments). β-Lactams were not included in this study, because of the MRSA character of the strain. However, previous work in our laboratory has demonstrated that β-lactams in general, and cloxacillin and carbapenems in particular, regain full activity (both in terms of maximal relative efficacy and relative potency when tested against MRSA phagocytosed by THP-1 macrophages).[5,6] This surprising effect has been ascribed to the acidic pH prevailing in the phagolysosomes where the

bacteria sojourn and thrive, which causes penicillin-binding protein (PBP) 2a to undergo a conformational change from a closed to an open state that allows its acylation by β-lactams.[7]

We wondered whether a similar mechanism would also apply to SCVs. For this purpose, we examined and report here on the activity of three β-lactams (cloxacillin, meropenem and doripenem) against the intracellular forms of the *menD* and *hemB* mutants of the COL strain. Vancomycin was used as an anti-MRSA antibiotic acting also on cell wall synthesis, but through a distinct mechanism. While the activity of all three β-lactams was restored against the phagocytosed *hemB* mutant and its parental COL strain as anticipated, we made the unexpected observation that the same antibiotics became almost 100 to 900-fold more potent against the phagocytosed *menD* mutant than against its parental strain or the *hemB* mutant. A similar, although less impressive effect (10-fold increase) was also seen with a menadione-dependent SCV of the OM1a methicillin-susceptible *S. aureus* (MSSA) strain compared with its parental strain. We explain our observations as resulting from the combination of the direct effect exerted by acidic pH on the susceptibility of MRSA to β-lactams and of the hypersusceptibility of the *menD* mutant to the oxidative species produced by phagocytic cells.

Materials and methods

Antibiotics and main reagents

The following antibiotics were obtained as the branded products commercialized in Belgium for human use: gentamicin as Geomycin® and vancomycin as Vancocin® (both distributed in Belgium by GlaxoSmith-Kline, Wavre); meropenem as Meronem® (AstraZeneca, Brussels); and doripenem as Doribax® (Janssen-Cilag, Beerse, Belgium). Cloxacillin was purchased from Sigma–Aldrich (St Louis, MO, USA). Pooled human serum from healthy volunteers (used for opsonization) was purchased from Lonza Ltd (Basel, Switzerland) and stored at −80°C until use. Cell culture media and sera were from Invitrogen Corp. (Carlsbad, CA, USA), microbiological culture media from BD Bioscience (Franklin Lakes, NJ, USA) and other reagents from Sigma–Aldrich or Merck KGaA (Darmstadt, Germany).

Bacterial strains and susceptibility testing

We used two series of isogenic strains in this study, namely: (i) the *S. aureus* strain COL (wild-type hospital-acquired MRSA), its *menD* and *hemB* SCV mutants [constructed by allelic replacement with an *erm*(B) cassette-inactivated *hemB* gene and an *erm*(C) cassette-inactivated *menD* gene] and the *hemB* genetically complemented strain;[3,4] and (ii) a wild-type clinical MSSA isolate (OM1a) and its clonally related menadione-dependent SCV (OM1b), both recovered from a patient with chronic osteomyelitis.[8] MICs were determined following CLSI recommendations,[9] except that readings were made at 24 and 48 h for SCVs as previously described,[2] and incubations were made in Mueller–Hinton broth adjusted at either pH 7.4 or 5.5 (to mimic the pH of the extracellular medium and of the phagolysosomes, respectively).

Stability of β-lactams in broth at neutral and acidic pH

The chemical stability of cloxacillin and meropenem was tested after 24 h of incubation in Mueller–Hinton broth at pH 7.4 or adjusted at pH 5.5, using an HPLC assay [stationary phase: Lichrospher® 100 RP-18, 25×4 cm, 5 μM (Merck, Darmstadt, Germany); mobile phase: 25:75 (v/v) acetonitrile/25 mM phosphate buffer, pH 6.5; UV detection: 220 nm; linearity zone: 32–1000 mg/L (R^2=0.999) for both cloxacillin and meropenem].

Cells, cell infection and influence of N-acetylcysteine (NAC)

All experiments were conducted with human THP-1 cells [ATCC TIB-202 (ATCC, Manassas, VA, USA)], a myelomonocytic cell line displaying macrophage-like activity,[10] with culture conditions, cell infection and assessment of antibiotic activities performed exactly as described earlier.[2,11] Antibiotic activity was also examined in the presence of 25 mM NAC (Sigma–Aldrich), used as a scavenger of oxidant species produced by phagocytic cells.[12–14]

Determination of free concentrations of β-lactams in cell culture medium

β-Lactams were incubated for 2 h at 37°C in standard culture medium (RPMI-1640 plus 10% fetal bovine serum), after which free (unbound) antibiotic was separated by ultracentrifugation through a semi-permeable membrane with cut-off at 30 kDa (Amicon® Ultra-0.5 30K, EMD Millipore, Billerica, MA, USA). Free and total antibiotic concentrations were then measured by a disc-plate microbiological assay using Antibiotic Medium 11 at pH 7.95 and *S. aureus* strain ATCC 25923 as the test organism [zone of linearity of diameter versus $\log_{10}$ concentration: 4–512 mg/L for cloxacillin (R^2=0.963); 1–512 mg/L for meropenem (R^2=0.999); and 0.25–512 mg/L for doripenem (R^2=0.978)].

Influence of H_2O_2 on antibacterial activity

Bacteria were pre-exposed to 10 mM H_2O_2 in the dark (concentration selected to minimally affect bacterial viability),[15] after which the excess of H_2O_2 was destroyed by 100 mU/mL catalase. Bacteria were then pelleted, resuspended in fresh broth and either used directly for determination of MICs following CLSI recommendations or exposed to antibiotics for 5 h and plated for colony counting (assessment of bactericidal effect).

Curve-fitting and statistical analyses

Curve-fitting analyses were made using GraphPad Prism® version 4.03 (GraphPad Software, San Diego, CA, USA). Statistical analyses were made with GraphPad Instat version 3.06 (GraphPad Software). Hill equations were fitted to the data, allowing calculation of the following pertinent pharmacological descriptors, as described and discussed in our previous publications:[2,11] (i) the relative minimal efficacy [E_{min}; cfu increase in $\log_{10}$ units at 24 h compared with the original inoculum, as extrapolated for an infinitely low concentration of antibiotic (this parameter describes the actual bacterial growth in the absence of antibiotic)]; (ii) the relative maximal efficacy (E_{max}; cfu decrease in $\log_{10}$ units at 24 h compared with the original inoculum, as extrapolated for infinitely high antibiotic concentrations); and (iii) the drug static concentration (C_s, concentration of antibiotic resulting in no apparent bacterial growth compared with the original inoculum, as determined by graphical interpolation).

Results

Susceptibility testing

Table 1 shows the MICs of the three β-lactams and of vancomycin for the strains investigated, as determined in broth at pH 7.4 or 5.5 [to mimic the extracellular medium and the

Table 1. MICs of antibiotics and influence of medium supplementation with MSB or haemin (24/48 h)[a]

		MIC (mg/L)								
		MRSA COL						MSSA OM1		
			menD mutant		*hemB* mutant				OM1b	
Antibiotic	pH	wild-type	control	+MSB[b]	control	+haemin[b]	*hemB*gc	OM1a	control	+MSB[b]
Cloxacillin	7.4	128	128	128	128	128	128	0.25	0.125	0.25
	5.5	1/2	128	128	128	0.25	1/4	0.125	0.125	0.125
Doripenem	7.4	16/32	16/32	16/64	32/64	8/16	8/32	0.06	0.03	0.03
	5.5	0.5/2	8	8	8	0.25	0.5/1	0.03	0.03	0.03
Meropenem	7.4	32	32/64	32/64	32/64	16/32	16/32	0.125/0.25	0.06	0.125/0.25
	5.5	1/2	4/8	4/8	4/8	0.125/0.25	1	0.125	0.06	0.125
Vancomycin	7.4	1/2	1	1	1/2	1	1	1	0.5	0.5
	5.5	1/2	1	1	1	1	1	1	0.5/1	0.5

[a]Only values that were different at 48 h and at 24 h are indicated.
[b]2 mg/L.

phagolysosomal environment (infected intracellular compartment),[2] respectively]. For the COL strain and its *menD* and *hemB* mutants, β-lactam MICs were high at neutral pH (in accordance with their MRSA character). They were markedly reduced at acidic pH for the wild-type strain, its *hemB* mutant grown in haemin-supplemented broth and the genetically complemented *hemB* strain (*hemB*gc). In contrast, the MICs of cloxacillin were unchanged at pH 5.5 versus pH 7.4 for the *menD* and *hemB* mutants grown in non-supplemented medium and for the *menD* mutant cultured in medium supplemented with menadione bisulphite (MSB), while the MICs of the carbapenems were only partially reduced for these strains. For the OM1a MSSA strain, the MICs of all β-lactams were low and not different from those observed for the OM1b SCV, with no influence of pH or of supplementation with MSB. The vancomycin MICs were all low and ± 1 $\log_2$ dilution for all strains and in all conditions.

Influence of pH on β-lactam stability

To check that the huge difference in activity observed for some of the strains at neutral and acidic pH could not be attributed to chemical degradation at neutral pH, we compared the stability of cloxacillin and meropenem as an example carbapenem in Mueller–Hinton broth at pH 7.4 or 5.5 after 24 h of incubation at 37°C, using a drug concentration of 200 mg/L. Cloxacillin degradation reached only 8.8%±0.7% and 2.2%±1.4% at neutral and acidic pHs, respectively, and that of meropenem reached 23.9%±0.1% and 14.3%±0.0% in the same conditions. As the MICs and other activity parameters were determined on a $\log_2$-scale basis, these minor changes in drug concentration over time cannot account for the results observed.

Activity of antibiotics against intracellular S. aureus

Each strain was used to perform concentration-dependent experiments, with the change in cfu from the post-phagocytosis value determined after 24 h of incubation. For all strains, the data could be analysed and used to fit a Hill equation. Graphical representations are shown in Figure 1 (top panels) for the COL strain, the *menD* SCV [as such or with supplementation with MSB (*menD*s)], the *hemB* SCV and the corresponding complemented strain (*hemB*gc). Numerical values (regression parameters and pharmacological descriptors) are presented in Table S1 (available as Supplementary data at *JAC* Online). For β-lactams, no or only modest differences were seen between the COL strain, its *hemB* mutant and the *hemB*gc strain. Conversely and most strikingly, the *menD* mutant showed (i) a much lower intracellular growth (lower E_{min}) and (ii) a considerably increased potency (lower C_s) compared with its parental COL strain. Supplementation with menadione (*menD*s strain) reduced this effect, but the response was still significantly different from that of the COL strain. Vancomycin was also more potent against the MRSA *menD* strain compared with the parental strain, but the difference was less marked than that observed with β-lactams and was no longer seen after supplementation with menadione. Similar observations were made for the MSSA strain OM1a and its *menD* SCV mutant OM1b, although the difference in the relative potency was less important than with the MRSA strains and was entirely suppressed by the addition of menadione (OM1bs strain) (see Figure S1 and Table S2, both available as Supplementary data at *JAC* Online). Noteworthily, these differences in intracellular potencies were not directly correlated with commensurate differences in MICs (measured at pH 5.5). Thus, although β-lactams have the same MIC for the *menD* strain compared with the *hemB* and supplemented *menD* (*menD*s) strains, the latter two required higher β-lactam concentrations for controlling intracellular growth (see Figure S2, available as Supplementary data at *JAC* Online, where data of Figure 1 and Figure S1, available as Supplementary data at *JAC* Online are presented as a function of multiples of the MIC at pH 5.5). Most interestingly, the static concentration of β-lactams towards the MRSA *menD* mutant was 400 to 3300-fold lower than its MIC measured at acidic pH. This suggests that another factor specific to the intracellular environment contributed to the increased intracellular activity of β-lactams against this strain.

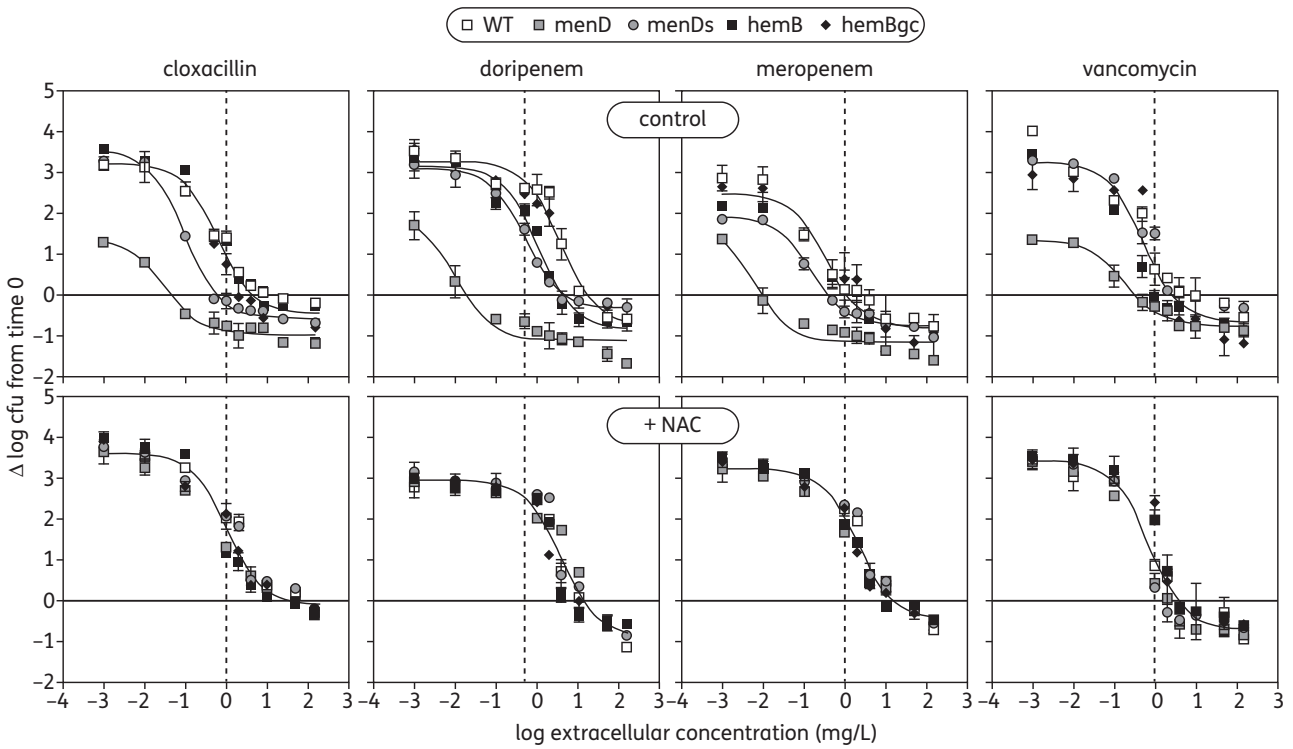

Figure 1. Concentration–response curves of cloxacillin, doripenem, meropenem and vancomycin against the intracellular forms of isogenic strains of *S. aureus* with an MRSA phenotype [wild-type (WT), *menD* mutant in control conditions (menD) or in medium supplemented with 2 mg/L MSB (menDs), *hemB* mutant (hemB) and the *hemB* genetically complemented mutant (hemBgc)]. Infected cells were incubated in the presence of increasing concentrations of antibiotics (total drug) for 24 h in control conditions (top panels) or in the presence of 25 mM NAC (bottom panels). The ordinate shows the change in the number of cfu (log scale) per mg of cell protein as compared with the initial inoculum. The continuous horizontal line corresponds to an apparent static effect. The abscissa shows the drug extracellular concentration. The broken vertical line corresponds to the MIC for the parental strain as measured at pH 5.5 (24 h reading). All values are means ± SD of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Experiments have been reproduced three times with similar results. For clarity, individual curves are shown only for strains with data significantly differing ($P < 0.05$) from one another by repeated measures ANOVA followed by Tukey's multiple comparison test. When not statistically different, data from different strains were analysed together to calculate a single Hill function. Individual regression parameters and pharmacological descriptors are shown in Table S1, available as Supplementary data at *JAC* Online.

Determination of the free concentration of β-lactams

Because protein binding in human serum is known to be high and concentration-dependent for cloxacillin,[16] but minimal for carbapenems,[17] we measured the free fraction for these antibiotics under the conditions of our experiments (RPMI-1640 medium supplemented with 10% fetal bovine serum) at three concentrations (total drug) in the range of the human C_{max} (total drug). The carbapenem (meropenem and doripenem) free fraction was >80% at all three concentrations, and that of cloxacillin was 47%, 60% and 78% at 8, 16 and 32 mg/L (see Figure S3, available as Supplementary data at *JAC* Online). These differences are probably negligible compared with the magnitude of changes of activity observed.

Influence of NAC on antibiotic activity against intracellular S. aureus

In a concurrent investigation, we have shown that the oxidative burst produced by phagocytic cells could play an important role in the bactericidal effect of antibiotics towards SCVs.[18] We therefore examined the influence of NAC, a scavenger of oxidant species,[12–14] on the intracellular activity of β-lactams and of vancomycin against the *menD* strain in comparison with the other SCV strains and their parental wild-type bacteria. In preliminary experiments, we checked that NAC did not modify the MIC of antibiotics (no change noted; data not shown). We then performed concentration– response experiments for each strain in the presence of NAC. The data are shown in Figure 1 (bottom panels) for the MRSA COL strain and the corresponding SCVs, and in Figure S1, available as Supplementary data at *JAC* Online for the MSSA OM1a strain, with the corresponding numerical values of the pharmacological descriptors presented in Tables S1 and S2, available as Supplementary data at *JAC* Online. All strains behaved alike in the presence of NAC for each antibiotic tested and, specifically, with no statistically significant difference between the *menD* SCV and the OM1b SCV versus their corresponding COL and OM1a parental strains. Thus, not only the higher potency (lower C_s) of β-lactams (and vancomycin to a lower extent) towards the *menD* SCVs was no longer observed, but the intracellular growth of these strains (E_{min}) was brought to the same level as that of the parental

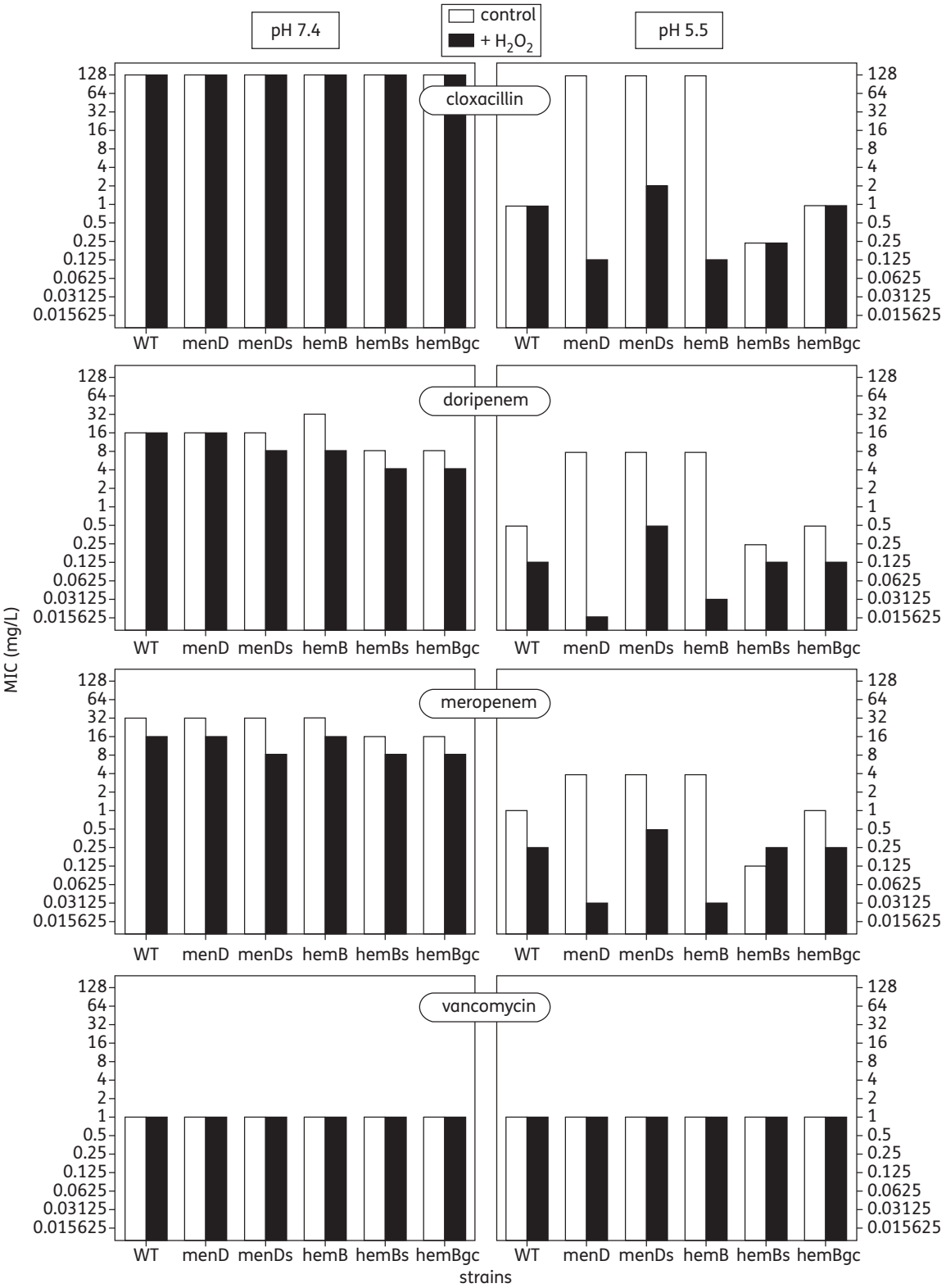

Figure 2. Influence of pH and H_2O_2 on the MICs of cloxacillin, doripenem, meropenem and vancomycin for the isogenic strains of *S. aureus* with an MRSA phenotype [wild-type (WT), *menD* mutant in control conditions (menD) or in medium supplemented with 2 mg/L MSB (menDs), *hemB* mutant in control conditions (hemB) or in medium supplemented with 2 mg/L haemin (hemBs) and the *hemB* genetically complemented mutant (hemBgc)]. MICs were read after 24 h of incubation in broth at pH 7.4 (left-hand panels) or 5.5 (right-hand panels) for bacteria that were pre-incubated (black bars) or not (white bars) with 10 mM H_2O_2 for 30 min prior to antibiotic addition. Experiments have been reproduced three times with similar results.

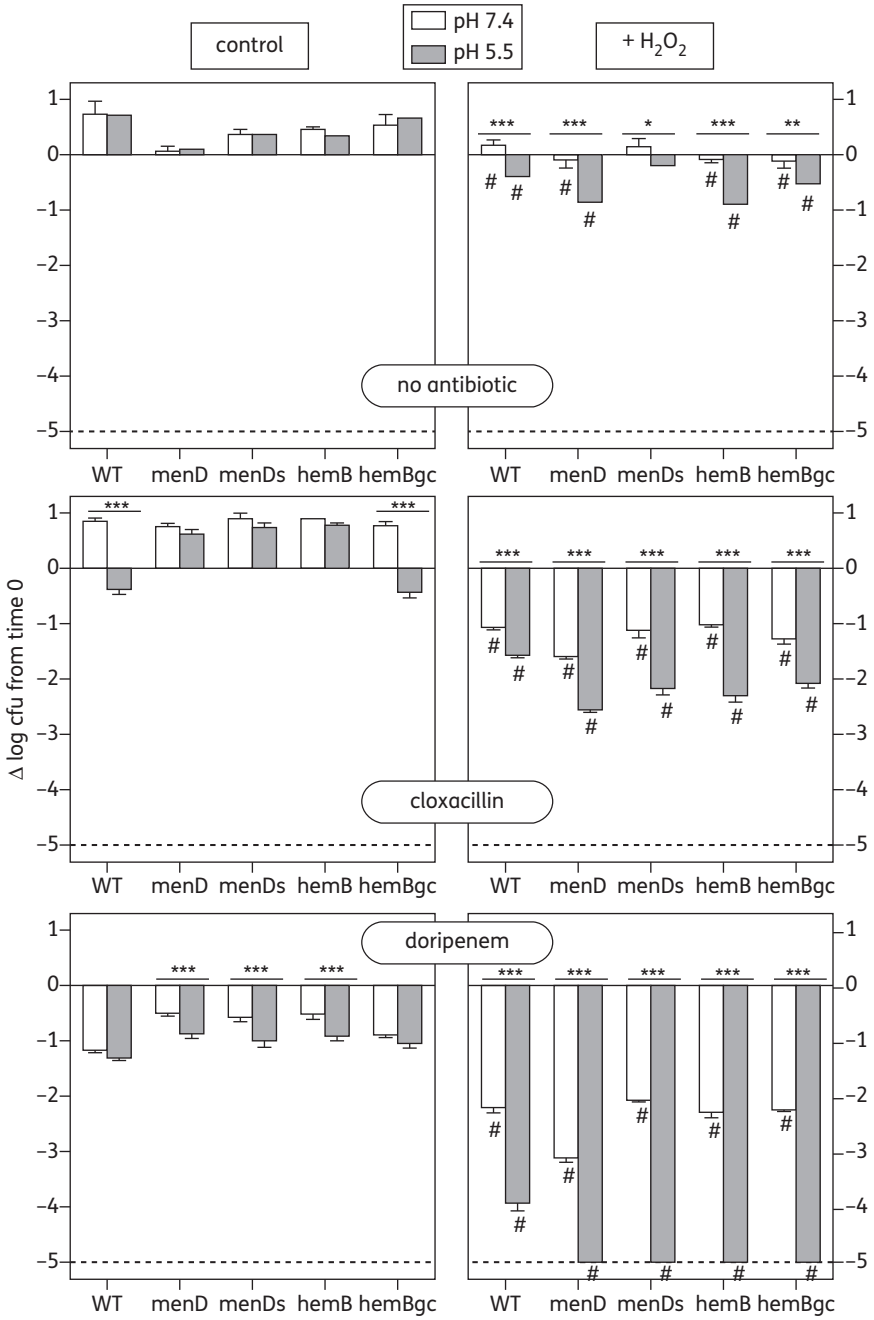

Figure 3. Influence of pH and H_2O_2 on the activity of antibiotics against the isogenic strains of *S. aureus* with an MRSA phenotype [wild-type (WT), *menD* mutant in control conditions (menD) or in medium supplemented with 2 mg/L MSB (menDs), *hemB* mutant (hemB) and the *hemB* genetically complemented mutant (hemBgc)]. Bacteria were pre-exposed (right-hand panels) or not (left-hand panels) to 10 mM H_2O_2 for 30 min, then incubated in broth at pH 7.4 (white bars) or 5.5 (grey bars) in the presence of antibiotics for 5 h at concentrations mimicking their human C_{max} (cloxacillin, 8 mg/L; doripenem, 23 mg/L; meropenem, 50 mg/L; and vancomycin, 50 mg/L). The ordinate shows the change in bacterial counts from the initial inoculum. The continuous horizontal line corresponds to an apparent static effect and the broken horizontal line corresponds to the limit of detection. All values are means ± SD of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Experiments have been reproduced two times with similar results. Statistical analysis (ANOVA with Tukey's *post hoc* test): comparisons between pH 7.4 and pH 5.5 for each individual strain (*$P<0.05$, **$P<0.01$ and ***$P<0.001$); and comparisons between control conditions and pre-incubation with H_2O_2 for each individual strain at a given pH (#$P<0.05$).

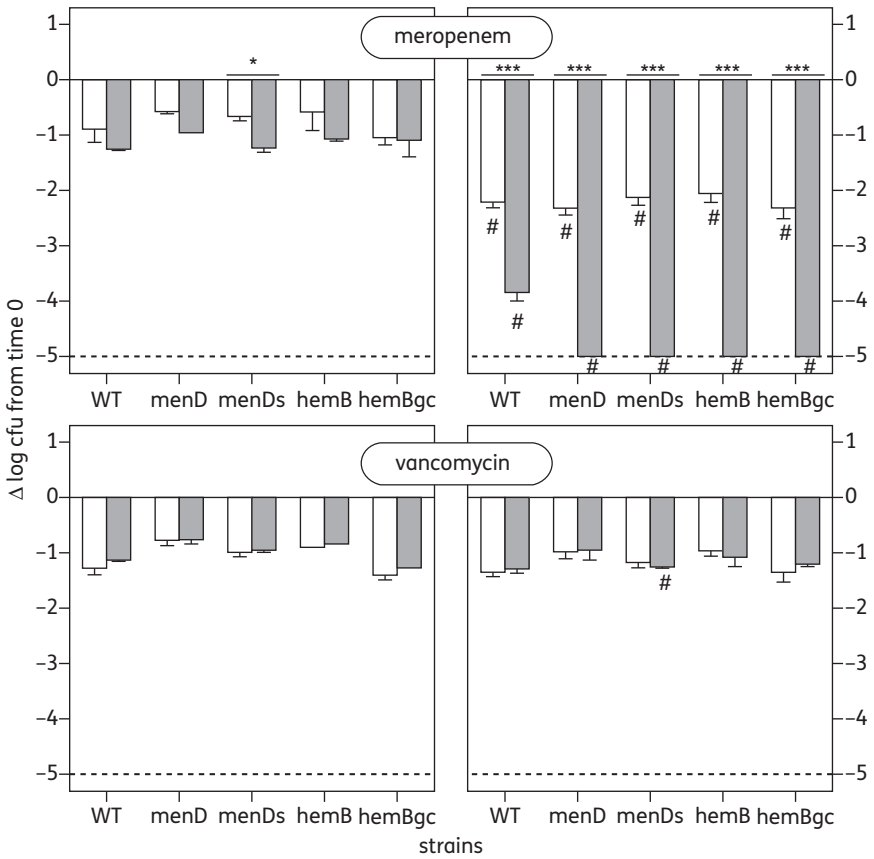

Figure 3. (Continued).

strains and of the other mutants. With the other strains, NAC also slightly reduced the relative potency of cloxacillin and meropenem, but barely affected that of vancomycin and of doripenem.

Influence of H_2O_2 on antibiotic activity against extracellular MRSA

As the previous experiment suggested a key role of oxidant species produced by THP-1 cells in the higher susceptibility of the *menD* MRSA strain towards β-lactams, we examined the effect of a 30 min pre-incubation of all MRSA strains in the presence of 10 mM H_2O_2 on the MICs of these antibiotics. Figure 2 shows that this pre-incubation did not modify the MICs of β-lactams when measured at neutral pH. At acidic pH, no or only a slight effect was seen for the wild-type strain. In contrast, major changes (7–10 dilutions) were seen for the *menD* and *hemB* mutants when tested at pH 5.5. Most conspicuously, the MICs of β-lactams for both SCVs were lower (2–3 $\log_2$ dilutions) than those for the wild-type strain measured at acidic pH. This decrease in MIC was less important for the *menD* strain grown in medium supplemented with menadione (*menD*s strain) and was not observed any longer for the *hemB* mutant when gown in medium supplemented with haemin (*hemB*s strain) or when genetically complemented (*hemB*gc strain). No effect of H_2O_2 on MICs was seen for vancomycin at either pH 7.4 or 5.5.

To further document the effect of H_2O_2 on bacterial killing, we also evaluated its influence at neutral and acidic pH on bacterial counts. To this effect, bacteria were exposed to H_2O_2 for 30 min and thereafter to antibiotics for 5 h [in the absence of H_2O_2; each antibiotic was used at a concentration mimicking its human C_{max} (note that the C_{max} value of cloxacillin was lower than its MIC at neutral pH)]. The results are shown in Figure 3. In the absence of H_2O_2, cloxacillin was ineffective and killing was limited to ~1–1.5 $\log_{10}$ cfu for doripenem, meropenem and vancomycin. Exposure to H_2O_2 only resulted in a very modest killing (≤1 $\log_{10}$ cfu) compared with controls, which was slightly more important for the *menD* and *hemB* mutants at acidic pH. In contrast, the combination of pre-exposure to H_2O_2 and incubation with antibiotics resulted in an enhanced killing for all three β-lactams at pH 7.4, and an apparent eradication for doripenem and meropenem with the SCV mutants at pH 5.5. No effect of H_2O_2 was seen for vancomycin. Testing the antibiotics at a concentration corresponding to their MICs showed similar differences in killing between H_2O_2-exposed and control bacteria (Figure S4, available as Supplementary data at *JAC* Online).

Discussion

SCVs are usually considered as poorly susceptible to antibiotics and refractory to their action when intracellular. Quite unexpectedly, the present data show that the intracellular environment

actually makes a menadione-dependent (*menD*) MRSA SCV hypersusceptible to β-lactams. Noticeably, this strain shows the same intracellular susceptibility as MSSA OM1b SCV (see C_s values in Tables S1 and S2, available as Supplementary data at *JAC* Online), and both are much more susceptible in cells than their corresponding parental strains. Thus, two mechanisms must be envisaged, namely a restoration of susceptibility to β-lactams for the MRSA *menD* SCV and another additional effect that benefits both MRSA and MSSA SCVs.

We showed previously that the activity of β-lactams can be restored against normal phenotype MRSA strains when these are phagocytosed by THP-1 cells, due to the acidic pH that prevails in phagolysosomes.[5,6] This pH effect has been reproduced in acellular systems and shown to be related to a pH-induced conformational change of PBP 2a.[7] This was not the case for the SCVs tested in this study, since acidifying the pH of the broth did not reduce their MICs. We have no simple explanation for this lack of restoration in broth, but suggest that it is linked to the SCV character of the strains, since a marked decrease in MICs could be obtained at acidic pH for the *hemB* SCV after haemin supplementation or complementation. For the *menD* SCV, MSB supplementation was not sufficient to restore the susceptibility to β-lactams at acidic pH, although it allowed for restoration of growth. This may be due to the fact that MSB supplementation does not fully reverse the SCV phenotype of this strain.[2] It shows, nevertheless, that growth impairment *per se* is not the main reason for lack of restoration of susceptibility. A most interesting aspect in our work is actually that restoration of the susceptibility of *menD* and *hemB* SCVs to β-lactams could be obtained by the combined effects of the addition of H_2O_2 and a decrease of pH. This identifies oxidative stress as a potential additional mechanism that could play a critical role intracellularly beyond the effect of pH.

After phagocytosis, *S. aureus* is exposed to the release of reactive oxygen species that are part of the natural defence of phagocytes against invading bacteria.[19–21] Oxidant stress reduces the NADH pool inside bacteria, with, as a consequence, a reduction in the ATP content and growth rate.[22,23] NADH serves as a cofactor for the reduction of menadione in menaquinone, which itself transfers electrons to the haem of cytochromes, providing the energy required for ATP release from the F_0F_1-ATPase.[22] The enzymes responsible for the synthesis of menaquinone and haem, respectively, are defective in *menD* and *hemB* mutants. This may explain why these strains can become incapable of coping with oxidant stress. SCVs, indeed, are known to be highly susceptible to oxidant agents, such as tellurite or selenite.[24] β-Lactams induce the production of oxidant species in bacteria,[25,26] which makes the following sequence of events quite likely: (i) after phagocytosis, *menD* SCV quickly becomes exposed to an oxidative stress, which it can only partially resist (hence its slower growth rate); (ii) β-lactams, because of the combined effect of oxygen reactive species (including H_2O_2) and of the low pH (as demonstrated in broth), will then exert an intense bactericidal effect in spite of the MRSA character of this strain. The increased potency of β-lactams towards the *menD* SCV is no longer observed if NAC is added to the incubation medium, which further supports the role of oxidant species. In this context, a clear correlation has indeed been demonstrated between the resistance of *S. aureus* to oxidative stress and to β-lactams,[27] and, conversely, between its susceptibility to H_2O_2 and to oxacillin.[28] The *hemB* SCV strain will not demonstrate a similar increase in intracellular susceptibility, because the intracellular milieu provides sufficient amounts of haem-like compounds to cause a local reversal of its phenotype. We show, indeed, that the intracellular behaviour of the *hemB* strain is similar to that of the parent strain, with no further modification by the addition of haemin or by complementation.[2] Vancomycin is also reported to trigger the production of oxidant species in bacteria, but only at high concentrations.[26] This is consistent with the lack of effect of NAC on vancomycin intracellular activity in our conditions.

As a conclusion, this paper documents for the first time that the menadione-dependent SCV mutant of an MRSA *S. aureus* strain is much more susceptible to β-lactams in the intracellular environment than are MRSA and MSSA strains with a normal phenotype, because of the combination of local acidic pH and exposure to oxidative burst. While the molecular mechanism(s) of this cooperation remain(s) to be determined, our observations may open research perspectives for the understanding of the metabolic defects occurring in these strains and, possibly, for the management of the infections they cause.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figures S1 to S4 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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DISCUSSION

« Everything should be as simple as it can be,
But not simpler »

ALBERT EINSTEIN

Staphylococcus aureus, a major cause of community- and hospital-acquired infections, has developed resistance to most classes of antibiotics. Methicillin-resistant *S. aureus* (MRSA) strains appeared in the hospital environment after introduction of semi-synthetic penicillins. Compounding the problem of genetically determined transmissible antibiotic resistance is the development of phenotypically resistant, slow-growing subpopulations in chronic bacterial infections. These particular forms are usually associated with a worsening of the disease prognosis. New antibiotics and initiative therapeutic options are urgently needed to improve the management of bacterial infections and a major challenge is to find drugs that act against SCVs.

The present study was therefore designed to get further knowledge of the intracellular fate of two genetically defined menadione- and hemin-dependent SCVs and their isogenic MRSA counterpart, in order to investigate their antibiotic pharmacodynamic profile in human professional or non-professional phagocytes, and to delineate the most rational drugs to use in our current or forthcoming arsenal.

1. Main findings of this work

At the end of this four-year project, our work has permitted to fill an important lack of data in this context and to make some unexpected observations. These main conclusions can be summarized as follows:

1.1. Intracellular Fate of Menadione- and Hemin-Dependent Mutants

In spite of the fact that menaquinone and heme are both required for aerobic and anaerobic electron transport, we observed a very different behavior between menadione- and hemin-dependent SCVs in phagocytic cells. Even if the internalization rate was similar for both SCVs in phagocytic cells, the intracellular growth of our strains appeared completely different. Indeed, the intracellular survival of the *menD* mutant was very slow, but restored by menadione bisulfite supplementation. In contrast, the *hemB* mutant grew at the same rate as the parental strain after 24 hours of infection, without any benefit of a hemin complementation of the medium. We have interpreted this contrasting behavior as denoting a difference in the intracellular availability of menadione and hemin-like substances in the intracellular environment. It has been indeed shown that intracellular levels of menadione are 100-fold lower than the concentration needed to reverse the SCV phenotype in menadione-dependent mutants (250).

Moreover, we also observed that the *menD* mutant was much more sensitive to reactive oxygen species (ROS) than the *hemB* mutant and the parental strain, in a model of THP-1 macrophages. Using N-acetylcystein to inhibit oxidant species production or H_2O_2 to increase ROS level, we indeed demonstrated that the intracellular slow growth was directly linked to hypersusceptibility of the menadione-dependent SCV to the oxidative burst. To explore the possible reasons for these differences, ROS interference with the metabolism of both menadione- and hemin-dependent mutants need to be further examined in details.

1.2. Intracellular Activities of Antibiotics

When comparing the *menD* and *hemB* mutants of the COL strain (Table 5), we showed that the antibiotic pharmacodynamic profile was markedly different against intracellular forms of SCVs in THP-1 cells according to their metabolic defect.

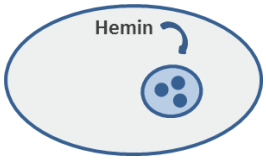
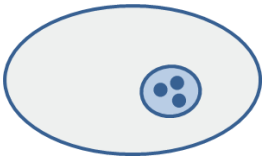
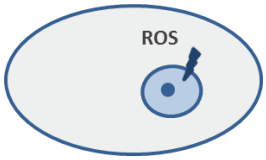
Pharmacological parameters	**Hemin-dependent SCV** 	**Normal Phenotype** 	**Menadione-dependent SCV** 
Potency C_s	=	<	
Minimal Efficacy E_{min}	=	>	
Maximal Efficacy E_{max}	=	=	

Table 5. Comparison of the way that auxotrophism from hemin or menadione affects the pharmacodynamic profile of antibiotics, as deduced from our work

The difference can also be extended to a thymidine-dependent SCV studied previously in our laboratory (186).

Actually, antibiotic activity was (i) globally lower for a thymidine-dependent strain in comparison to the isogenic parental strain, but not restored by thymidine supplementation, (ii) totally similar for the *hemB* mutant and its parental strain and (iii) consistently associated with a much lower minimal efficacy and a slightly increased potency against the *menD* mutant than against the parental strain, but restored by menadione supplementation. Taken together, these data lead thus to revisit the general concept that, intracellularly, SCVs are less susceptible to antibiotics than a normal phenotype strain. A strong influence of auxotrophism on antibiotic response is thus suggested.

1.3. Role of Reactive Oxygen Species in Antibiotics Action

We have demonstrated a critical role of ROS in response to antibiotics depending on the phenotype of the strain on the one hand, and on the antibiotic investigated on the other hand. The *menD* mutant was clearly much more susceptible to oxidative burst than the other strains in the intracellular environment, which markedly improves antibiotic potency towards this strain as compared to the parental strain. Boosting ROS production by activating cells also improves antibiotic activity against other strains, to an extent reflecting the way antibiotic cooperate with ROS to kill bacteria.

1.4. MRSA and β-lactams

We have confirmed that, as other MRSA strains, SCVs become susceptible to β-Lactams intracellularly. However, in sharp contrast to the mechanism described for normal phenotype strains, for which the restoration of activity is merely attributed to acidic pH (151), we demonstrated here that cooperation between vacuolar acidic pH and oxidant species was needed to obtain the same effect against SCVs.

1.5. Clinical Interest of our Results

The altered phenotype of SCVs is associated with a reduced production of virulence factors and a decreased susceptibility to a series of antibiotics, allowing their enhanced intracellular survival (170; 289). All these characteristics present a considerable dilemma to clinicians, since therapeutic options are limited.

As previously stated, thymidine-, hemin- and menadione-dependent SCVs display contrasting behaviors with respect to their response to antibiotics, in direct relation with differences observed in

their intracellular fate. By systematically comparing a large series of antimicrobial agents in a same model, the studies performed here allowed us to delineate drugs that may offer an advantage for the eradication of intracellular foci. In particular, we have highlighted that oritavancin, a promising drug in development, and moxifloxacin were the most effective antibiotics, as previously described in our laboratory against intracellular *S. aureus* and thymidine-dependent SCVs (10; 186).

2. Interests and Limits of our Intracellular Infection Models

2.1. Cell types

Unlike some other intracellular models which use animal cell lines, these cells are from human origin and therefore may be more appropriate to study models of human infections. THP-1 cells have been successfully used in various studies conducted to characterize interactions between *S. aureus* and phagocytes, in a clinical context (73; 206; 100), and to analyze the potential relationship between intracellular pharmacokinetics and pharmacodynamics of antibiotics, or the influence of the intracellular environment on intracellular activity (196; 188). The cell type used constitutes therefore an appropriate model for our project.

As most phagocyte-infected models described in the literature used short incubation times (259; 288; 87; 188), our experiments take also advantage of a successful 24-hours maintained model of intracellular infection. THP-1 cells indeed allow *in vitro* evaluation of intracellular activity against bacteria in active stage of multiplication, which is not the case after 5 hours of incubation where bacteria are still in lag phase of growth (247; 10).

2.2. Bacterial strains

Using some laboratory-generated strains is a first essential step for the development of a new pharmacodynamic model. In order to detail pharmacological responses of menadione- and hemin-dependent SCVs to several drugs, we indeed opted for stable strains. We controlled the specificity of our observations by comparing SCV strains and an isogenic strain and/or a genetically-complemented strain. Our observations may not be directly extended to SCV clinical isolates but they nevertheless allow us to draw provisional conclusions of the potential interest of drugs against SCVs. Yet, they do not represent at all strains collected from clinics. Therefore, recent clinical isolates, essentially differing from laboratory-generated strains by the expression of virulence factors or antibiotic

resistance mechanisms, should be included in our studies to better define the efficiency of antimicrobial agents and/or delineate new specific approaches for these particular isolates.

2.3. Pharmacological Evaluation of Antibiotic Activity

The general aim of our work was to study the intracellular activity of antibiotics against both menadione- and hemin-dependent strains, in order to determine some important pharmacological parameters, such as their minimal or maximal efficacies (E_{min}, E_{max}) and their potency (C_s). However, our experimental set-up presents some limitations that should be improved in the future.

A first limitation is that the intracellular accumulation and subcellular disposition of antibiotics in infected cells has not been measured. A series of studies have been performed in our laboratory to investigate the accumulation and/or the distribution of the antibiotics used in the present study. Yet, high concentrations were generally used because of the limited sensitivity of the technique used for detecting them. In most cases, however, there was a fair degree of linearity in the concentration-accumulation responses and similarity in drug disposition between non-infected and infected cells (280). Furthermore, these studies have rarely compared accumulation in activated or non-activated THP-1 cells.

A second limitation in a pharmacodynamic perspective is that all our experiments were made with drug concentrations remaining constant throughout the observation period. Unless using continuous infusion, this is obviously very different from the *in vivo* situation where concentrations fluctuate over time.

A third limitation concerns the fact that our culture medium contains a small proportion of serum (10%) of non-human origin. However, some of the drugs used in the present work show a high degree of protein binding in human serum (oritavancin, for example). As, only the free fraction will probably be able to penetrate the cells, it is therefore difficult to make a direct parallelism between the extracellular concentrations used in our model and those to which cells will be exposed *in vivo*.

A fourth limitation is that our experiments were limited to 24 hours and could not evaluate the persistence for several days, due to the risk of contamination by extracellular bacteria in samples subjected to low concentrations of antibiotics. So, in order to get a more complete view of the infection process, these infection models will need to be complemented with *in vivo* animal studies.

A fifth limitation concerns the host inflammatory response to infection that cannot be mimicked by *in vitro* studies. Cytokines are endogenous chemical mediators which play an important role in orchestrating the inflammatory cascade of the human body. The production of pro-inflammatory and anti-inflammatory cytokines is strictly controlled by complex feedback mechanisms. Pro-inflammatory cytokines are primarily responsible for initiating an effective defense against exogenous pathogens. However, overproduction of these mediators can be harmful and may ultimately lead to shock, multiple organ failure, and death. In contrast, anti-inflammatory cytokines are crucial for down-regulating the exacerbated inflammatory process and maintaining homoeostasis for proper functioning of vital organs, but excessive anti-inflammatory response may also result in the suppression of body immune function. Moreover, integrated and direct interactions between the invading infectious agents and the soluble mediators of the host's innate immune system have been demonstrated in the literature (303; 37). Some bacteria indeed express specific receptors and use cytokines as growth factors (212; 130) or as altered virulence factors (158).

3. Perspectives

3.1. Pharmacokinetic Studies

There is a clear link between cellular pharmacokinetics and pharmacodynamics because the capacity of drug to rejoin the infected compartment is a prerequisite to activity. Completing this work by studying the pharmacokinetics of antibiotics in the eukaryotic cells used in the project would be of high interest, particularly to compare (i) infected versus non-infected cells, and (ii) monocytes and macrophages (283).

3.2. Impact of Reactive Oxygen Species

Our work has suggested that ROS may be important for some antibiotics to express an intracellular activity. Yet, we have not measured the level of ROS produced by the cells we used. There are a number of well-established methods available to measure intracellular ROS. Most widely used methods involve oxidizable fluorescent dyes, carboxyl derivatives, and other derivatives of fluorescein (248; 219; 251; 296; 175; 190). In addition, there are several chemiluminescent substances that are very sensitive and have been used in measuring intracellular ROS (90; 20; 140). These methods could be applied here to compare ROS production in the different cell types, as well as the influence of infection, antibiotics, or N-acetylcystein on this production.

3.3. Infections, Inflammation and Antibiotics

There is growing evidence that certain antibiotics exert their beneficial effects not only by killing or inhibiting the growth of bacterial pathogens but also indirectly by immunomodulation. The anti-inflammatory properties of macrolides in chronic inflammatory pulmonary disorders were recognized more than 20 years ago and have been well documented in the last decade (192; 221; 249). Recent data suggest that several antibiotics such as tetracyclines (150), cephalosporins (234; 162) and, to a lesser extent, fluoroquinolones (299; 22) may have a beneficial immunomodulatory effect. Moreover, the bactericidal antibiotics rifampin, daptomycin, clindamycin and aminoglycosides kill bacteria without releasing high quantities of pro-inflammatory cell wall components, reducing mortality and long-term sequelae in experimental bacterial sepsis, plague and meningitis (269; 284; 101). Clinically, macrolides have been well established as an adjunctive treatment to β-lactams in pulmonary diseases. For other indications, appropriate clinical trials are necessary before using the immunomodulatory properties of antibiotics in clinical practice.

3.4. Antibiotic Combinations

Therapeutic strategies against multi-resistant *S. aureus* have pointed out the interest of using antibiotic combinations for initial empirical treatment of critically ill patients. Antibiotic combinations have a long been used to provide a broader spectrum of antimicrobial activity. However, it is sometimes used with specific intent of (i) obtaining a synergistic antimicrobial effect, (ii) allowing low-doses to reduce the toxic side-effects, and (ii) preventing or minimizing the like hood of emergence of multi-resistant strains.

Moreover, available data have suggested that combining antibiotics can improve intracellular activity against both normal and SCV phenotypes of *S. aureus* strains (9; 7; 6; 187). It would therefore be worthwhile to evaluate whether combination of antibacterial agents may improve intracellularly the relative potency and/or the maximal efficacy of a series of commonly used antibiotics against menadione- and hemin-dependent SCVs.

3.5. Static versus Dynamic Models

The usual *in vitro* estimations of antimicrobial activity (MICs, dose- and time-kill studies) determined at constant drug concentrations (static conditions) do not take into account variations in drug concentrations occurring over time upon intravenous or oral administration. *In vitro* dynamic

models have been developed, in which a system renewing the medium in order to adjust concentrations over time allows to mimic human pharmacokinetics of antibiotics. These models have been successfully applied in comparative studies with many antimicrobial used against extracellular bacteria (21; 75). It would be interesting to set up this type of model to examine intracellular infections.

3.6. *In Vivo* Activity of Antibiotics

Extrapolation to the *in vivo* situation cannot be made safety at this stage. Beside the importance of pharmacokinetic variations highlighted before, *in* vitro models poorly take into account the role of defense mechanism, and their cooperation with antibiotics, in the control of infection. But few experimental animal models have been published specifically looking for activity against intracellular bacteria. Recently, a remarkable animal model of mixed extracellular and intracellular peritoneal infection has been developed. In this model, mice are infected by peritoneal infection of *S. aureus,* treated with antibiotics, and finally sacrificed (233). Antibiotic activity is then determined in the peritoneal fluid (total activity) and after centrifugation (extracellular activity in the supernatant and intracellular activity in the pellet of peritoneal macrophages). Interestingly, this model generates data that are highly supportive of those obtained in our *in vitro* model.

4. Rational Treatment for the Management of SCVs

Our experimental data have highlighted the interest of the following antibiotics for the control of intracellular SCVs:

Moxifloxacin, a fourth-generation fluoroquinolone, displays a rapid bactericidal effect against both extra- and intracellular bacteria by cooperating with reactive oxygen species. Moreover, these molecules are distributed both in the lysosomes and in the cytosol (35; 246). However, this class of antibiotics should be used judiciously because raising resistance, especially MRSA, is often associated with cross-resistance to other antibiotics (e.g. cephalosporins and aminoglycosides).

Oritavancin, a lipoglycopeptides in development, is rapidly bactericidal against both the extracellular and intracellular forms of SCVs and its activity is not modified by acidic environment or oxidative burst. Moreover, it reaches high level of accumulation in the lysosomes of cells (281). Taken together, these observations suggest that it may become the most widely used choice for infections involving intracellular reservoirs.

β-lactams proved efficacious against intracellular MRSA SCVs, due to a cooperation between acidic environment and reactive oxidant species. Our observation may stimulate future research, including *in vivo* studies aimed at delineating the potential interest of including β-lactams in the treatment of MRSA infections in tissues where pH is acidic.

Of note, rifampin is generally considered as one of the antibiotics of choice for the treatment of intracellular infection and is shown here to produce a fast extra- and intracellular effect. However, its poor safety profile and the easy selection of resistance by target mutation constitute a main limitation to its large use. Combination of rifampin with other molecules has been shown to decrease emergence of resistance, increase intracellular penetration and enhance antimicrobial activity of either agent (182).

5. SCVs & Clinical Perspectives

At the end of this work, we have collected a series of unexpected results about the internalization and the intracellular fate of genetically stable menadione- and hemin-dependent SCVs of the COL parental strain. We have also studied the pharmacodynamic profile of different anti-staphylococcal agents against extracellular and intracellular bacteria, in professional and non-professional phagocytes. These data have allowed revisiting a series of concepts regarding antibiotic activity against SCVs.

As a conclusion to this work, we have globally reviewed *in vitro* data, animal studies and clinical reports describing antibiotic activity against thymidine-, menadione- and hemin-dependent SCVs of *Staphylococcus aureus* to put our data in a more clinically-oriented perspective.

REVIEW PAPER

Antibiotic activity against Small Colony Variants of *Staphylococcus aureus:* Review of *in vitro*, animal, and clinical data

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ABSTRACT

The pathogen *Staphylococcus aureus* uses various strategies to persist in the host. One of them consists in switching to a Small Colony Variant (SCV) phenotype. Phenotypically, SCVs are characterized by a slow growth rate, an atypical colony morphology and unusual biochemical features, constituting a real challenge for identification by the clinical microbiology laboratory. In parallel, their metabolic defects alter their susceptibility to antibiotics. Combined with a high propensity to survive intracellularly, and, for some of them, to form biofilms, this poor susceptibility to antibiotics contributes to therapeutic failures. This paper reviews available literature on antibiotic activity against SCVs of *S. aureus in vitro*, in animal models and in the clinics. *In vitro*, aminoglycosides and antifolate agents show high MICs towards electron transport defective and thymidine-dependent SCVs, respectively. The other antibiotic classes usually show MICs comparable to those measured against parental strains, but are less bactericidal. Intracellularly, auxotrophs for thymidine, hemin or menadione show contrasting behaviors with respect to their response to antibiotics, which can be explained by differences in their intracellular fate. In animal models, SCVs often persist, including in metastatic localizations, in spite of administration of active antibiotics. In the clinics, several case reports mention the selection of SCVs after prolonged administration of antibiotics like aminoglycosides and antifolate

agents but also many other classes. Apparent eradication is obtained after several weeks or even months of aggressive polytherapy combined, when applicable possible, with surgical intervention. Further research is thus warranted for optimizing the treatment of these difficult forms of infection.

INTRODUCTION

Small-Colony Variants (SCVs) of *Staphylococcus aureus* are found in antibiotic-refractory infections such as osteomyelitis, chronic airway infections in cystic fibrosis patients, and device-related infections (see[1] for review). These naturally-occurring variants gain a survival advantage by their ability to persist within eukaryotic cells, which protects them from host defenses and antibiotics.[2-4] SCVs are characterized by non-pigmented, non-hemolytic colonies approx. 10 times smaller than the normal phenotype. This tiny size is often due to auxotrophy for distinct growth factors such as menadione, hemin and/or thymidine.[1-4]

Two major types of SCVs are found in clinical isolates, namely electron transport defective SCVs and thymidine-auxotrophs (Figure 1 illustrates the way these auxotrophisms may affect susceptibility to antibiotics).

An interrupted electron transport is observed in auxotrophs for menadione or hemin, two components essential for the formation of menaquinone and cytochromes, respectively.[5,6] The subsequent decrease in transmembrane potential, which is required for the penetration of cationic antimicrobial compounds,[5,7-9] impairs the activity of aminoglycosides and antifolate antibiotics.[2,10] Gentamicin treatment can select for these SCVs.[10] Aminoglycoside-selected SCVs can harbor cross-resistance to fusidic acid, due to combined mutations in the *rplF* gene encoding the ribosomal protein L6 and in genes required for hemin or menadione biosynthesis.[11]

Thymidine dependence relies on mutations in thymidylate synthase (*thyA*), the enzyme responsible for the conversion of dUMP to dTMP.[12] As sulfonamides and diaminopyridines act upon the biosynthetic pathway of tetrahydrofolic acid, a byproduct of the reaction, thymidine-dependent SCVs often emerge after long-term treatment with trimethoprim/sulfamethoxazole in cystic fibrosis or other patients, and are resistant to these agents.[2]

Worryingly also, exposure to antiseptic agents used in healthcare like the biguanide triclosan can select for an SCV phenotype that does not show any particular auxotrophism but is resistant to this biocide.[13]

As normal phenotypes, SCV can also present classical mechanisms of resistance to antimicrobial agents. Poor intrinsic susceptibility to specific antibiotics combined with acquired resistance have thus become an important problem for effective treatment. This paper reviews the current literature describing antibiotic activity against *S. aureus* SCVs, from *in vitro* and animal models to clinical data.

IN VITRO STUDIES

Susceptibility to antibiotics

Routine *in vitro* susceptibility methods have been developed and approved for testing rapidly growing bacteria. Because SCVs fail to meet these properties, MIC data need to be interpreted with caution.[14] Table 1 presents a synoptic view of susceptibility data for SCVs, classified according to their MSSA or MRSA character and to their auxotrophism. No large epidemiological survey is available so far, but only anecdotal reports for specific strains. As anticipated from the mechanism leading to auxotrophism, only aminoglycosides and

antifolate agents suffer from an almost systematic loss of activity in menadione- or hemin-dependent-, or thymidine-dependent SCVs, respectively. One clinical isolate with SCV phenotype has been found highly resistant to rifampin, due to a mutation in *rpoB* and not to the SCV character.[15] Another study reports also increased MICs to tigecycline in a collection of 48 SCVs isolated from Cystic Fibrosis patients.[16] Otherwise, MICs are globally similar for SCVs and their normal phenotype counterparts. Yet, pharmacodynamic studies suggest that the bactericidal activity of antibiotics may be affected for many drugs against SCVs, as demonstrated for daptomycin, for which bactericidal effects were obtained for prolonged exposure at higher concentrations,[17] and for vancomycin or β-lactams, which showed a reduced efficacy against menadione- or hemin-dependent SCVs.[14,18] For cell wall active agents, this effect may rely on the slow multiplication rate of SCV.

In comparative studies examining several antibiotics against SCVs, fluoroquinolones (moxifloxacin) appeared consistently highly effective against thymidine-, menadione-, or hemin-dependent SCVs, as well as gentamicin against the thymidine-dependent strain, and rifampin and daptomycin against the menadione- and hemin-dependent ones.[18,19] Another study shows that ciprofloxacin MICs were higher for SCVs than for isolates with normal phenotype, while other fluoroquinolones (moxifloxacin, levofloxacin, finafloxacin) did not show marked differences.[20] Of particular interest, the enhanced activity of finafloxacin at low pH might facilitate SCV eradication in acidic environments occurring in patients with osteomyelitis, skin infections, abscesses and cystic fibrosis lung infections.[20] Among other investigational agents, two membrane active drugs, the lipoglycopeptide oritavancin[18,19] and the dicationic porphyrin XF-70,[21] proved as bactericidal against SCVs as against their parental normal-phenotype strain. At a still earlier stage in discovery, tomatidine, the aglycon form of the tomato secondary metabolite tomatine described as an antimicrobial saponin, shows lower MICs against menadione- and hemin-dependent SCVs than against normal phenotype strains but is only bacteriostatic. This activity seems associated with the dysfunctional electron transport system in these SCV variants.[22] Tomatidine is therefore synergistic with aminoglycosides against electron-defective SCVs.[23]

Antimicrobial peptides are an integral part of the host defense against invading microorganisms. Unfortunately, it has been shown that hemin- and menadione-dependent SCVs can emerge upon exposure to subMIC concentrations of protamine[7] and that both types of SCVs are resistant to lactoferrin B.[24] In addition, higher MICs are seen against host cationic peptides like thrombin-induced platelet microbicidal protein (tPMP).[9,25]

Based on these *in vitro* studies, it remains difficult to define optimal therapy for infections due to *S. aureus* SCVs. Reversion to the normal phenotype has been observed in several *in vivo* and *in vitro* models of persistent infection.[26] Revertants might also occur upon *in vitro* testing and may render these microorganisms more susceptible to antibiotics, thereby misrepresenting actual values. In the case of menadione auxotrophs, this reversal can also be obtained *in vivo* by administering vitamin K to patients.[27]

Activity against intracellular bacteria

SCV easily persist intracellularly[3,4] and can even be induced in the intracellular milieu.[28] Studying antibiotic activity against intracellular SCVs is therefore particularly relevant. Several *in vitro* models using human or animal cells have been developed to test intracellular activity.

In models of human monocytes, a hemin-dependent SCV shows an intracellular growth similar to that of a normal phenotype strain, suggesting it finds inside the cells the heme-like compounds required for its growth. On the contrary, a thymidine-dependent SCV grows

slower, and a menadione-dependent strain does not grow over a 24 h incubation time,[18,19] which is supposed to affect their response to antibiotics.

Systematic comparisons of anti-staphylococcal agents have therefore been performed in this model using a pharmacodynamic approach allowing characterizing antibiotic potency and efficacy. The three types of SCV display contrasting behaviors. Against the stable thymidine-dependent SCV isolated from a cystic fibrosis patient, vancomycin, oxacillin, fusidic acid, clindamycin, linezolid, and daptomycin were much less active than quinupristin/dalfopristin, moxifloxacin, rifampin, and oritavancin. Yet, for all drugs, the maximal efficacy was markedly reduced against the thymidine-dependent SCV as compared to that observed against the normal-phenotype and revertant isogenic strains, probably related to its slower growth.[19] Against the hemin-dependent SCV derived from the COL MRSA strain, oritavancin and moxifloxacin were also much more effective than vancomycin, gentamicin, daptomycin, or rifampin, and their activity was undistinguishable from that observed against the parental strain, in line with the restored intracellular growth of this SCV.[18] Against the menadione-dependent SCV also derived from the COL MRSA strain, the maximal efficacy of antibiotics remained unaffected, which is surprising in view of its slow intracellular growth. Yet the parameters that are affected are rather the amplitude of the dose-response curves (which is reduced) and the potency of antibiotics (which is increased).

Among other agents highly active extracellularly, XF-70 showed a rapid bactericidal effect at low concentrations against an MSSA and its hemin-dependent SCV phagocytized by human polymorphonuclear neutrophils.[21] In accordance with its bacteriostatic character, tomatidine only inhibits the intracellular replication of SCVs within polarized cystic fibrosis-like epithelial cells.[22]

Because of the difficulty to eradicate intracellular SCVs, two strategies have been evaluated to improve antibiotic activity.

The first one consists in associating antibiotics with different modes of action. Several studies suggest indeed that combinations do improve intracellular killing of SCVs, especially when they include rifampin or other bactericidal agents like oritavancin.[29,30]

A second strategy consists in reinforcing cell defense mechanisms. When human monocytes are activated in macrophages by phorbol 12-myristate 13-acetate, intracellular growth of menadione- or hemin-dependent SCVs and of their normal parental strains is reduced. This does not affect the maximal efficacy of antibiotics but rather increases their potency (lower concentration needed to reach a static effect intracellularly). Interestingly, this effect is however not systematic, being observed (a) for antibiotics like gentamicin or moxifloxacin, the MIC of which is reduced in the presence of H_2O_2, but not for oritavancin or vancomycin, the MIC of which is not affected by H_2O_2 and (b) for the hemin-dependent mutant and its parental strain, but not for the menadione-dependent mutant.[31,32] These data therefore suggest a useful cooperation between antibiotics and host defenses for antibiotics that act in synergy with reactive oxidant species. Conversely, antibiotics for which cell activation has minimal effect may remain as effective when host defenses are weakened. The menadione-dependent mutant may escape the effect of cell activation, antibiotics showing already higher potency towards this strain in non-activated cells.[18] This observation suggests that the menadione-dependent strain is hypersusceptible to oxidant species, which is further corroborated by its unanticipated susceptibility to β-lactams (see Figure 1 for an illustration of the potential link between menadione-dependence and susceptibility to oxidant species). β-lactams regain activity against normal phenotype MRSA intracellularly, due to an opening of the conformation of the PBP2a occurring at the acidic pH prevailing in phagolysosomes.[33] Interestingly enough, this effect is exacerbated for the menadione-dependent SCV of the COL MRSA strain, which was 100 to 900-fold more susceptible to β-lactams when inside the cells than its parental strain.[31] A same trend is also

observed for an MSSA strain, but the shift in potency is less marked. *In vitro* studies suggest that this high potency is due to a cooperation between acidic pH and oxidant species because it can be reproduced when measuring MICs at acidic pH after pre-exposure to H_2O_2.

Activity against biofilms

Biofilm is another form of persistent infection that is also difficult to eradicate.[14] A recent study suggests that menadione-dependent SCVs are more prone to form biofilms *in vitro* than thymidine-auxotrophic ones.[34] This is consistent with the fact that menadione-dependent strains are mainly found in osteomyelitis or device-associated infections that are often biofilm-related. The situation may however be different *in vivo*, since biofilms are also frequent in cystic fibrosis patients, who are rather infected by thymidine-dependent SCVs. In this case, the switch to a high biofilm producer SCV phenotype can be induced by quorum sensing molecules produced by *Pseudomonas aeruginosa* also present in the respiratory tract of these patients.[35]

Exposure to antibiotics may induce biofilm formation. Subinhibitory concentrations of gentamicin trigger not only SCV emergence but also biofilm formation in *S. aureus*, due to the activation of the alternative transcription sigma factor B (SigB).[36] On the contrary, non–auxotrophic SCV selected by triclosan appear as weak biofilm producers.[37]

Very few studies have examined antibiotic activity against SCVs growing in biofilms. Biofilms of the reference MSSA strain ATCC29213 are much more resistant to the action of oxacillin, cefotaxime, amikacin, ciprofloxacin, or vancomycin, none of them being able to reduce bacterial counts at high multiples of their respective MICs.[38] Surviving bacteria within the biofilm harbor a persister phenotype, but only ciprofloxacin selects also for SCVs within the biofilm. These SCVs do not seem associated with increased resistance within the biofilm as they easily revert to a normal phenotype upon subculture. A stable menadione-dependent mutant of the same strain shows however a higher propensity to form biofilm; its sensitivity to antibiotics is reduced in broth but has not been examined in biofilms in one study,[39] whereas another study showed a profound decrease in antibiotic susceptibility in the surface-associated form as compared to the planktonic.[14] These studies were carried out with a single reference strain and need to be extended to more strains, including clinical isolates.

ANIMAL MODELS

A few animal models have been developed to study the fate of SCVs as well as their response to antibiotics. They suggest in many cases, but not systematically, that SCVs may be more difficult to eradicate and contribute therefore to the persistence of infection.

In rabbit endocarditis models, both hemin- and menadione-dependent mutants of the 8325-4 strain are equally able to establish the infection, but only the hemin-dependent mutant achieves a same bacterial density in the spleen or the kidneys as its parental strain, which is not the case for the corresponding menadione-dependent mutant.[8] In concordance with observations performed in infected cells, this again suggests that target organs may have been replete with hemin during the course of endocarditis as a consequence of hemorrhagic necrosis, restoring the wild-type phenotype. Oxacillin reduces bacterial counts in all target tissues for animals infected with the parent strain or the hemin-dependent mutant but only in vegetations and not in kidneys and spleen for animals infected by the menadione-dependent mutant, probably related to is low multiplication rate.[8] In another study, gentamicin treatment easily selects for SCVs that are themselves able to re-establish the infection and to colonize blood, heart valve vegetations, spleen, kidney, and liver as

efficiently as the parental strain, but they are less virulent.[40] A β-lactam is effective in this model; combination with an aminoglycoside is useful against the normal phenotype strain but not against the SCV.

In a mouse mastitis model, the cephalosporin cephapirin shows a reduced ability to control the infection caused by the hemin-dependent mutant of the strain Newbould 305 as compared to its isogenic parent.[41] This occurs despite the facts that both strains display similar MICs and that the SCV mutant shows a lower propensity to colonize the mammary glands.

In a rabbit model of chronic osteomyelitis, vancomycin loaded in a hydroxyapatite cement proves highly effective to treat the infection caused by *S. aureus* SCVs isolated from patients with osteomyelitis, none of the infected animals treated showing signs of infection after 42 days thanks to the slow release of high concentrations of antibiotic.[42]

In a mouse peritonitis model allowing for testing simultaneously activity against extracellular and intracellular bacteria, colonization of both the extracellular and intracellular compartments were lower for a menadione-dependent SCV than for its parental counterpart, leading to lower signs of sickness. Yet, metastatic spread to the kidneys and persistence at 96 h was observed for the SCV.[43] Linezolid or dicloxacillin were able to control both intra- and extracellular infections caused by either phenotype but not to clear SCVs from the kidney after a single dose. Parallel experiments performed in the THP-1 *in vitro* model, show, as described above, a reduced intracellular growth for the menadione-dependent mutant, an increased potency for antibiotics against this strain, but no change in maximal efficacy, which reached about 1-log reduction form the initial inoculum, as also observed *in vivo.*

CLINICAL DATA

There are no large clinical trials examining therapeutic options for SCVs infections, but only case reports or studies on small series describing successful or unsuccessful approaches. Table 2 summarizes these studies, describes the antibiotics used prior SCV identification and for their subsequent treatment. Globally, SCVs have been isolated after long and/or unsuccessful antibiotic exposure and needed aggressive and prolonged polytherapy for their eradication. Effective regimens often include rifampin or a fluoroquinolone, as well as quinupristin/dalfopristin in one specific case,[34] which is consistent with their high intrinsic activity *in vitro*. β-lactams (for MSSA) or glycopeptides (for MRSA) are also often administered, although considered as less active against SCVs based on *in vitro* testing.[14,18] When applicable, surgical debridement or removal of infected devices are probably key determinants in clinical success. Two studies mention the administration of vitamin K aimed at reversing the SCV phenotype (see Table 2). Globally, however, antibiotic choices remain largely empirical. At the present time, no guideline has been proposed for treating infections associated to this particular phenotype. In spite of apparent favorable clinical and microbiological responses, patient's careful follow-up remains essential because SCV infections have been associated with recurrence after intervals as long as 54 years.[27]

Notably, prolonged treatment may also lead to selection of resistance, further complicating treatment. Thus, a remarkable adaptive response of *S. aureus* to antimicrobial challenge during chronic infection was demonstrated for an SCV isolated from a patient with persistent and recurrent methicillin-resistant *S. aureus* (MRSA) bacteremia who received an apparently extensive and appropriate antimicrobial therapy combining rifampin, ciprofloxacin, and vancomycin (thereafter replaced by linezolid)[15] (see Table 2). The isolated SCV shows indeed resistance to linezolid (23S RNA ribosomal methylation), rifampin (mutation in *rpoB*), fluoroquinolones (mutation in *parC*), and β-lactams (plasmid-encoded β-

lactamase). Likewise, thymidine-auxotrophs of *S. aureus* have been shown to be hypermutable and therefore might be more likely to acquire mutational antimicrobial resistance than normal colony phenotypes.[44] This hypermutability may explain the emergence of resistance to rifampin and daptomycin during treatment in a clinical case report.[34] Yet, emergence of resistance during treatment is not systematically associated to selection of SCV. No correlation was found for example between treatment-related selection of macrolide-resistant *S. aureus* in cystic fibrosis patients receiving long-term azithromycin and SCV isolation.[45]

CONCLUSION

Although clearly challenging for both the microbiologist and the clinician, SCVs of *S. aureus* remain an ill-explored field, at least with respect to the more appropriate therapeutic options to prevent their emergence on the one side and to eradicate them when present on the other side. While long-term therapy with gentamicin and anti-folate agents is clearly associated with their selection, clinical reports suggest that other drugs may also be incriminated. In vitro susceptibility testing should also be performed in conditions allowing examining SCV sensitivity (48 h incubation). Because clinical investigations are probably difficult to perform, *in vitro* or animal pharmacodynamic models may be of great help to determine the conditions of antibiotic exposure selecting for SCVs (in the line of studies demonstrating conditions of exposure selecting for resistance), or to define antibiotic regimens or drug combinations that are more prone to act upon these slow-growing, metabolically-defective strains. Strategies aimed at favoring their reversion or taking advantage of their metabolic defects may also be wise to investigate in the future.

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The authors have no conflict of interest to declare.

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Table 1: MICs of antibiotics against *S. aureus* with normal or SCV phenotype [a]

MIC values (mg/L) of antibiotics according to phenotype[a]																
Class	Antibiotic	Strains														
		wild-type			SCV and auxotrophism [b]											
					Not Specified				MSSA				MRSA			
		Not Specified	MSSA	MRSA	Not Specified	men	hem	thy	Not Specified	men	hem	thy	Not Specified	men	hem	thy
Aminoglycosides	Amikacin	2 [40]	<0.031-2 [39,40]	1 [40]	**64** [40]					**64** [39]	0.5-2 [40]					
	Gentamicin	<0.125-4 [2,16,40,46]	<0.031-8 [4,14,22,30,39,41,46,47]	0.25 -1 [18,48]	**<0.125->128** ; 0.75-**4;** **16** [2,16,40]	**16** [46]			**8->8**; **8** [22,47]	**8;** **12** [14,39,46]	**0.5**; 2-4; **4**; **4-8** ; 16; [4,22,30,41,47]	0.125; 4 [19,30]	0.3 [15]	0.5; **32** [18,48]	**1;** **32** [18,48]	
	Kanamycin		0.25 [4]	2-4 [48]							**2** [4]			**64-128** [48]	**64-128** [48]	
	Tobramycin			0.5-1 [48]										**32** [48]	**32** [48]	
Antifolates	Trimethoprim		2 [47]						1-**8** [47]		**8** [47]					
	Trimethoprim-Sulfamethoxazole	0.004-32 [2,16]	0.06; 0.064 [47,49]		0.023->32; **> 32** [2,16]				0.06-**0.5** [47]		**0.5** [47]	**>32** [49]				
Penicillins	Penicillin	0.016–24 [2]			0.016–32 [2]											
	Cloxacillin		0.25 [31]	128 [31]						0.125 [31]				128 [31]	128 [31]	
	Nafcillin	0.5 [40]			0.5 [40]											
	Oxacillin	<0.06-2 [30]	0.06-0.5 [8,14,19,22,30,39,41,47]		<0.06-**>64** [30]			0 .125; 0.5 [19,30]	0.06-0.12 ; 0.12-**1** [22,47]	0.25; 0.5; **2** [8,14,39]	0.03-0.06 ; 0.06 ; 0.5 [8,22,30,41,47]					
	Piperacillin	0.064–32 [2]			**0.19**–32 [2]											

Cephalosporins	Cefuroxime	0.5–3 [2]	1-2 [50]	4->128 [50]	**3-8** [2]	1-2 [50]				2->128 [50]		
	Cefotaxime		1 [39]				**4** [39]					
	Ceftazidime											
	Ceftobiprole		0.13-0.5 [50]	0.5-2 [50]		0.13-0.25 [50]				0.5-4 [50]		
	Cephapirin		0.12 [41]					0.06 [41]				
Carbapenems	Doripenem		0.06 [31]	16 [31]			0.03 [31]				32 [31]	16 [31]
	Meropenem		0.125 [31]	32 [31]			0.06 [31]				32 [31]	32 [31]
Fluoroquinolones	Ciprofloxacin	0.032-32 [2,16]	0.12-0.5 [20,22,39,41]	0.5-32 [15,20]	0.03->32 ; 0.064–4 [2,16]	0.12 [22]	0.5 [39]	0.06 ; 0.12-0.25 ; 0.5 [20,22,41]	0.5 [20]	2 ; 8-32 [15,20]		
	Levofloxacin		0.25-0.38 [20]	3- >32 [20]				0.25 [20]	0.25 [20]	4- >32 [20]		
	Moxifloxacin		<0.03-0.25 [19,20,50]	0.03-4 [18,20,50]		<0.03-0.13 [50]		0.094 [20]	0.094 ; 0.125 [19,20]	2-8; 1-8 [20,50]	0.125 [18]	0.125 [18]
	Finafloxacin		0.19-0.38 [20]	1.5-4 [20]				0.5 [20]	0.25 [20]	1-6 [20]		
Quinolones	Fleroxacin		1 [14]				1 [14]					
Glycopeptides	Vancomycin	0.25-2 [16]	0.5-2 [14,19,22,30,31,39,41,47,51]	1-4 [15,31,51]	0.125-**2** [16]	1; 2 [22,47]	0.5 ; 1 ; 2 [14,31,39]	0.5 ; 2 [22,30,41,47,51]	0.5; 2 [19,30]	8 [15]	1 [31]	1; [31,51]
	Teicoplanin			3 [15]						8 [15]		
	Telavancin		0.125 [19]						0.125 [19]			
	Oritavancin		0.03 [19]	0.25 [18]					0.015 [19]		0.125 [18]	0.03 [18]

Lipopeptides	Daptomycin		0.125-1 [17,19,30]	0.19-1 [15,17,18]			1; 2 [17,30,50]	0.125; 0.5 ; [19,30]	0.38 [15]	0.5 [18]	0.5 [18]; 2 [17]
Ansamycins	Rifampin		0.002-0.125 [19,30,41]	0.016-0.023 [15,18]		0.03-0.008 [47]	0.008-0.015 ; 0.015; 0.06 [30,41,47]	*0.0005* [19]	**> 256** [15]	0.016 [18]	0.016 [18]
Macrolides	Erythromycin		0.25-0.5 [22,41]			0.12 [22]	**1-2** [41]				
Lincosamides	Clindamycin		0.06 -0.125 [19,30]				0.25 [30]	0.125; 0.25 [19,30]			
Oxazolidinones	Linezolid	0.25-4 [16]	0.5-4 [19,50]	0.5-1 [15,50]	0.25-4 [16]	0.5-**4** [50]		2 [19]	1-2; 2; [15,50]		
Other antibiotics	Fusidic Acid		0.125 [19]					0.03 [19]			
	Quinupristin-Dalfopristin		0.5 [19]					0.5 [19]			
	Tigecycline	0.015-0.5 [16]	0.125 [19]	0.19 [15]	0.015-**2** [16]			0.125 [19]	0.19 [15]		
	XF-70		0.5-1 [52]	0.25-1 [52]			0.25-0.5 [52]				0.25-1 [52]
Other agents	Tomatine		>16 [22]			>16 [22]	>16 [22]				
	Tomatidine		>16 [22]			*0.12* [22]	*0.12* [22]				
	Lysostaphin		2 [41,47]			1-4 [47]	1 [41,47]				
	Lactoferrin B		16-64 [24]	256 [24]	**>256** [24]		**>256** [24]	**>256** [24]		**>256** [24]	**>256** [24]

[a] Only studies comparing normal phenotype and SCV strains have been included in this table

[b] Values in bold correspond to MICs that are at least 2 dilutions higher than those of the corresponding parental strain with normal phenotype; Values in italics correspond to MICs that are at least 2 dilutions lower than those of the corresponding parental strain with normal phenotype

[c] men: menadione-dependent; hem: hemin-dependent; thy: thymidine-dependent

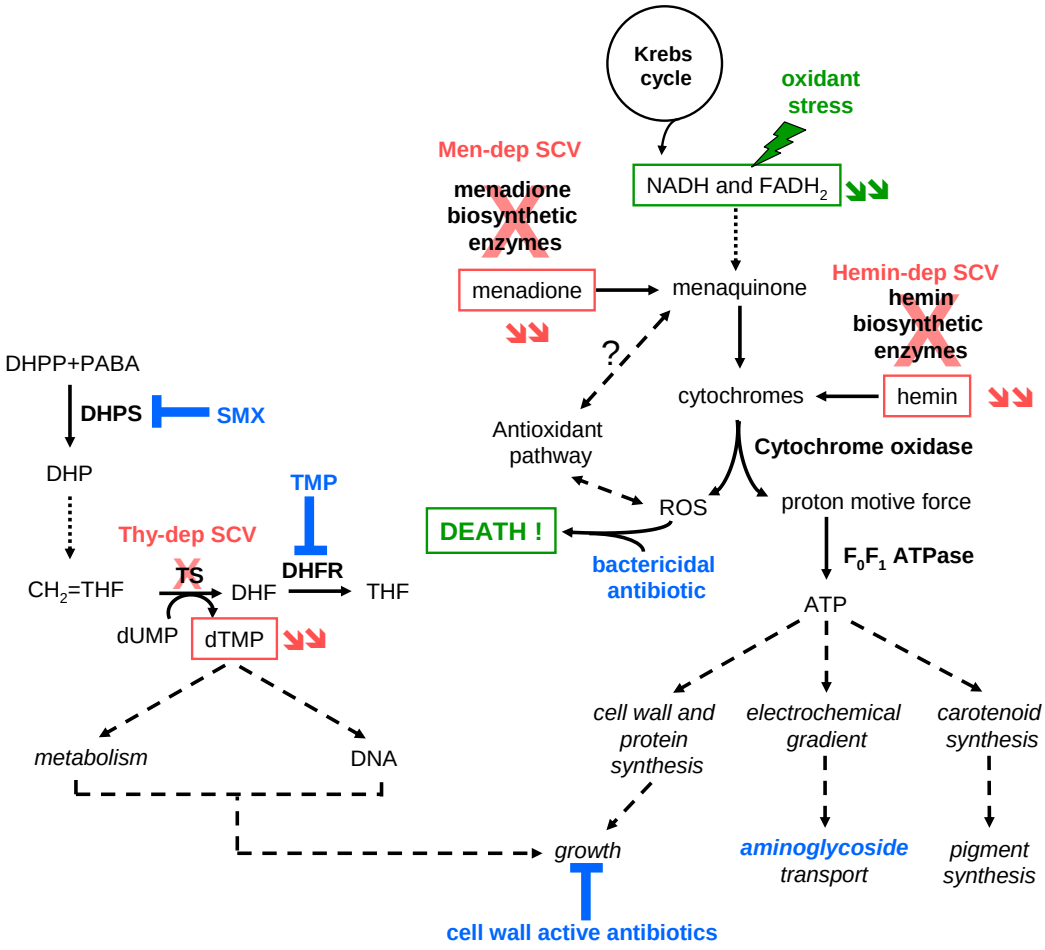
Table 2 : Summary of case reports of infections by SCVs of *S. aureus*, with description of antibiotics received before and after isolation of the SCV and of treatment outcome

Infection	Previous treatment	SCVs characteristics	Treatment	Clinical outcome (time after diagnosis of SCV)	Reference
Bacteriemia	Ciprofloxacin, rifampin,vancomycin, linezolid	MRSA, resistant to fluoroquinolones, rifampin, linezolid Not hemi- or menadione-dependent	Linezolid + TMP-SMX[a]	Unknown	15
Osteomyelitis	Cefotaxime, gentamicin beads		Surgical debridement coupled with antibiotic combination (cefotaxime + ciprofloxacin)	Cure (2 years)	53
Osteomyelitis (4 cases)	Gentamicin beads	Hemin or menadione-dependent		Failure	10
Sternoclavicular arthritis		Hemin or menadione-dependent; resistant to gentamicin	Intraveinous nafcillin followed by oral rifampin; Cloxacillin; oral vitamin K	Cure (1 month)	54
Prostethic joint infection (5 cases)	Rifampin + levofloxacin in 5/5 after flucloxacillin (2/5) or vancomycin + cefepime (1/5)	1/85 resistant to sulfamethoxazole/trimethoprim; 1/5 resistant to rifampin	Flucloxacillin + rifampin (1/5); levofloxacin + rifampin (1/5); flucloxacillin (1/5); flucloxacillin followed by levofloxacin + rifampin (1/5); penicillin + levofloxacin (1/5)	Cure or probable cure (3-23 months)	55
Recurrent abscess in AIDS patient	Clindamycin	thymidine-dependent, MRSA, resistant to erythromycin, clindamycin, ciprofloxacin, gentamicin, TMP-SMX	vancomycin	Failure	56
Abscess	Clindamycin	Hemin or menadione-dependent Resistant to penicillin, ampicillin and tetracycline	Flucloxacillin + rifampin	Cure (4 weeks)	57

Infection of the peritoneal dialysis exit site	Ciprofloxacin, vancomycin, amoxicillin/clavulanic acid, cephalexin	MRSA			[58]
Endocarditis related to a pacemaker-lead infection.	Aminoglycoside and vancomycin	Hemin-dependent; resistant to rifampin	Flucloxacillin	Cure (7 months)	[59]
Endocarditis in hemodialysis patient with pacemaker	Cefuroxime, rifampin, fusidic acid, dicloxacillin, vancomycin		Intravenous cefuroxime and vancomycin for 4 weeks followed by prophylactic oral cefuroxime	Cure	[60]
Left ventricular assist device infection and prosthetic valve and pacemaker endocarditis	Vancomycin, rifampin, gentamicin, daptomycin, and TMP-SMX	Thymidine-dependent; MRSA; resistant to tobramycin, amikacin, kanamycin, rifampin, daptomycin, erythromycin, levofloxacin, and TMP-SMX	Replacement of infected prosthetic tricuspid valve and left ventricular assist device + vancomycin, gentamicin, quinupristin-dalfopristin, rifampin,TMP-SMX	Cure (> 7 months)	[34]
Brain abscess	Meropenem, clindamycin and gentamicin; intrathecal gentamicin	Hemin-dependent; MRSA	Combination of vancomycin and rifampin followed by prolonged treatment with teicoplanin	Cure (3 months)	[61]
Meningitis and ventriculoperitoneal shunt infection	Ciprofloxacin, vancomycin	MRSA	Combination of ciprofloxacin, vancomycin, and rifampin	Cure (4 months)	[62]
Multiorgan infection	Amoxicillin/clavulanate, ampicillin, gentamicin, cefotaxime, doxycycline, vancomycin	MSSA, gentamicin-resistant	Combination of oxacillin, rifampin, TMP-SMX, and vitamin K	Cure (3 months)	[63]

[a] sulfamethoxazole/trimethoprim

Figure 1. Illustration of the mechanisms leading to SCVs phenotype in *S. aureus* and of their link with reduction in susceptibility to specific antibiotic classes (adapted from references[1,5]). Electron transport deficient SCVs show alterations in the pathways leading to the synthesis of menadione or hemin, with as a consequence a reduction in the amount of ATP produced. This leads to a reduced growth rate, which may affect the efficacy of antibiotics active upon dividing bacteria, as cell wall active agents, and to a reduction in transmembrane potential, which impairs aminoglycoside uptake. Menadione-dependent SCVs are hypersusceptible to oxidant species, possibly because of a reduced electron transport and an alteration of the induction of antioxidant pathways (shown to be menaquinone-regulated in gram-negative bacteria[64]). Thymidine-dependent SCVs are unable to convert dUMP into dTMP (using dihydrofolate [DHF] as a cofactor) due to mutations in thymidylate synthase (TS), hence a depletion in dTMP. These strains are unsusceptible to antifolate agents that act on successive steps in this pathway, namely to sulfamides like sulfamethoxazole (SMX), which inhibit t he dihydropteorate synthase (DHPS) producing dihydropteorate (DHP) from dihydropteridinepyrophosphate (DHPP) and para-aminobenzoic acid (PABA), and to diaminopyridines like trimethoprim (TMP), which inhibit the dihydrofolate reductase (DHFR) catalyzing the reduction of DHF in tetrahydrofolate (THF). They also show a reduced growth rate. Globally also, antibiotics may be less bactericidal towards electron-transport deficient SCV due to reduced ROS (radical oxidant species) production.[65]



ANNEXES

1. Introduction

As mentioned earlier, Small-colony variants (SCVs) of *Staphylococcus aureus* can be isolated from the chronically infected airways of patients suffering from Cystic Fibrosis (CF). We have then started working on a human bronchial epithelial cell line, the CFBE41o-, to use a more relevant cell type in relation to CF. For now, we have obtained preliminary data documenting the intracellular survival of our strains and their susceptibility to antibiotics at fixed concentration in isogenic epithelial cell lines differing in their CFTR expression.

1.1. Cystic Fibrosis

Cystic fibrosis (CF), one of the most prevalent genetic diseases of the Caucasian population, is caused by the functional defect of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR). CFTR, a member of the ABC transporter family, is a protein kinase A (PKA)-regulated chloride channel and regulator of numerous epithelial transporters (225; 224). The multifaceted clinical manifestations of CF are, in part, attributed to the impaired transepithelial salt and water movement, culminating in recurrent bacterial infection, colonization, and hyper-inflammation of the lung, representing the major cause of morbidity in CF (27). The mechanism of the CF lung disease, though not completely elucidated, can be attributed to the combination of defective airway surface liquid hydration and mucocilliary clearance, excessive mucus secretion, and Na^+ resorption (27).

The most prevalent disease-causing mutation is deletion of a single phenylalanine residue at position 508 (F508del [145; 279]). Wild-type (WT)-CFTR moves out of the endoplasmic reticulum (ER) to the Golgi apparatus, where it acquires complex-glycosylation, and is targeted to the apical plasma membrane, where it functions as a chloride channel. In contrast, F508del-CFTR is retained in the endoplasmic reticulum, presumably due to misfolding. The CFTR molecules retained in the ER are immature core-glycosylated and are degraded by the ER-associated degradation pathway. CFTR is then ubiquitinated and retrotranslocated to the cytosol, where it undergoes proteasomal degradation (121; 95).

While the role of CFTR in epithelial cell function has been the subject of extensive research, its role in other cells is largely unknown. However, it was recently shown that CFTR function was required not only in endothelial cells for hypochlorous acid production within phagolysosomes allowing an effective bacterial killing (276; 61; 198), but also in smooth muscle where it plays an

unexpected but fundamental role in the autonomic and hormonal regulation of the vascular tone (226).

CF has a unique set of bacterial pathogens that are frequently acquired in an age-dependent sequence. The pattern of age-specific prevalence as well as overall prevalence of these pathogens in the CF population is demonstrated in Figure 1 from the Cystic Fibrosis Foundation Patient Registry data (51). Of the organisms causing infection in CF, only *Staphylococcus aureus* may be pathogenic in immune-competent individuals. *Pseudomonas aeruginosa*, *Burkholderia cepacia complex*, non-typeable *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, and *Achromobacter xylosoxidans* are all considered opportunistic pathogens. All of these organisms can variably be associated with pulmonary exacerbations in CF.

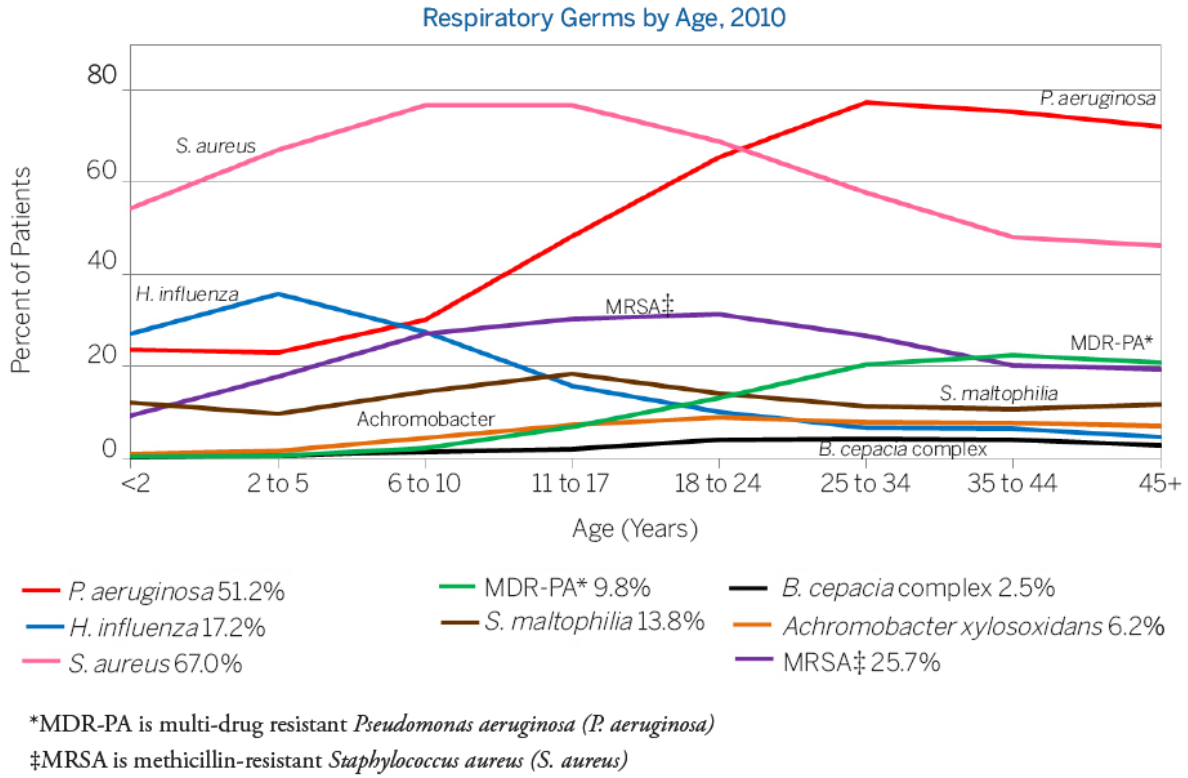


Figure 1. Age-specific prevalence of airway infections in patients with CF (51). Organisms reported to the U.S. Cystic Fibrosis Patient Registry, 2010. Overall percentage of patients (all ages) who had at least one respiratory tract culture (sputum, bronchoscopy, oropharyngeal, or nasal) performed in 2010 that was positive for the following organisms: *P. aeruginosa* (red line), 51.2%; *S. aureus* (pink line), 67.0%; MRSA (purple line), 25,7%; *H. influenzae* (blue line), 17.2%; *S. maltophilia* (brown line), 13.8%; *Achromobacter xylosoxidans* (orange line), 6.2%; *B. cepacia complex* (black line), 2.5%.

The importance of non-fermentative Gram-negative species is well known, but an additional matter of concern for CF patients is the emergence of MRSA (26; 56; 80; 265). The potential negative

effect of MRSA on pulmonary function has been demonstrated in ample numbers of patients (55; 173; 70; 223) but further studies are needed to verify possible diverse pathogenic effects of MRSA on the lung in relation to different molecular characteristics of MRSA strains. As for other pathogens, eradicating MRSA from the airways of patients affected by CF can theoretically benefit the patient and reduce the possibility of diffusion of these bacteria.

In addition, it has been recently shown that *P. aeruginosa* simultaneously suppresses the growth and enhances the aminoglycoside resistance of *S. aureus* by producing an anti-staphylococcal substance, 4-hydroxy-2-heptyl-quinoline-N-oxide (HQNO), a compound found in the sputum of infected CF patients. Moreover, prolonged exposure to HQNO selects for aminoglycoside-resistant genetic variant (SCV) *S. aureus* (116).

1.2. CFBE41o-

The CFBE41o− cell line was originally derived from a bronchial tissue isolate of a CF patient homozygous for the F508del CFTR mutation and immortalized (105; 148; 97; 258). In order to develop stable cell lines, WT- or F508del-CFTR cDNA was stably introduced into the parental CFBE41o^{-} cells by electroporation. RT-PCR analysis shown that recombinant WT- and F508del-CFTR transcripts were detected in transduced cell lines whereas expression of endogenous CFTR in parental cells was below the level of detection (named KO CFTR [13]).

Confocal microscopy data derived from human epithelial cell line expressing WT-CFTR showed a diffuse staining of CFTR predominantly associated with the apical membrane, in contrast to KO- or F508del-CFTR cells (Figure 2).

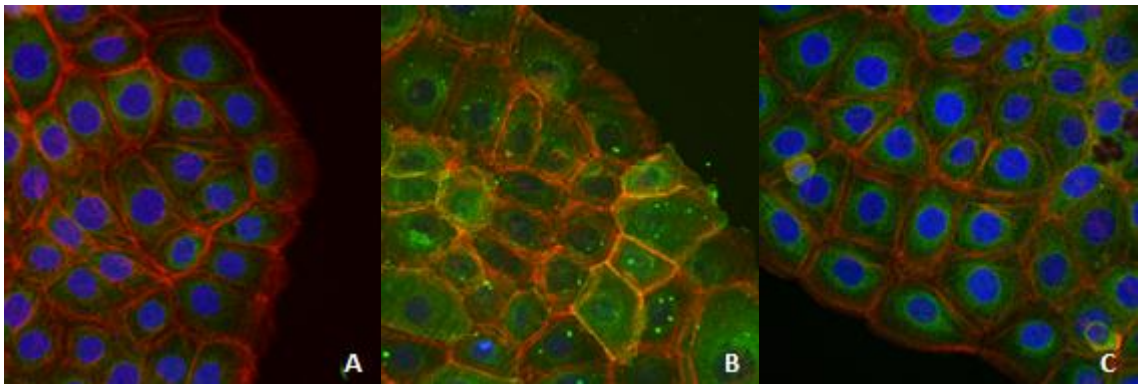


Figure 2. Localization of CFTR in all CFBE41o– cell lines by confocal microscopy. CFTR protein was detected in cells fixed with 0.8% formaldehyde in acetone and permeabilized with saponin (174) using a CFTR monoclonal antibody (clone24-1) for CFTR, in green. Actin was stained with rhodamine-

labeled phalloidin, in red, nucleus with TO-PRO-3, in blue ([Garcia, not published data]). No specific signal in KO cells (A), localization at the pericellular membrane in WT cells (B) and perinulear localization in F508del cells.

1.2.1. Impact of CFTR on Bacterial Internalization

One function of CFTR is to recognize the presence of many pathogens including *Pseudomonas aeruginosa* (5), *Burkholderia cepacia complex* (271) or *Chlamydia trachomatis* (2) and initiate rapid innate immune responses to quickly eliminate them. But data are still lacking for *S. aureus* and its SCVs forms.

For *P. aeruginosa***,** bacterial recognition is mediated via binding of the lipopolysaccharide outer core to a portion of the first extracellular domain of CFTR comprising amino acids 108-117 (208; 209). This interaction leads to (i) the release of interleukin-1β within a few minutes (222), (ii) nuclear translocation of nuclear factor (NF)-κB within 10–15 min (237), (iii) epithelial cell ingestion of *P. aeruginosa* over the next 3 h (45; 77; 208), (iv) cytokine gene transcription, translation and protein secretion, critical for neutrophil migration into the lung and subsequent phagocytosis of remaining bacteria, (v) and the apoptosis of epithelial cells over 4–24 h, associated with the resolution of infection (34). Part of the epithelial cell response to *P. aeruginosa* infection involves the rapid formation of detergent-resistant membrane micro-domains (“lipid rafts”) containing CFTR (102). One hundred fifty or more different proteins are recruited to lipid rafts within 15 min of *P. aeruginosa* infection of cells expressing WT-CFTR (146), and one of these proteins (the major vault protein) is critical for *P. aeruginosa* uptake by epithelial cells and clearance from the lung following infection. Another prominent protein recruited to these rafts is caveolin-1, the major structural component of the morphologically distinct subclass of lipid rafts termed caveolae due to their unique flask-like shape in the plasma membrane (68; 202). Cells unable to express wild-type (WT)-CFTR are deficient in all of these rapid responses.

1.2.2. Impact of CFTR on Antimicrobial Agents

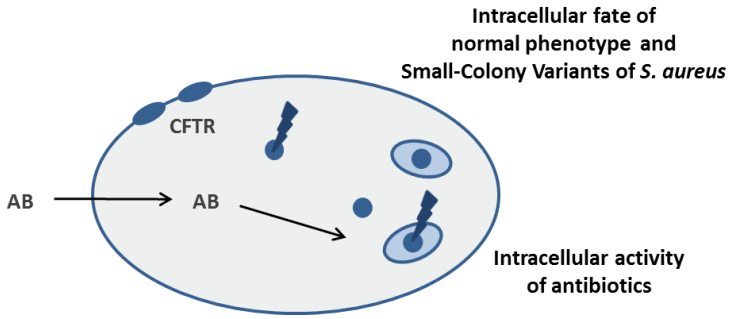
The literature has reported an inverse expression relationship between members of ATP-Binding Cassette (ABC) superfamily, and more especially between CFTR and P-glycoprotein (160; 30; 277). This reciprocal expression may have an impact on pharmacokinetics and pharmacodynamics of antibiotics. This mechanism could (i) modify transport of the antibiotics described as substrates of

ABC transporters, (ii) alter their concentration in the extra-and intracellular compartments, and thus (iii) compromise their activity.

1.2.3. Impact of CFTR on Bacterial Killing

Recent studies indicate that CF neutrophils have an impaired ability to chlorinate and kill bacteria (197). These findings suggest that the defective CFTR present in the CF neutrophil phagolysosomal membrane limits the concentration of chloride anion in the phagosomal compartment, which in turn compromises their ability to produce hypochlorous acid within the organelle. This defect is further exacerbated in a chloride-deficient environment, which ultimately undermines the overall antimicrobial capacity of CF neutrophils.

2. Additional Results: SCVs in Bronchial Cells



The infection of non-phagocytic host cells by *Staphylococcus aureus* and more particularly by Small-Colony Variants (SCVs) contributes to the persistence of this pathogen in the lungs of cystic fibrosis (CF) patients.

The literature describes that the CFTR chloride channel (i) modulates the internalization by epithelial cell of many other pathogens (e.g. *Pseudomonas aeruginosa*) acting as a cell-surface receptor, and (ii) influences the pharmacokinetic profile of antibiotics, as a member of the ATP-Binding Cassette superfamily.

The aim of this study was therefore to compare the intracellular fate of *S. aureus* and its SCV derivatives in airway epithelial cells expressing different CFTR genotype and to evaluate the intracellular activity of antibiotics against these particular forms. For sake of homogeneity with the previous study, we have used the same bacterial strains, although thymidine-dependent mutants are more often isolated in CF patients than hemin-dependent or menadione-dependent strains.

INTRACELLULAR ACTIVITY OF ANTIBIOTICS AGAINST MENADIONE- AND HEMIN-DEPENDENT SMALL-COLONY VARIANTS OF *STAPHYLOCOCCUS AUREUS* IN MODELS OF BRONCHIAL EPITHELIAL CELLS EXPRESSING DIFFERENT CFTR GENOTYPE

2.1. Introduction

Cystic fibrosis (CF) is one of the most common lethal genetic diseases affecting Caucasian population. This autosomal recessive disorder is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated chloride channel expressed in epithelia of multiple organs. The absence or dysfunction of CFTR has an impact on multiple organs and mainly affects respiratory and digestive systems. Ultimately, the majority of CF patients succumb from respiratory failure subsequent to chronic bacterial infections (18; 28). *Pseudomonas aeruginosa* and *Staphylococcus aureus* are currently the most common pulmonary pathogens in these patients.

Focusing on *S. aureus*, antibiotic treatment failure is frequently observed in CF patients and is often associated with relapsing infections (11; 13). These are thought to reemerge from bacteria persisting inside host cells (17). It is also suggested that the intracellular fate of *S. aureus* in CF epithelial cells may differ from that seen in non-CF cells (12). The ability of *S. aureus* to persist within epithelial cells may therefore represent an important factor contributing to the specific persistence of *S. aureus* in CF patients.

Small-Colony Variants (SCVs) are associated with *S. aureus* chronic infections and are frequently isolated from the airway of CF patients (1; 22; 27). SCVs have a dysfunctional oxidative metabolism or a lack in thymidine biosynthesis causing an alteration in the expression of virulence factors, a slow growth and a loss of colony pigmentation (27). Due to these particular characteristics, SCVs have an increased ability to invade and persist within non-phagocytic host cells (27; 31).

In this thesis, we have studied the intracellular persistence of a normal-phenotype *S. aureus* and its menadione- and hemin-dependent derivatives and the pharmacodynamic profile of a series of antibiotics in human THP-1 monocytes. We have now developed and characterized another *S. aureus* infection model using bronchial cell lines with different CFTR genotype. We then investigated the influence of CFTR expression on the intracellular fate of our strains and their susceptibility to several anti-staphylococcal agents, using CFBE41o- isogenic cell lines with different CFTR genotype.

2.2. Materials & Methods

2.2.1. Antibiotics and Main Reagents

The following antibiotics were obtained as the branded products commercialized in Belgium for human use: gentamicin as Geomycin® and vancomycin as Vancocin® (both distributed in Belgium by GlaxoSmithKline s.a./n.v., Genval), meropenem as Meronem® (AstraZeneca, Brussels) and rifampin as Rifadine (Merrell Dow Pharmaceuticals Inc., Strasbourg, France). Moxifloxacin was obtained as microbiological standards from Bayer HealthCare (Leverkusen, Germany). Pooled human serum from healthy volunteers was purchased from Lonza Ltd (Basel, Switzerland) and stored at - 80°C until use. Cell culture media and sera were from Invitrogen Corp. (Carlsbad, CA, USA), microbiological culture media, from BD Bioscience (Franklin Lakes, NJ, USA) and other reagents from Sigma-Aldrich or Merck KGaA (Darmstadt, Germany).

2.2.2. Bacterial Strains and Susceptibility Testing

We used three isogenic strains throughout this study, namely, *S. aureus* strain COL (wild-type [WT] hospital-acquired [HA] MRSA strain [8; 29]) and its *menD* and *hemB* SCV derivatives. These *hemB* and *menD* mutants were constructed by allelic replacement with an *ermB* cassette-inactivated *hemB* gene and an *ermC* cassette-inactivated *menD* gene, respectively (3; 34). MICs were determined by microdilution at neutral pH according to CLSI recommendations (2). Readings or colony counts were made after 24 or 48 h.

2.2.3. Cells, Cell Infection and Intracellular Activity of Antibiotics

Immortalized CFBE41o− bronchial epithelial cells were a generous gift of Dr. J.P. Clancy (University of Alabama, Birmingham, AL, USA). Wild type and F508del CFTR cDNA were stably transduced into CFBE41o$^-$ cells using TranzVector™ (Tranzyme, Inc., Birmingham, AL, USA). Expression of CFTR was coupled to the puromycin-N-acetyltransferase gene (*puro*) via the internal ribosomal entry site (IRES) of encephalomyocarditis virus, allowing for rapid selection of cells expressing CFTR in media containing puromycin. CFBE41o$^-$ cells were transduced at a multiplicity of infection of one followed by puromycin (4 µg/ml) selection. Puromycin-resistant cells were expanded and clones were selected for high-level expression (4).

Infection of CFBE41o- was performed as previously described in our laboratory for non-phagocytic cells (15). In comparison to macrophages, internalization was allowed to take place for 2 h with an initial inoculum of $1x10^8$ bacteria per mL (19). Antibiotics were used at a concentration corresponding to their human peak in serum. After a 24 h exposure, change in CFU from the post-phagocytosis inoculum and cellular protein content were measured in parallel.

2.2.4. Statistical Analyses

Statistical analyses for intra- and inter-assay were made with GraphPad Instat version 3.06 (GraphPad Software).

2.3. Results

2.3.1. Phagocytosis and Intracellular Growth

In a first series of experiments, we compared the capacities of the parental strain and its two SCV derivatives to infect CFBE41o- cells. Considering phagocytosis first (left panel), all strains were more avidly internalized in KO cells than in cells expressing WT- or F508del-CFTR. Moreover, the *menD* mutant was much more phagocytized than the *hemB* mutant or the parental strain in all cell types.

To assess intracellular growth (right panel), viable counts were measured after 24 h of incubation. Interestingly, the *menD* mutant seems to persist better than the parental strain in cells expressing a functional or mutated CFTR.

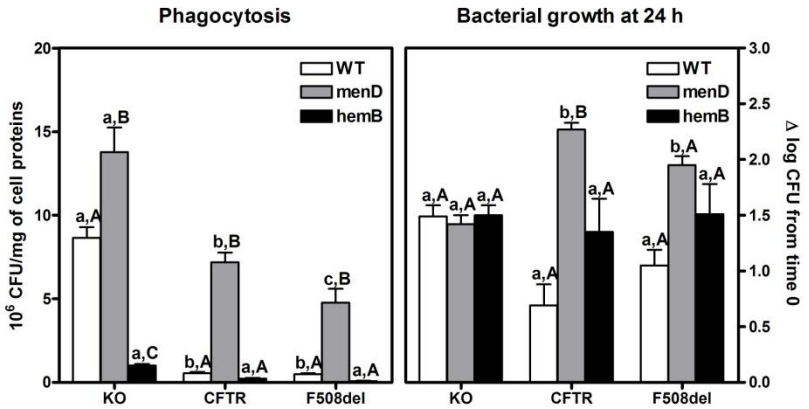


Figure 1. Comparative phagocytosis and intracellular survival of SCVs (*menD* and *hemB*) in bronchial epithelial cells, compared to the isogenic *S. aureus* normal-phenotype strain (WT). (Left) Enumeration

of cell-associated CFU after 2 h of phagocytosis by CFBE41o- cells with no expression (KO), or expression of wild-type (CFTR) or mutated (F508del) CFTR. (Right) Intracellular growth of strains after 24 h of incubation in the same cell lines. Values are expressed as the change in CFU (in log scale) per mg of cell protein compared to the initial inoculum. Results are means ± standard deviations of three independent determinations. Statistical analyses (with Tukey *post hoc* test only performed if $p<0.05$): values with different letters are significantly different from each other (lower case letters, comparison for a single strain in the different cell types; upper case letters, comparison of the different strains in each cell type).

2.3.2. Susceptibility Testing

Five antibiotics representative of the major classes of anti-staphylococcal drugs were previously studied against the same strains in a model of THP-1 cells and were then selected for the present study. The three strains were susceptible to all antibiotics except to β-lactams due to the MRSA phenotype of the COL parental strain. Of note only moxifloxacin showed a significant (2- log_2-dilution) increase in MIC against SCVs (Table 1).

Table 1. MICs of antibiotics against bacterial strains

Antibiotic	Cmax (mg/L)	MIC (mg/L)		
		WT	***menD***	***hemB***
RIF	18	0.02	0.02	0.02
MXF	4	0.03	0.125	0.125
GEN	18	0.25	1	0.125
VAN	50	1	1	1
MEM	50	32	32	32

2.3.3. Activity of Antibiotics at a Fixed Concentration against Intracellular SCVs

The activity of antibiotics against intracellular SCVs after 24 h of infection was then examined at a total drug concentrations mimicking their maximal concentration (C_{max} – Table 1) values in human serum (Figure 2 – Upper panel) or with an extracellular concentration of 10x MIC (Figure 2 – Lower panel). The most salient observation is that globally, all antibiotics were more active against the *menD* mutant in all three cell types. Meropenem was active intracellularly despite the MRSA character of the strains, as previously described (16; 10).

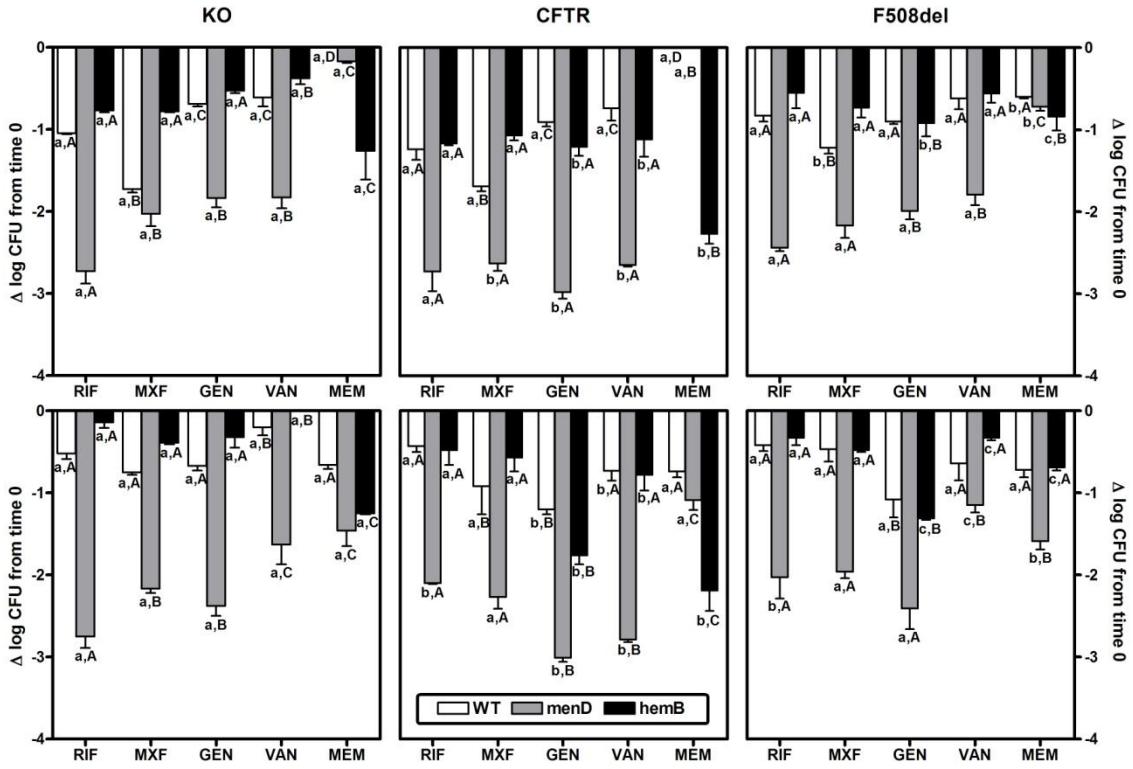


Figure 2. Comparative intracellular activity toward isogenic *S. aureus* strains with different phenotypes in CFBE41o- cells with no expression (KO), or expression of wild-type (CFTR) or mutated (F508del) CFTR. Infected cells were exposed for 24 h at a fixed extracellular concentration (total drug) of each antibiotic (corresponding to the human C_{max} [Table 1 - Upper panel] or 10x MIC [Lower panel]). The graph shows the change in the number of CFU per mg of cell protein over time (Δ log CFU from the initial inoculum). Data are means ± standard deviations ($n > 3$). Analysis of variance and multiple comparison by Tukey's test were used to determine significance; values with different letters are significantly different from one another for each strain (lower case letters, comparison of a single strain in the different cell types for each antibiotic; upper case letters, comparison of the different strain in each cell type for all antibiotics [$p<0.05$]).

In order to better evidencing differences between cell types, the same results were presented in figure 3 as the difference in intracellular inoculum for KO versus WT cells (left panel) or F508del versus WT cells (right panel). Globally, all antibiotics were less active in KO or F508del cells than in WT cells, except for meropenem against the *menD* strain, which was systematically slightly more active in cells expressing a functional CFTR.

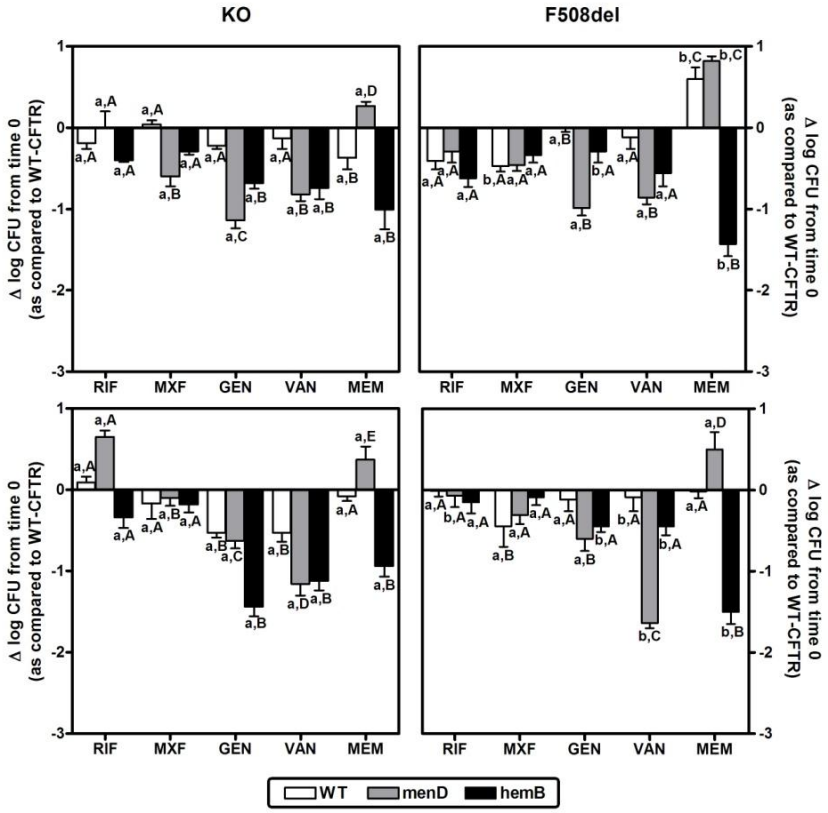


Figure 3. Comparative intracellular activity toward isogenic *S. aureus* strains with different phenotypes in CFBE41o- cells with no expression (KO), or expression of wild-type (CFTR) or mutated (F508del) CFTR. Infected cells were exposed for 24 h at a fixed extracellular concentration (total drug) of each antibiotic (corresponding to the human C_{max} [Table 1 - Upper panel] or 10x MIC [Lower panel]). The graph shows the change in the number of CFU per mg of cell protein over time expressed as a ratio (Δ log CFU from the initial inoculum [expressed as a ratio of WT- vs. KO or F508del CFTR]). Data are means ± standard deviations (n > 3). Analysis of variance and multiple comparison by Tukey's test were used to determine significance; values with different letters are significantly different from one another for each strain (lower case letters, comparison of a single strain in the different cell types for each antibiotic; upper case letters, comparison of the different strain in each cell type for all antibiotics [$p<0.05$]).

2.4. Discussion

Previously, we have examined the intracellular fate of menadione- and hemin-dependent SCVs and their intracellular susceptibility to antibiotics in models of phagocytic cells. The present data extend this work to non-phagocytic cells, in order to obtain relevant information in a CF context. However, these preliminary results should be interpreted with caution and further investigations are clearly required.

Considering first internalization, one of the most striking observations of this study is that the *menD* mutant showed a higher internalization rate in all cell types, in comparison to the *hemB* mutant and the parental strain. These data suggested the possibility that up-regulation of bacterial adhesins may be higher in the *menD* mutant than in the *hemB* mutant. Even if it is well known that SCVs exhibit increased expression of the fibronectin-binding proteins and clumping factor allowing these variants to be well adapted for efficient attachment to host cells (32; 22; 21), the expression of bacterial adhesins by distinct SCVs is poorly documented. In the literature, few studies have been conducted comparing hemin- or menadione-dependent strains.

When observing the impact of CFTR expression on bacterial invasion, it appeared that all strains were more internalized by KO cells than those presenting a functional or mutated CFTR. Several studies have suggested that CFTR was implicated in the internalization of *Pseudomonas aeruginosa* (25; 24). Here, we demonstrated the inverse phenomenon for *S. aureus* and its SCV forms. Although the underlying mechanism to this increased internalization in the absence of CFTR is unknown, we may suggest that the *S. aureus* cellular receptors, such as $\alpha_5\beta_1$ integrin, could be differentially distributed or expressed in KO cells, in comparison to those expressing a functional or mutated CFTR. This is however contradictory with data having shown that CF cells express an increased number of asialylated glycolipids, which are known to serve as receptor for many pulmonary pathogens, including *S. aureus* (14; 30; 20). Yet, several investigators have also observed that the level of differentiation of cells used for bacterial invasion experiments may alter the interaction between pathogens and host cells (5; 9; 23; 26), suggesting that the model used is probably critical.

After 24 hours of infection, we also observed an identical behavior for *S. aureus* and its SCV derivatives in KO cells, where then level of persistence appeared similar for all strains. However, in cells expressing a functional or mutated CFTR, this survival appeared (i) reduced for the normal phenotype, (ii) increased for the *menD* mutant and (iii) not changed for the *hemB* mutant. As demonstrated in our previous work, pH environment and redox state of cells could influence the intracellular survival of our strains. In the literature, recent studies in CF KO mice have demonstrated that CFTR participates in phagosomal pH control of murine alveolar macrophages (7; 6). Additional studies also suggested that dysfunctional CFTR leads to an increase in mitochondrial oxidative stress inter alia characterized by increased levels of reactive oxygen species (ROS) in CFTR-deficient lung epithelial cells (33). Both these environmental parameters should thus be explored in the three CFBE41o- cell lines.

Moving now to intracellular activities, we noted that all antibiotics were more effective against the *menD* mutant than the others strains, in all cell types. But neither the intracellular bacterial growth nor the different CFTR genotype of the cells can explain this observation. More strikingly, we showed that all drugs were more active against SCVs in cells expressing a functional CFTR than a mutated or no CFTR. The reason for this difference is still unclear. Based on the work presented in this thesis, we may think again of variations in the production of ROS between these cell lines, or in the accumulation level of antibiotics. But whatever the underlying mechanism, this observation may contribute to explain the difficulty of eradicating these particular forms in CF patients.

However, the model used in the present study has some features limiting its significance, such as a lack of dynamic exploration (i.e. fixed concentrations of antibiotics maintained throughout the infection period and few concentrations used). Further experimental studies are thus required to better understand the mechanisms underlying these preliminary observations.

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3. Discussion

3.1. Non-Professional versus Professional Phagocytic Cells

It is probably premature to try comparing the intracellular fate of our strains and the intracellular activity of antibiotics in professional phagocytes versus non-professional phagocytes. Actually, a lot of characteristics including the expression of CFTR, the lysosomal pH, or ROS production should be examined in our cell lines, as they seemed to markedly affect SCVs behavior.

Of note, several recent reports indeed suggested that CFTR might be of pivotal importance in the normal function of immune cells, such as macrophages. Studies in CF knockout mice demonstrated that CFTR participates in phagosomal pH control of murine alveolar macrophages thereby CFTR-deficient macrophages failed to acidify lysosomes and phagolysosomal compartments and displayed an altered bactericidal activity (61; 60; 309). Additionally, defects in the ROS-mediated killing of *Pseudomonas aeruginosa* by murine CFTR-deficient alveolar macrophages have been recently reported (309). Finally, a contribution of CFTR in the production of different cytokines by macrophages has been lately described (31).

The expression level of CFTR in THP-1 cells and its impact on lysosomal pH or ROS production seem essential to determine, to possibly highlight some similarities and/or differences between monocytes and macrophages, on the one hand, and between both phagocytic cells and epithelial cells, on the other hand.

3.2. Interests and Limits of our Intracellular Infection Model

CFBE41o- with different CFTR genotype are representative cell types of the infected target tissues. However, even if SCVs are observed in the sputum of 70% of patients suffering from cystic fibrosis, 2/3 of these strains harbor a thymidine-dependent phenotype and 1/3 shows dependence to menadione, hemin or double auxotrophism. These latter phenotypes are indeed more frequently found in osteomyelitis or device-related infections. To complete our study, we should include in our experiments a thymidine-dependent SCV, on the one hand, and use some more relevant cells for infections associated to menadione- or hemin-dependent strains, such as osteoblasts or endothelial cells.

Furthermore, the use of isogenic cells allowed us to define the role of one specific parameter for each cell type. Nonetheless, the model of bronchial epithelial cell lines could still be improved. For example, polarization would better reproduce physiological conditions making these cell-based assays more biologically relevant.

3.3. Perspectives

Recent studies have indicated that CFTR processing and activity are strongly influenced by the cell model system under study (195; 154), with enhanced maturation and surface stability demonstrated in polarizing models compared to a non-polarized environment (287). Polarizing CFBE41o- cells should provide a model more representative of the *in vivo* situation.

Moreover, polarized cells have the ability to maintain a specific biochemical composition of the apical and basolateral plasma membrane domains while selectively allowing transport of proteins and lipids from one pole to the opposite by transcytosis. This organization permits regulated vectorial transport of molecules (67; 171; 184; 164; 180). The stabilized expression of uptake and efflux transporters in polarized cells is therefore to take into account to determine the pharmacological profile of antibiotics.

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