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Intracellular *Staphylococcus aureus*, an emerging link to persistent and relapsing infections: factors influencing the activity of antimicrobials against intracellular *S. aureus*

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La phrase la plus excitante à entendre en sciences,
celle qui annonce de nouvelles découvertes,
n'est pas « Eureka » (j'ai trouvé), mais
plutôt « tiens, c'est marrant … »

Isaac Asimov

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Finalement, je dédie cette thèse à ma petite-sœur, Stéphanie, qui nous a quittés bien trop tôt.

INDEX

ABBREVIATION LIST

ABC	ATP-Binding Cassette
AST	Antimicrobial Susceptibility Testing
CAPD	Continuous Ambulatory Peritoneal Dialysis
CAT	Chloramphenicol Acetyl-Transferase
CBIP	Centre Belge d'Information Pharmacologique
CDC	Center for Disease Control
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
FACS	Fluorescence Activated Cell Sorting
FDA	Food and Drug Administration
FEM	Factor Essential for Methillin resistance
FnBPs	Fibronectin Binding Proteins
hVISA	Hetero-Vancomycin Intermediate Resistant *Staphylococcus aureus*
IL	Interleukin
LLO	ListerioLysin O
LPG	Lysine-Phosphatidyl-Glycerol
mep	Multi-drug Export Protein
MICs	Minimal Inhibitory Concentrations
MRSA	Methicillin Resistant *Staphylococcus aureus*
MRPs	Multi-drug Resistant Proteins
MSSA	Methicillin-Sensitive *Staphylococcus aureus*
MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MLS_B	Macrolides – Lincosamides – Streptogramins B
PBPs	Penicillin Binding Proteins
P-gp	P-glycoprotein
PVL	Panton-Valentine Leukocidin
rpoB	RNA polymerase β-subunit
SCVs	Small Colony Variants
SSSS	Staphylococcal Scalded Skin Syndrome
ST	Sequence type
SSSI	Skin and Soft-Structures Infections
TNF	Tumor Necrosis Factor
TRAIL	Tumor necrosis factor-Related Apoptosis Inducing Agent
TSS	Toxic Shock Syndrome
TSST-1	Toxic Shock Syndrome Toxin 1
UDP	Uridine Diphosphate
VISA	Vancomycin Intermediate *Staphylococcus aureus*
VRSA	Vancomycin Resistant *Staphylococcus aureus*

INTRODUCTION

I – MICROBIOLOGICAL PROPERTIES

The term *Staphylococcus* is derived from the Greek expression staphyle (bunch of grapes) and was chosen by Sir Alexander Ogdson because of its characteristic arrangement into clusters. Microscopically, *Staphylococcus aureus* is a non-motile Gram-positive organism characterized by individual cocci. It is distinguished from other staphylococcal species (*S. epidermidis* and *S. saprophyticus*) on the basis of the gold pigmentation of its colony and positive reactions in the following tests: (i) coagulase, a test differentiating *S. aureus* from coagulase-negative *S. epidermidis*; and (ii) acid production by mannitol fermentation.

1. Important microbiological determinants of *Staphylococcus aureus*

1.1. Genome

The genome consists of a single circular chromosome (2.7 to 2.8 mega-base pairs; G + C content of about 33 %), coupled with an assortment of extra-chromosomal accessory genetic elements (conjugative and non-conjugative plasmids, mobile elements, bacteriophages,…).[161]

Diversification within the *S. aureus* population is achieved through a combination of mutations, recombinations and horizontal gene transfers. This pathogen demonstrates therefore a remarkable ability to acquire potentially useful genes from a variety of organisms, facilitating the evolution of many virulent and drug-resistant strains.[108]

1.2. Cell-wall constituents

The peptidoglycan, the major cell-wall constituent, consists of alternating polysacharide subunits of N-acetylglucosamine and N-acetylmuramic acid, and pentapeptide sides chains linked to the muramic acid residue[216]. Other important cell-wall constituents include (i) a group of phosphate-containing polymers designed as teichoic acids, which are covalently bound to pepdidoglycan (cell-wall teichoic acids) or linked to the lipid of the bacterial cell membrane (membrane teichoic acids)[128], and (ii) major adhesins such as fibronectin-binding proteins, clumping factor, protein A and collagen-binding protein.[139]

1.3. Major toxins and virulence factors

The success of *S. aureus* as a pathogen is in large part the consequence of its versatile arsenal of toxins and virulence factors. Depending of the strains, *S. aureus* is capable of secreting membrane-damaging toxins (α-δ haemolysins, Panton-Valentine leukocidin), epidermolytic toxins or enterotoxins (enterotoxins A-E, Toxic Shock Syndrome Toxin 1 [TSST-1]).

II – DISEASES CAUSED BY *STAPHYLOCOCCUS AUREUS*

In an elegant series of clinical observations and laboratory studies in the early 1880's, Ogston described staphylococcal diseases and their role in sepsis and abscess formation. More than 100 years later, *Staphylococcus aureus* continues to pose an increasing threat worldwide. This dangerous pathogen is commonly implicated in a wide range of infectious illnesses (Table 2.1), ranging from superficial would infections to life-threatening cardiovascular infections, septicaemia and toxic-shock syndrome.[139] It is also a major food poisoning bacterium.

TABLE 2.1. Clinical manifestations associated with *Staphylococcus aureus* infections

Types of infections	
Direct infection	
(a) Skin infections	folliculitis, carbuncle, impetigo, cellulitis, abcess, …
(b) Deep infections	arthritis, osteomyelitis
Blood stream infections	bacteriemia, sepsis metastatic infections : endocarditis, meningitis, osteomyelitis, arthritis, pericarditis, lung abscess, …
Toxin-mediated diseases	food poisoning scaled skin syndrome toxic shock syndrome with multiple organ failure

1. Carriage of *Staphylococcus aureus*

Nasal carriage of *S. aureus* appears to play a key role in the epidemiology and pathogenesis of infection. While 20 % of the population never carries this pathogen, 20 % and 60 % of individuals are, respectively, persistent and intermittent carriers. Prevalence and incidence of *S. aureus* nasal carriage vary according to the population[126,127,248], with increasing carriage rates for physicians, nurses, hospital ward attendants, patients with diabetes mellitus, patients undergoing chronic hemodialysis or continuous ambulatory peritoneal dialysis (CAPD), intravenous drug addicts, patients with dermatologic diseases.

2. Staphylococcal human infections

Staphylococcus aureus has ability to survive in various environments. In particular, this bacteria shows high tolerance to variations of pH (Fig.2.1), which confers an advantage for colonizing body sites characterized by mildly acidic pH (such as skin, mouth, vagina and urine).

2.1. Skin-related infections

Staphylococcus aureus has long been recognized as a leading cause of skin and soft-tissues infections including abscesses, atopic dermatitis, carbuncles, cellulitis, furuncles,

folliculitis, impetigo, pemphigus, psoriasis, … [226] In particular, necrotizing soft-tissue infections may be rapidly fatal because of the toxin-induced circulatory collapse.

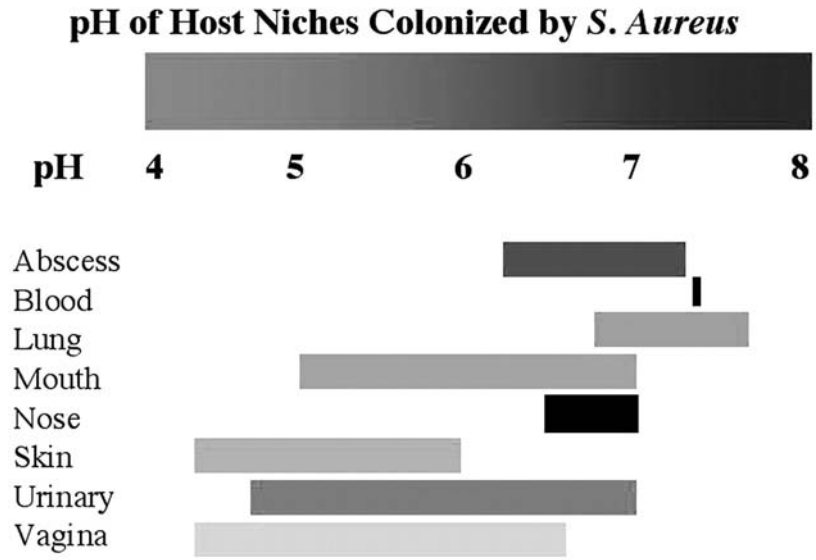


Fig.2.1. pH ranges of various staphylococcal niches in the human host. Abcess, pH 6.2 to 7.3; blood, pH 7.4; lung, pH 6.8 to 7.6; mouth, pH 5 to 7; nose, pH 6.5 to 7; skin, pH 4.2 to 5.9; urinary tract, pH 4.6 to 7; and vagina, pH 4.2 to 6.6 (Weinrick et al, 2004[276]).

2.2. Deep-seated infections

Following bacterial dissemination, *S. aureus* is able to cause deep-seated infections such as inflammatory reactions in the joint space (septic bursitis, arthritis) or severe bone infections (osteomyelitis).[146]

2.3. Bacteraemia

According to the National Nosocomial Infection Surveillance system of the US Centers for Disease Control and Prevention (CDC), 16 percent of hospital-acquired cases of bacteraemia in the United States were due to *Staphylococcus aureus* from 1991 to 1995. The frequency of *S. aureus* MRSA bacteraemia has, however, increased dramatically in United States and in Europe over the few years (see for review: [76,88]). This increasing frequency, coupled with the growing emergence of antibiotic resistance, has therefore renewed interest in this serious staphylococcal infection.[25,193]

2.4. Metastatic infections

Once it enters the blood, *Staphylococcus aureus* is one of the most lethal human pathogens with the unique ability to cause difficult-to-threat endovascular infections (heart valve infections), pneumonia, meningitis, lung abscesses, pericarditis, …

2.5. Toxin-mediated diseases

Among the predominant bacteria involved in food-borne diseases, *Staphylococcus aureus* is the leading cause of gastroenteritis resulting from the consumption of enterotoxins-contaminated food.[134] Symptom-onset is abrupt and the disease may be severe enough to warrant hospitalization, but is usually self-limiting and does not require specific therapy.

In contrast, staphylococcal scalded skin syndrome (SSSS; Ritter syndrome) and toxic shock syndrome (TSS) are more severe. Staphylococcal scalded skin syndrome is a rare but well-described disorder in neonates and young children and results from the colonization or infection with a strain of *S. aureus* producing epidermolytic toxins.[188] It ranges in severity from trivial focal skin blistering to extensive, life-threatening exfoliation. Toxic shock syndrome is one of the most feared staphylococcal manifestations and results from the colonization or infection with a strain of *S. aureus* producing the protein TSST-1. The key features of this infection are widespread erythroderma occurring in association with profound hypotension and multiple organ dysfunction.[217]

III – LIFE ON THE INSIDE, MICROBIAL STRATEGIES OF *S. AUREUS*

The diversity of bacteria-host interactions is outstanding. These interactions range from mutualistic symbiotic interactions that benefit both parties to unstable relationships in which pathogens kill rapidly host cells. Chronic and relapsing infections lie somewhere between these two extremes. In the case of *S. aureus,* it has been shown that this pathogen has ability to invade *in vitro* many host cells, including both professional (neutrophils[92], monocytes[227] and macrophages[22]) and non-professional phagocytes (endothelial cells[96,142,267], osteoblasts[114], keratinocytes[152], fibroblasts[253],…). This unusual microbial strategy, which is illustrated in figure 3.1, provides a niche protecting the bacteria from humoral responses and antimicrobials.

1. Intracellular life-style of Staphylococcus aureus

1.1. Adherence of the bacteria to host cell

Bacterial adherence to host cell is a prerequisite for invasion. For this purpose, *S. aureus* expresses an array of adhesins designed as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). These proteins include fibronectin-binding proteins (FnBPs), fibrinogen-binding proteins, elastin-binding adhesion, collagen-binding adhesion and finally, a broad-specificity adhesion (MAP) (see for review[4]).

1.1.1. Fibronectin-binding proteins (FnBPs)

Fibronectin Binding Proteins (FnBPs) are likely the most important MSCRAMMs in staphylococcal host cell invasion. Interactions appear to occur through a bridging model where fibronectin is bind by staphylococcal FnBPs as well by eukaryotic integrins. Using FnBP mutants and their co-isogenic parental strains, it has been established that FnBPs are critically required for the adherence of the pathogen to bovine mammary epithelial cells[66], human endothelial cells[189], mouse fibroblasts[78], human osteoblasts[2], murine peritoneal macrophages[228], human keratinocytes[125]. Additionally, transformation of non-invasive species of *Staphylococcus carnosu*s and *Lactococcus Lactis* with FnBP-encoding plasmids conferred an invasive potential to these organisms.[235]

1.1.2. Integrins

The relevance of β1-integrins in the internalization process has been well described. Using mutational analysis, Van Nhieu and colleagues have demonstrated the critical role of their cytoplasmic domains for staphylococcal internalization[263]. Additionally, the utilization of

$\alpha_5\beta_1$-integrins raised-antibodies is associated with a decreased internalization of *S. aureus* by keratinocytes[125].

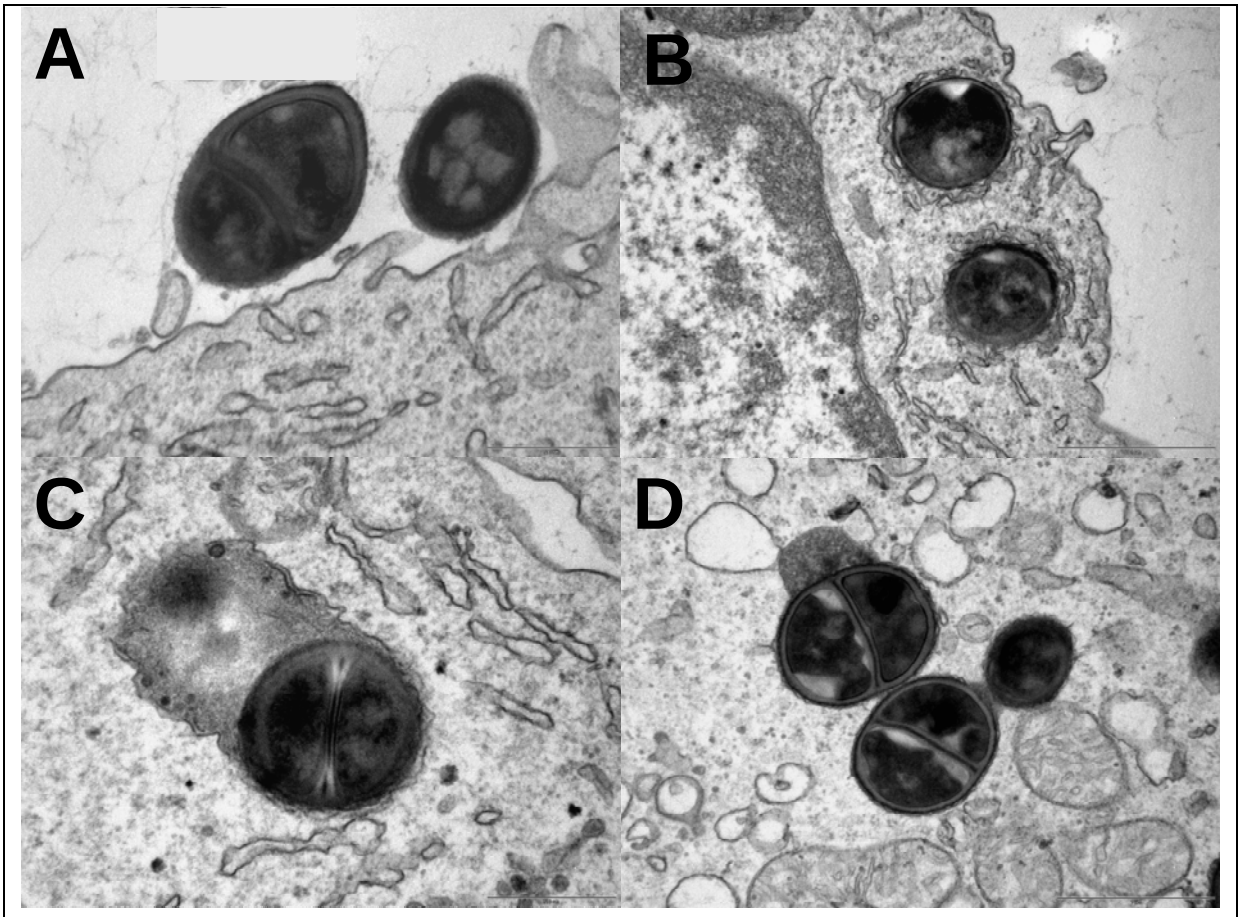


Fig.3.1. Infectious cycle of *Staphylococcus aureus* illustrating the adhesion to host cells (macrophages) (A) or the residence within membrane-bounded vacuoles (B-D).

1.2. Invasion and residence within phagosomes

Internalized bacteria are generally processed within membrane-bounded vesicles that mature through a series of fusion steps yielding to mature phagolysosomes (characterized by a low pH and containing lysosomal hydrolases). Internalization process can be (i) passive and directed by the host cell (phagocytosis; professional phagocytes) or (ii) triggered by the induction of membrane ruffling and macropinocytosis (non-professional phagocytes) using receptor-mediated endocytosis.

1.3. Escape of the bacteria from the phagosomes

Intracellular bacteria have evolved mechanisms to evade destruction occurring after internalization (Fig.3.2). Some pathogens (like *Mycobacteria tuberculosis, Legionella pneumophila*, and *Brucella abortus)* are able to subvert host vesicle trafficking, preventing therefore the effective fusion of phagosomes with lysosomes. Other bacteria (like *Shigella*

flexneri, *Listeria monocytogenes*, and *Rickettsia spp*.) can escape from phagosomes into the cytosol. In the case of *Staphylococcus aureus*, it has been established that this pathogen has ability to escape the phagolysosomal pathway in certain non-profesionnal phagocytes, including endothelial cells (Human umbilical vein endothelial cells [HUVEC][153]), epithelial cell lines (cystic fibrosis tracheal epithelial cell line [CFT-1 cells][116]; bovine mammary epithelial cell line [MAC-T][24]), immortalized cell line from cervical carcinoma [HeLa cells][218]) and bones cell line (embryonic chick osteoblast)[202]. To the present day, the specific pathways by which *S. aureus* subverts host cell trafficking remain elusive.

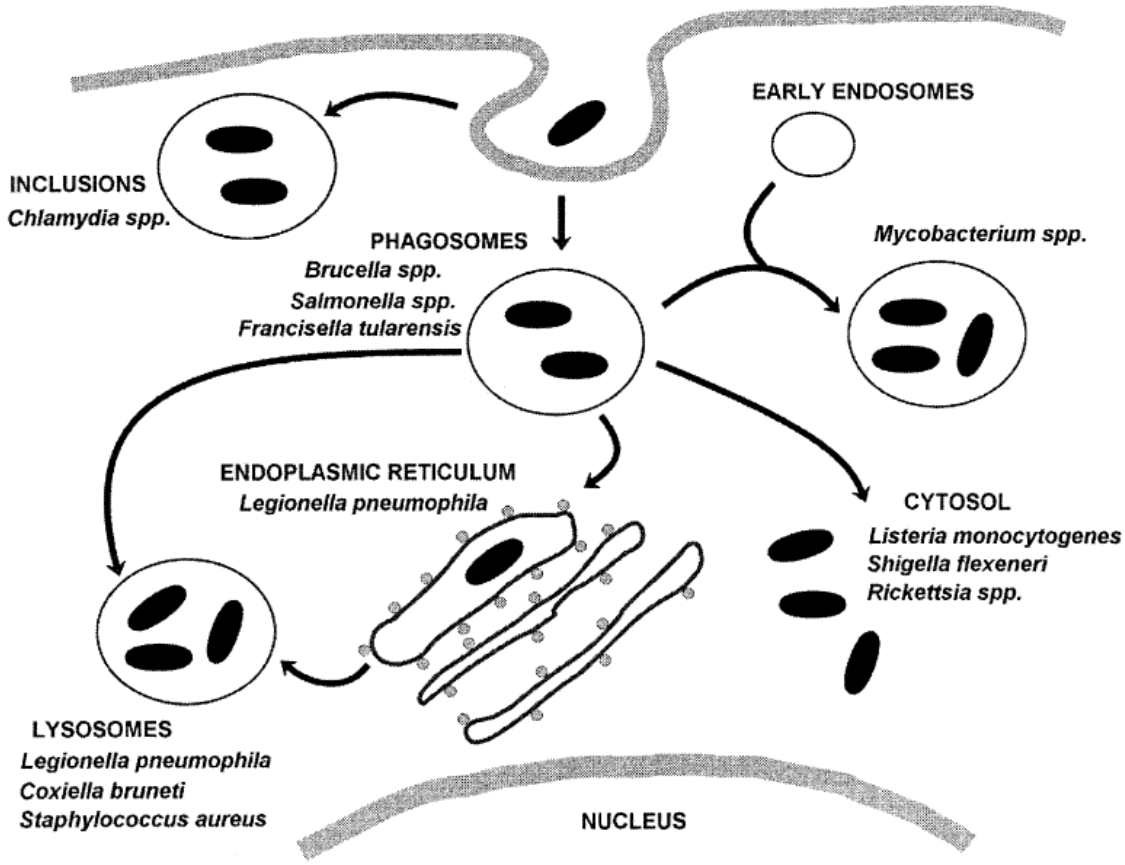


Fig.3.2. Pictorial description of the various pathways followed by intracellular bacteria to evade cellular mechanisms of destruction after phagocytosis.
Some bacteria remain localized within phagosomes, that are (i) unable to fuse with other vacuoles (*Brucella spp., Salmonella spp., and Francisella tularensis*), (ii) able to fuse with newly formed endosomes but not with lysosomes (*Mycobacterium*), or (iii) able to fuse with the endoplasmic reticulum in a form of anormal autophagy (*Legionella pneumophila*). Other bacteria, such as *Listeria monocytogenes, Shigella flexeneri,* and *Rickettsia spp,* escape hastily from phagosomes to avoid both acidification and the subsequent sequestration within phagolysosomes. Finally, some bacteria (such as *Staphylococcus aureus* or *Coxiella burnetii*) may resist to the fusion of phagosomes with lysosomes and multiply within the phagolysosomal apparatus (Carryn et al, 2003[48]).

1.4. Intracellular persistence

Molecular detail governing the intracellular persistence of *S. aureus* for extended period is still largely unknown. However, it has been shown that shortly after its internalization by human lung epithelial cells, the bacterial gene expression profile of this pathogen dramatically changes (Fig. 3.3). Firstly, and in contrast to other microorganisms known to actively multiply within cells (such as *Listeria monocytogenes*, *Shigella*, or *Salmonella*), intracellular *S. aureus* transiently shuts down most of its metabolic functions. Profound shut-down of gene expression is observed for genes involved in the production of energy (citrate and ATP cycles, pyruvate metabolism, and oxidative phosphorylation) or in the cell division machineries (transpeptidases, genes involved in the synthesis of teichoic acid, …).[85]

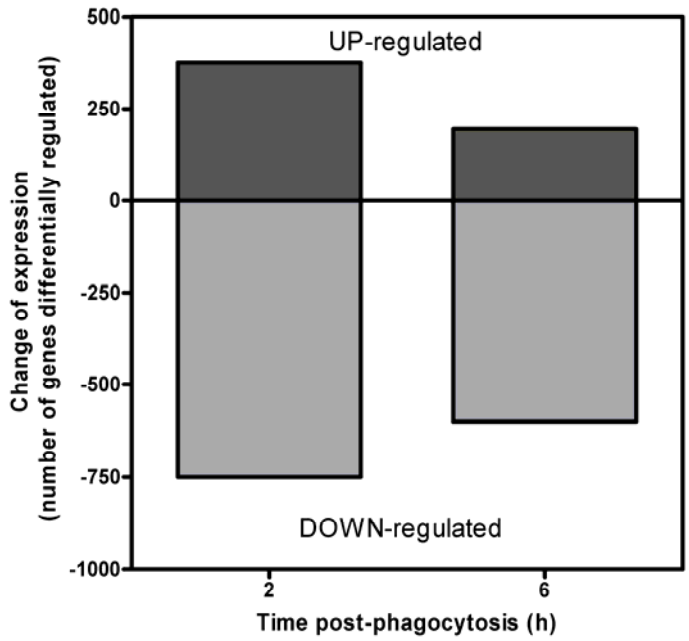


Fig.3.3. Global view of *S. aureus* whole genome expression upon internalization in human lung epithelial cells. The graph represents the number of genes found differentially regulated compared to extracellularly (Adapted from Garzoni et al, 2007)[85].

Secondly, the expression of several toxin genes known to affect host cell integrity (such as the cytolysin alpha-haemolysin [*hla*]) also appears strongly regulated intracellularly. Importantly, their expression is highly modulated through a complex network of regulatory molecules (including two-components systems and global regulators [such as *agr* or *sar]*)[163,175], contributing not only to a rapid adaptation of the bacteria to environmental changes, but also to the regulation of toxins and cell-surface components expression during bacterial growth. In the model described by Garzoni and colleagues, internalized bacteria are associated with a moderately increased expression of RNAIII, a compound known to

induce *agr* expression, resulting consequently in the reduced expression of *hla* that potentially mediates the induction of apoptosis and cellular destruction[85].

Thirdly, whereas major metabolic pathways are severely down-regulated, numerous genes involved in virulence are up-regulated. Among the most significantly up-regulated genes, Garzoni and colleagues have found extensive modifications for genes encoding fibronectin binding proteins (*fnbpA, fnbpB*) or the intercellular adhesion proteins (*icaB*)[85].

Taken together, these results support that *S. aureus* extensively reprograms its transcriptome once it reaches the intracellular environment. Internalization of *S. aureus* appears, therefore, to require (i) only a limited number of bacterial factors (since most bacterial genes were down-regulated), which may explain the delay of intracellular bacterial replication, and (ii) a reduced expression of bacterial enzymes targeting the eukaryotic membrane (i.e. alpha haemolysin).

1.5. Bacterial induced-host damages

Staphylococcal infections are typically associated with the death of tissues. The ability of *S. aureus* to induce apoptosis has been noticed in a variety of cell types including epithelial cells[24], endothelial cells[153], keratinocytes[171], osteoblasts[249], neutrophils[237] and macrophages[211]. In particular, Wesson and colleagues have demonstrated that *S. aureus* mediated-apoptosis in bovine mammary epithelial cells involved caspase-8 and caspase-3, two key components associated with death receptors induced-apoptosis.[277] Induction of apoptosis by *S. aureus* might play an important role in the microbial pathogenesis by providing a route to the pathogen for its effective dissemination to distant sites.

2. Small Colony Variants of *Staphylococcus aureus*

2.1. Main characteristics

Small Colony Variants (SCVs) of *Staphylococcus aureus*, which are commonly related to persistent and recurrent illnesses (pulmonary infections associated with cystic fibrosis, chronic osteomyelitis or keratosis follicularis [Darier-White disease])[268,269,274], represent naturally occurring subpopulations of *S. aureus* that were first described in the early 1900's. Although several genetic and metabolic defects may contribute to the expression of this phenotype, a frequent characteristic of these slow-growing, pinpoint bacteria is their auxotrophic phenotype (Table 3.1). The two most frequently described groups of metabolic defects are those that lead to (i) a deficient electron transport pathway (due to defects in the biosynthesis of menaquinone [menaquinone pathway] or cytochrome

[hemine pathway], affecting thereby oxidative phosphorylation and ATP generation)[213,270] and (ii) an impaired thymidine biosynthesis.[36]

TABLE 3.1. Typical features of *S. aureus* SCVs (adapted from Von Eiff et al[272])

Properties	Normal *S. aureus* phenotype	SCV phenotype
Morphology	Yellow-oranges colonies ∅ 4-8 mm	Pinpoint colony ∅ 1 mm
Auxotrophism	No	Hemin ; Hemin/Menadione Thymidine
Toxins activity: - **α-haemolysin** - **β-haemolysin**	 Normal Normal	 reduced reduced
Enzymes activity : - **Coagulase** - **Catalase**	 Normal Normal	 3-4 fold delayed Reduced
Biochemical reactions	Normal	Delayed and/or changed
Growth rate on solid media	12-18 h	Delayed (48 h)

2.1.1. SCVs with defects in electron transport

Alteration in electron transport chain is a common phenotypic characteristic of SCVs. Using specifics mutants (*hemB* or *menD* mutants) and the phenotype microarray technology, Von Eiff and colleagues have systematically detailed the physiological changes occurring in these mutants.[273] Compared to their parental strain, the *hemB* mutant is defective in utilization of a variety of carbon sources, including Krebs cycle intermediates and compounds that ultimately generate ATP via electron transport. The phenotype of the *menD* mutant is similar to that of the *hemB* mutant, but defects in carbon metabolism are more pronounced than those seen with the *hemB* mutant. Of particular relevance, carbohydrates that provided ATP in the absence of electron transport allow the growth of both mutants.

2.1.2. SCVs with defects in thymidine biosynthesis

The phenotype of thymidine-auxotrophic SCVs, which are frequently isolated from patients suffering of cystic fibrosis, is nearly identical to that of SCVs characterized by a deficient electron transport (Fig.3.4). Nearly all thymidine-dependent SCVs have emerged due to a long-term treatment with trimethoprim, an agent interfering with the tetrahydrofolic acid pathway.[37,272] Tetrahydrofolic acid is a co-factor for the thymidylate synthase, which catalyses the last step of the biosynthesis of thymidine (i.e. the formation of dTMP from dUMP). While wild-type *S. aureus* are normally susceptible to trimethoprim (because they need to produce dTMP to build up their DNA), Thymidine-auxotrophic SCVs are resistant to this agent because they use the dTMP found in the environment.

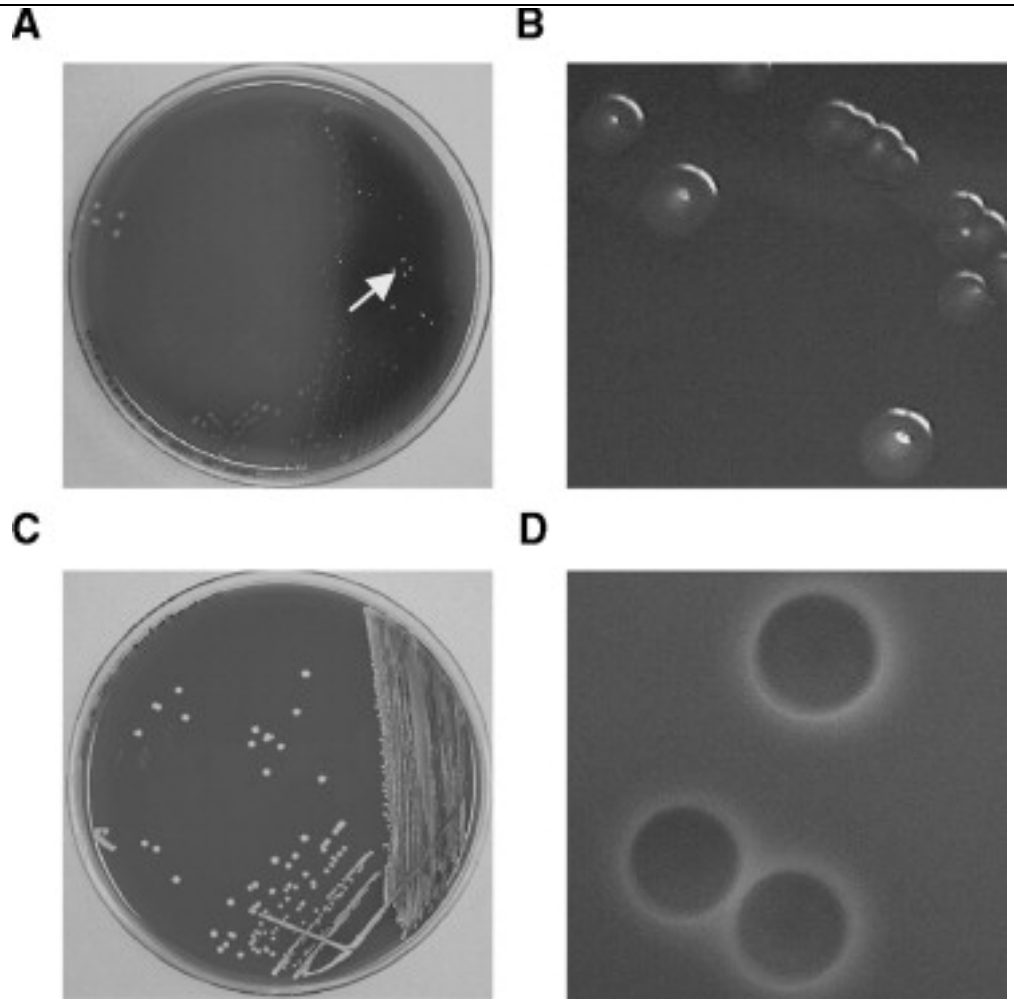


Fig.3.4. Phenotype of a thymidine-auxotrophic SCV (A, B) and its corresponding, large-colony revertant (C,D) (Besier et al, 2007[36]).

Besier and colleagues have shown that alterations of the thymidylate synthase might play a major role in the occurrence of thymidine-auxotrophic SCVs.[36] Sequence analysis of the gene encoding the thymidylate synthase (*thyA* gene) of six clinical isolates of thymidine-auxotrophic *S. aureus* has revealed that all isolates had mutations in the *thyA* sequence (such as (i) truncation by nonsense or frame-shift mutations, (ii) deletions or (iii) amino acid substitutions within the *thyA* gene product). Moreover, while deletions and frame-shift mutations results in the formation of a stable SCV phenotype, a nonsense mutation or a single nucleotide substitution is associated with an instable SCV phenotype. Of particular relevance also, construction of a genetically well-defined *thyA* mutant results in the formation of a thymidine-auxotrophic SCV phenotype.

2.2. Survival advantages of SCVs

The formation of SCVs has important pathogenic implications. Firstly, because the defects and the ensuing SCV phenotype are often reversible, these mutants have abilities to

revert to a highly virulent phenotype when antibiotic therapy is interrupted. This reversion in phenotype may lead to the observation of mixtures of SCVs and normally growing bacteria, which makes the isolation of SCVs difficult and therefore, explains why these organisms are often underdiagnosed.

Secondly, SCVs are characterized by an increased ability to survive within eukaryotic cells. Of particular importance, Vaudaux and colleagues have demonstrated that *hemB* mutants are associated with an enhanced surface expression of fibronectin-binding proteins compared to its isogenic parent[265], which may explain why SCVs mutants are often more internalized within cells (Nguyen et al, unpublished data). An additional key property of SCVs is their decreased overall metabolism and their reduced production of cytotoxins (such as the alpha-haemolysin), which may explain why eukaryotic cells are largely unaffected by these enzymes.

Finally, *in vitro* and *in vivo* studies have documented that exposure to different classes of antibiotics frequently contributes to the selections of SCVs. Because of their reduced growth rate and their defects in metabolic processes, SCVs are commonly associated with an increased resistance to cell-wall active agents (such as β-lactams), aminoglycosides or trimethoprim-sulfamethoxazole. The reduction of susceptibility to aminoglycosides is caused by a decrease in membrane potential, which reduced consequently the uptake of these agents within the bacteria. Clinically, gentamicin seems to promote the selection of SCVs, since electron transport-defective SCVs were frequently isolated in orthopaedic patients treated with gentamicin-containing beads.[199] In another clinically relevant situations, thymidine-dependent SCVs mutants were observed in patients suffering of cystic fibrosis or pneumocystosis and receiving a long-term trimethoprim-sulfamethoxazole therapy.[37,272]

3. Clinical relevance of *S. aureus* intracellular forms

If the ability of *Staphylococcus aureus* to persist in various *in vitro* models is widely established, the capacity of this pathogen to escape from host-cell defences and persist *in vivo* intracellularly is still an ongoing debate.

3.1. Recurent rhinosinusitis

Clément and colleagues have recently reported that isolates of *Staphylococcus aureus* may reside *in vivo* in human nasal epithelial cells, which may probably be related to the initiation of recurrent sinusitis[55]. This *in vivo* intracellular persistence was further documented in the same population by Plouin-Gaudon and colleagues, and seemed to constitute a

significant risk factor for recurrent episodes of rhinosinusitis refractory to antimicrobials and surgical therapy.

3.2. Bovine mastitis

S. aureus is also one of the most common causes of bovine mastitis, which is a disease responsible for important economic losses in dairy production (see for review: [34,124,197]). *In vitro* studies have shown that *S. aureus* adheres to mammary epithelial cells and extracellular matrix components, and invades various mammary cell lines. Using the Fluorescence Activated Cell Sorting (FACS) procedure, Hébert and colleagues have also demonstrated that *S. aureus* are found both in macrophages and alveolar cells isolated from milk samples of infected cow.[105]

3.3. Life-threatening infections

Fully evidence of such intracellular reservoirs is still lacking for life-threatening cardiovascular infections, severe skin and soft-tissues infections, cystic fibrosis and osteomyelitis, probably because of technical difficulties in localizing the highly focalised *S. aureus* reservoirs. Understanding the *in vivo* invasive potential of *S. aureus* should, therefore, open up new avenues to control this pathogen in these diverse settings.

3.3.1. Cardiovascular infections

Endovascular *S. aureus* infections are among the most difficult to threat. At the end of 1920's, Grant and colleagues have hypothesized that the development of infective endocarditis begins with the formation of non-bacterial thrombotic vegetations caused by congenital and rheumatic valvular heart disease.[89] Then, during an episode of bacteraemia, *S. aureus* binds to these vegetations and multiply within their interstices. Various reports indicate, however, that 31 to 70 % of *S. aureus* endocarditis occurs in patients having no known pre-existing heart disease.[122,243] The discovery that cultured endothelial cells phagocytize *in vitro S. aureus* have suggested that ingested bacteria play possibly a role in the initiation of infectious endocarditis and the persistence of septicaemia. Moreover, experimental data show that adhesins (bacterial surface components), platelets, plasma proteins, endothelial cells, and sub-endothelial tissue components are involved in the infection of the vessel wall (see for review: [236]). These findings support the concept that the intracellular life-style of *S. aureus* protects the bacteria from the lethal action of the immune host system and shelters it from the action of antibiotics.

3.3.2. Severe skin and soft-structures infections

Colonization of human skin by *S. aureus* is a common feature of a variety of dermatologic diseases, which is often followed by tissue invasion and cell damages. It has been shown that *S. aureus* has ability to invade *in vitro* keratinocytes, leading to both necrotic and apoptotic damages (independently of the production of alpha- or beta-haemolysins).[152]

3.3.3. Pulmonary infections associated with cystic fibrosis

Although the role of *S. aureus* in the progression of cystic fibrosis remains unknown, it has been reported that *S. aureus* may rapidly recolonized pulmonary tissues, soon after therapy. It has been suggested that *S. aureus* may persist within host cells of the lung[140], which may possibly explain the propensity of recurrent *S. aureus* infections in patients suffering of cystic fibrosis.[116]

3.3.4. Osteomyelitis

S. aureus is the major etiological agent of human osteomyelitis (see for review: [71]), which is characterized by a progressive inflammatory response resulting in extensive damages in bone tissues. *In vitro* studies have previously demonstrated the ability of this pathogen to persist and multiply inside cultured osteoblasts.[114,249] *S. aureus* invasion of osteoblasts is associated with the secretion of interleukin (IL)-6, IL-12, and colony-stimulating factors (see for review:[4]) that can potentially exacerbate the inflammatory response observed in the setting of osteomyelitis. Additionaly, *S. aureus* induces the expression of tumor-necrosis factor (TNF)-related apoptosis inducing agent (TRAIL) by infected osteoblasts, while non-infected osteoblasts do not express this molecule. These results are particularly important, because induction of TRAIL in osteoblasts following bacterial infection could therefore exert a positive osteoclastogenic effect resulting in the increase of bone destruction.[5,205] Finally, *S. aureus* inhibits bone-matrix synthesis, which is a characteristic commonly observed with osteomyelitis.

IV – OVERVIEW OF ANTI-STAPHYLOCOCCAL AGENTS

The discovery of antibiotics and their introduction into medical practice was a revolution that has significantly improved human health. However, multi-drug resistant *S. aureus* has increased in scope in the succeeding years, with the prospect of untreatable *S. aureus* infections. To the present day, glycopeptides remain the gold standard therapy for the treatment of infections caused by β-lactam resistant *S. aureus* (Methicillin-Resistant *S. aureus*) (Table 4.1), often in combination with rifampicin, fusidic acid or gentamicin.

Table 4.1. Antibiotics active against *S. aureus*.

Pathogen	Antibiotics
MSSA	β-lactams Penicillins (dicloxacillin, flucloxacillin, amoxicillin/clavulanate, ticarcillin/clavulanate, piperacillin/clavulanate) Cephalosporins (cephazolin, cephalexin) Clindamycin Co-trimoxazole (trimethoprim plus sulfamethoxazole)
MRSA (if susceptible to the following agents)	Glycopeptides (vancomycin, teicoplanin) Rifampicin Fusidic acid Fluoroquinolones (moxifloxacin, gatifloxacin) Last-line agents (linezolid, quinupristin-dalfopristin)

1. Cell-wall inhibitors

Table 4.2. List of the anti-staphylococcal agents that target the bacterial envelope

Target	Pharmacochemical class	Static or cidal Effect	Bacterial target
Cell-wall	**β-lactams**	Cidal	Penicillin-binding proteins
	Glycopeptides	Cidal	High affinity binding to the D-Ala-D-Ala C-terminus of the pentapeptide, blocking the addition of late precursors to the nascent peptidoglycan chain
	Lipopeptides	Cidal	Binding of daptomycin to bacterial membrane components leads to the loss of membrane potential
	Ceragenins (Cationic Steroid Antibiotics)	Cidal	Targeting of the membrane by ceragenins leads to the loss of membrane potential, permeabilizing efficiently the bacterial membrane

1.1. β-lactams antibiotics

Since 1928, when Alexander Fleming first noted the inhibition of *Staphylococcus* from a mold belonging to the genus *Penicillium*, β-lactams antibiotics have led the fight against bacterial infections. All of these drugs share a common structure and mechanism of action, but have evolved into four various classes with differing spectrum of antibacterial activity. They include penicillins (penicillin G, methicillin, oxacillin,…), cephalosporins (cephalexin,

cefuroxime, …), carbapenems (imipemen, meropenem,…), and the monobactam aztreonam.

1.1.1. Mode of action

β-lactams, an integral part of the armamentarium against *S. aureus* infections, achieve their antimicrobial effect by inhibiting enzymes responsible for catalysing vital stages of cell-wall biosynthesis (Table 4.2, Fig.4.1).[110] The understanding of their mechanism of action was therefore considerably advanced by the discovery of penicillins-binding proteins (PBPs with transpeptidase activities), which are membrane-bound enzymes involved in linking pentapeptide to the growing linear peptidoglycan (Fig.4.1).[39]

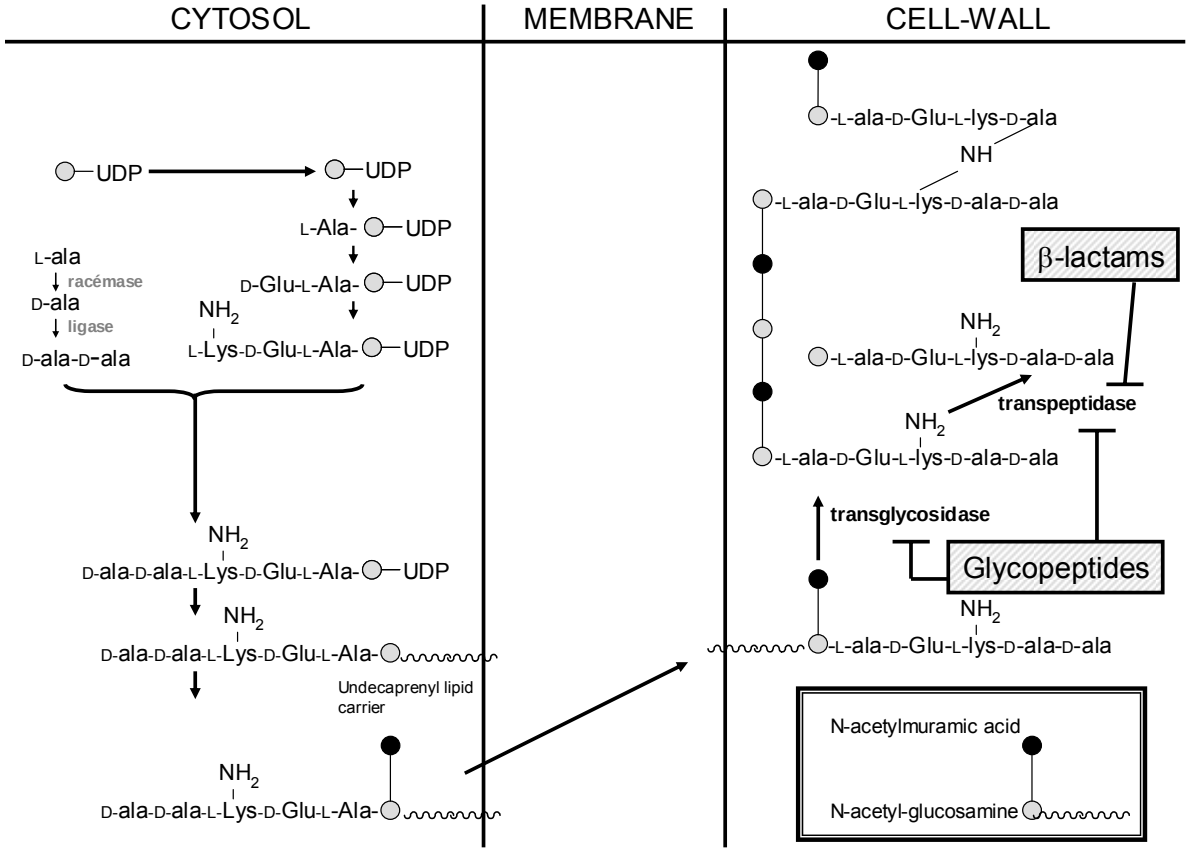


Fig.4.1. Mode of action of cell-wall active agents. Peptidoglycan units are formed in the cytosol of the bacteria, by binding of a short peptide to uridine diphosphate (UDP)-N-acetylmuramic acid. Before crossing the bacterial membrane, the peptidoglycan precursor is attached to a lipid carrier, followed by the binding to the N-acetylglucosamine. At the cell surface, peptidoglycan units are reticulated by the action of transglycosylases (catalysing the polymerisation between sugars) and of transpeptidases (catalysing the polymerisation between peptidic residues). (Adapted from Van Bambeke et al, *Infectious Diseases*, 1999).

Considering the Tipper-Strominger hypothesis[239] which emphasizes a structural similarity between β-lactams and the acyl-D-alanyl-D-alanine terminus of nascent

peptidoglycan strands, such inhibition is the direct result of the covalent binding of the antibiotic to one or more PBPs (which are listed in Table 4.3).

TABLE 4.3. Properties of penicillin-binding proteins of *Staphylococcus aureus*

Protein no.	Molecular mass (kilodaltons)[50]	Enzyme activities	Function or mechanism of resistance
1	85	Transpeptidase	Cell division (septation)[191]
2	81	Transpeptidase Transglycosylase	Cell division and β-lactam resistance[195,196]
2a (or 2')	78	Transpeptidase	Protein from extraspecies origin conferring β-lactam resistance [50,104]
3	75	Transpeptidase	Unknown
4	45	"Secondary" Transpeptidase	Secondary cross-linking [136]; presumed participation in the process of Vancomycin-intermediate resistance [74,283]

In addition, *S. aureus* produces also several hydrolases that specifically cleave various covalent bonds of the peptidoglycan. Despite the fact that the physiological functions of these enzymes remain largely unknown, it has been proposed that they play a key role in a variety of cell processes, including cell-lysis induced by β-lactams.[10,135]

1.1.2. Resistance to β-lactams antibiotics

1.1.2.1. β-lactamases-mediated resistance to β-lactam antibiotics

The introduction of penicillin in the early 1940s has dramatically improved the prognosis of patients with staphylococcal infections. However, as early as 1942, penicillin resistant *S. aureus* were first described, firstly in hospitals and subsequently in the community.[141] Penicillin destruction by bacterial β-lactamases is now one of the most widespread and serious forms of microbial resistance, with more than 90 percent of the isolates producing this enzyme, regardless of the clinical setting.[141]

Staphylococcal resistance to penicillin is mediated by the *blaZ* gene that codes for an extracellular penicillinase hydrolysing the β-lactam ring of penicillinase-susceptible β-lactams (penicillin, ampicillin, …). The expression of penicillinase in staphylococci is often inducible, owing to the presence of *blaZ* regulatory sequences, termed as (i) *blaI*, a gene coding *for the protein BlaI* that acts as a repressor of *blaZ* transcription*, (ii) blaR1*, a gene coding for the protein BlaR1 that acts as a signal transducer in the presence of β-lactams (by its potent anti-repressor effect on BlaI) (Fig.4.2). [91,94]

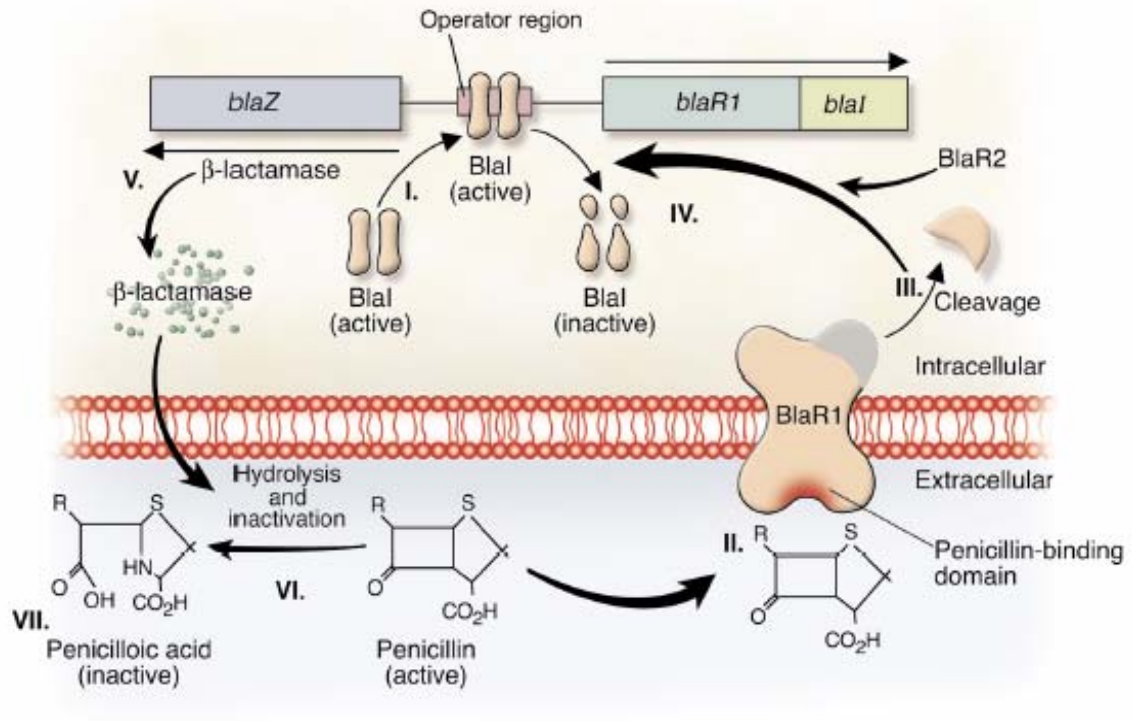


Fig.4.2. Induction of staphylococcal β-lactamase synthesis in the presence of penicillin. In the absence of β-lactam antibiotics, β-lactamase is expressed only at low levels. However, binding of penicillins to the transmembrane sensor-transducer BlaR1 stimulates BlaR1 autolytic activation, resulting in the cleavage of BlaI into inactive fragments and allowing the transcription of both *blaZ* and *blaR1-blaI*. (Lowy et al, 2003[141]).

1.1.2.2. Methicillin-Resistant *Staphylococcus aureus* (MRSA)

By 1960, many hospitals had outbreaks of virulent β-lactamase producing *S. aureus*. They were overcome with penicillinase-stable penicillins (such as methicillin or oxacillin) but the victory was only brief. In 1961, strains of Methicillin-Resistant *Staphylococcus aureus* (MRSA) were recognized. To the present day, MRSA isolates are found worldwide, and most are drug multi-resistant.[11]

1.1.2.2.1. Epidemiology of Methicillin resistance

MRSA has historically been considered as a problem within the hospital setting. However, a dramatic increase in the prevalence of MRSA infections has been noticed in recent years from nursing home facilities, as well as the community and the animals.

1.1.2.2.1.1. Hospital-acquired MRSA

The past decade has witnessed an alarming increase in the prevalence of methicillin-resistance worldwide. MRSA has become a major infection control problem in hospitals, mainly associated with intra- and inter-hospital dissemination of particular epidemic clone lineages of the *S. aureus* population. The Surveillance Network-USA, an

electronic surveillance system reporting resistance trends, shows that MRSA is prevalent in all regions of United States and implicated relatively frequently in hospital outbreaks. In Europe, the prevalence of MRSA infections (Fig.4.3) varies significantly among countries, with still lower MRSA infections in the northern European countries (0.6 % in Scandinavian countries, 1 % in the Netherlands) which has been attributed to the national "search and destroy" policy, which demands isolation and screening of patients at risk (Table 4.4) for MRSA carriage on admission to health-care facilities.[266]

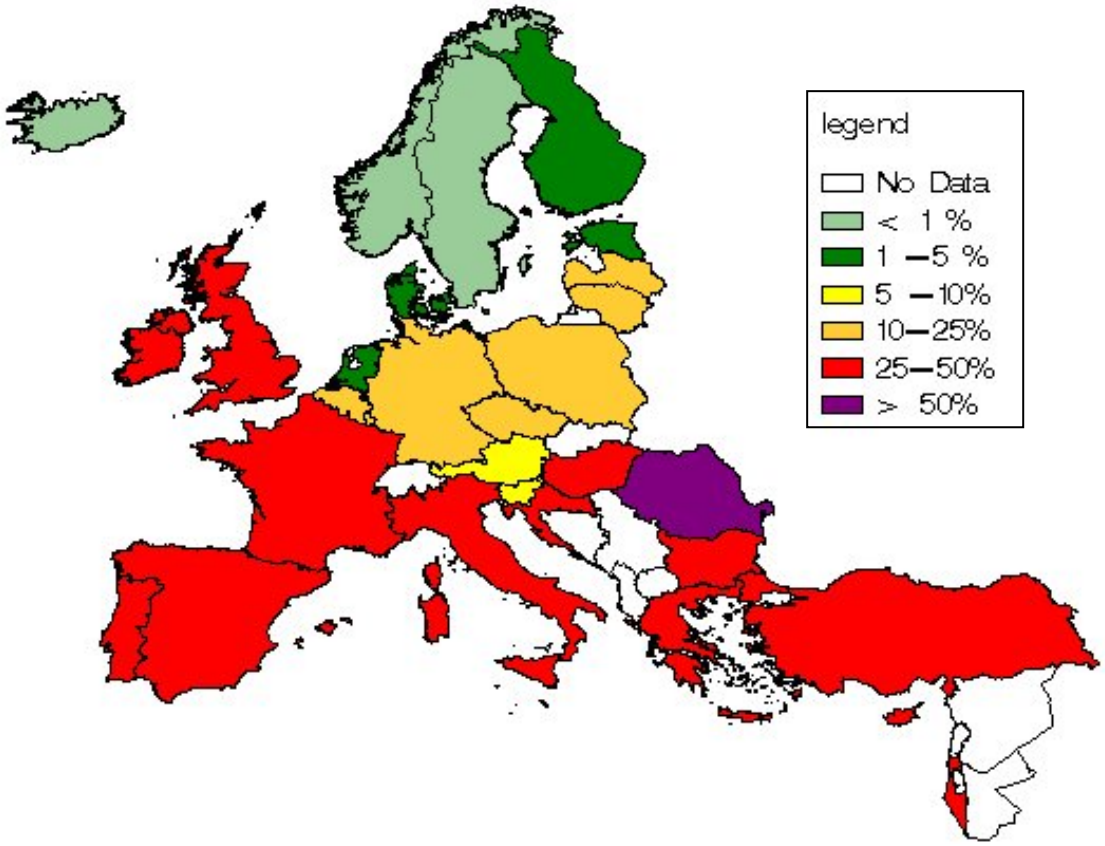


Fig.4.3. Prevalence of invasive isolates of MRSA in 2006 (European Antimicrobial Resistance Surveillance System; Annual Report 2006; http://www.rivm.nl/earss/).

Table 4.4. Risks factors associated with MRSA colonization and infections (adaptated from Segal-Maurer et al, 1996[222]; Bradley S.F., 1997[44]).

Risks factors associated with :
Hospital-acquired infections
Advanced age
Presence and size of wounds
Chronic underlying disease
Presence of invasive indwelling devices (including endotracheal tube)
Previous hospitalization or increased length of stay in hospitals
Long-term stay in an intensive care unit
Exposure to a colonized or infected patient.
Prior and prolonged antibiotic exposure

1.1.2.2.1.2. Nursing homes-acquired MRSA

Formerly confined to hospitals, MRSA has been isolated with increasingly frequency from nursing home patients (See for review: [44,56,164]). In the facilities studied, most nursing home residents acquire MRSA during a hospital stay and remain persistently colonized for months to years. Fortunately, although MRSA colonization is relatively common, rates of MRSA infection and attributable mortality appear to be low.

Moving now to the Belgian situation ([Dr B. Jans, national report from the National Surveillance of Health-care associated Infections), it has been shown that the overall prevalence of MRSA positive patients has dramatically increased from 1999 to 2005 (~ 5 % vs. ~ 18.9 %).

1.1.2.2.1.3. Community-acquired MRSA

Of particular concern, MRSA has now migrated into the community (see for review:[11,57,67,72,84,176]). The occurrence of truly community-acquired MRSA infections in otherwise healthy individuals without risks factors is increasing in many areas, particularly in the paediatric population.[166] Community-associated strains share some characteristics with hospital-acquired *S. aureus*, but also differ by the lack of traditional risks factors associated with health-care infections (Table 4.4), their intrinsic virulence (related to the production of PVL, a necrotizing leukocidin commonly associated with community-acquired staphylococcal skin infection and necrotizing pneumonia[264]) and their susceptibilities to antibiotics (resistance to only a fewer classes of antibiotics).

1.1.2.2.1.4. Animal MRSA

Recent reports have documented MRSA infections in animals such as horses, pets, dairy cows and pigs.[59,262,280] Of particular concern, MRSA strains of animal origin has been recently reported in pigs and pigs farmers from the Netherlands[262] and Belgium (report of the Centre Belge d'Information Pharmacologique [CBIP, www.cbip-vet.be]), suggesting the transmission of MRSA between animals and humans. Subsequent screening revealed that more than 20 % of pigs farmers and 39 % of slaughterhouse pigs were MRSA positive. More importantly, these isolates are unusual, because they could not be typed by pulsed-field gel electrophoresis (unknown change in the methylation system). All isolates belong also to the sequence type (ST) 398, which is a hitherto unusual type in humans.

The process by which these strains emerged in the animal population and subsequently in humans is unclear. Additional insight is needed into the transmission routes between animals and their carers, in order to find ways to control their transmission. Therefore, a concerted effort on the part of clinicians, infection control practitioners and veterinarians is urgently required to prevent further spread of these novel MRSA isolates.

1.1.2.2.2. Genetic basis of the methicillin resistance

1.1.2.2.2.1. *mecA* gene

The central genetic component of methicillin resistance, referred to the *mecA* gene in the literature, is not native to the *S. aureus* genome. This gene encodes for a 78-kDa Penicillin-Binding Protein (PBP) 2a (also referred to PBP2') which has unusually low-affinity for all β-lactam antibiotics conferring resistance to the entire family of β-lactams.

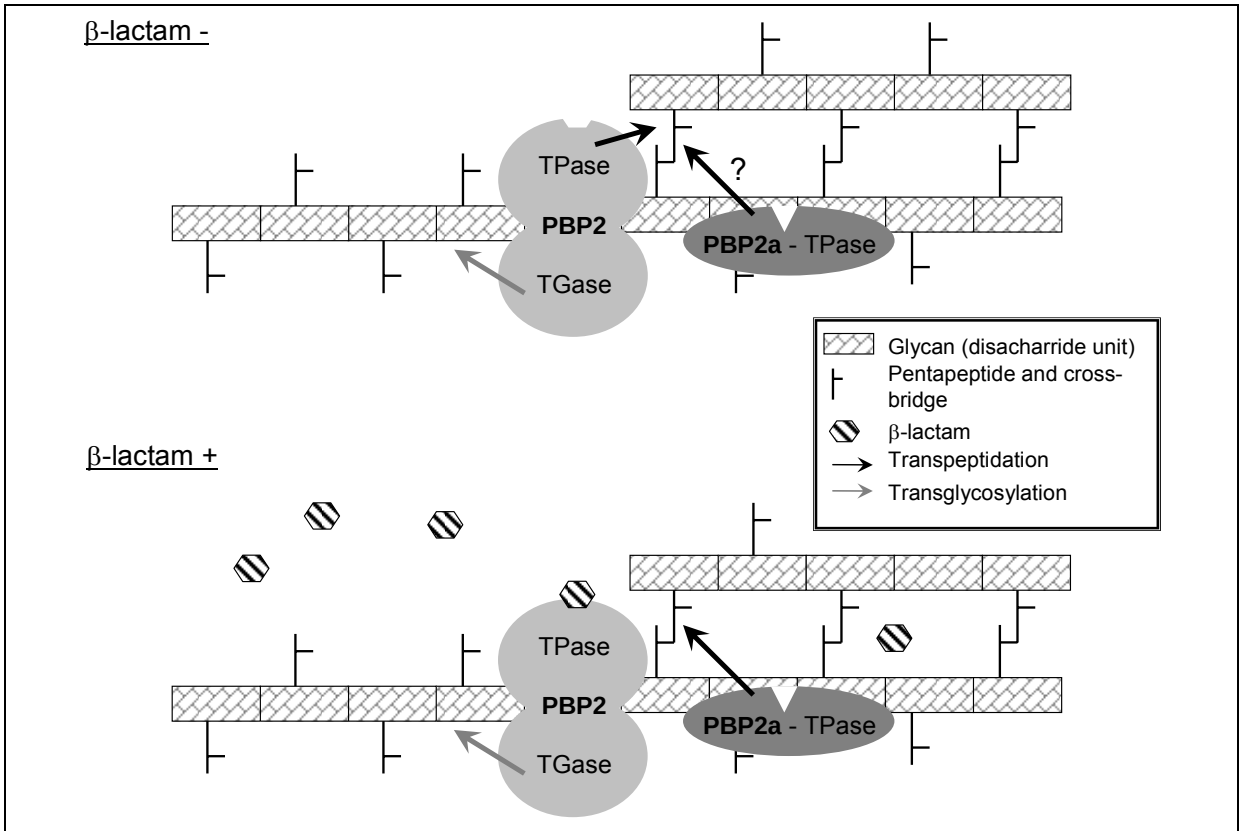


Fig.4.4. Cooperative functioning between the transpeptidase domain of PBP2a and the transglycosylase domain of PBP2 in MRSA. In the absence of antibiotics, it is assumed that both the transpeptidase and transglycosylase domains of PBP2 participate in the cell-wall biosynthesis of *Staphylococcus aureus* (the participation of the transpeptidase domain of PBP2a remains, however, elusive until now). Conversely, exposition of MRSA to β-lactams leads to the acylation of the transpeptidase domain of PBP2, which is no longer capable of performing its function of peptide cross-linking. However, it is assumed that the penicillin-insensitive transglycosylase domain of PBP2 remains active and cooperates with the transpeptidase activity of PBP2a for an effective cell-wall synthesis and bacterial growth of the organism in the presence of β-lactams. (Adapted from Pinho et al, 2001[195]).

Based on homologies with PBPs, PBP2a was suggested to have a transpeptidase activity.[238] In the current model of methicillin resistance, it was suggested that this low-affinity protein substitute to the function of native PBPs (PBP1, 2, 3 and 4) when these latter are acylated and rapidly inactivated by β-lactams (allowing the bacteria to accomplish vital functions). However, observations made recently by Pinho and colleagues strongly suggest that this current model needs to be completed. Firstly, they showed that transposon inactivation of PBP2 in the highly Methicillin-Resistant *S. aureus* COL cause a massive reduction of minimal inhibitory concentrations (MICs) of methicillin (800 vs 12 mg/L), suggesting that PBP2 is critical for the complete expression of methicillin resistance. Secondly, they found that the acquired PBP2a acts together with the native PBP2 to mediate oxacillin resistance by contributing complementary transpeptidase and transglycosylase activities, respectively (Fig.4.4).[195]

1.1.2.2.2.2. The Staphylococcal Cassette Chromosome mec (*SCCmec*)

The 2.1-kb *mecA* gene is located on a mobile genetic element, designated as the Staphylococcal Cassette Chromosome *mec* (*SCCmec*). Currently, five main SCCmec subtypes (types I to V) have been distinguished, ranging in size from 20.9 to 66.9 kb[101]. Sccmec types I (34.3 kb), IV (20.9 to 24.3 kb) and V (28 kb) encode exclusively resistance to β-lactam antibiotics. In contrast, SCCmec types II (53.0 kb) and III (66.9 kb) contain additional drug resistance genes on integrated plasmids (pUB110, kanamycin resistance; pI258, penicillins and heavy metals resistance; and pT181, tetracycline resistance) or transposon (Tn554, carrying the *ermA* gene, which is responsible for inducible macrolide, lincosamide and streptogramin resistance). Moreover, while hospital-acquired MRSA (HA-MRSA) typically have SCCmec subtypes I to III and rarely carry the gene for Panton-Valentin Leukocidin (PVL), types IV and V are largely associated with community-acquired MRSA (CA-MRSA) isolates and at least type IV frequently carries PVL gene.[60]

1.1.2.2.2.3. Regulatory elements of *mecA*

In addition to *mecA*, the staphylococcal *SCCmec* include also the divergently transcribed regulatory elements *mecR1-mecI.*[107] MecR1 has sequence homology to the staphylococcal penicillin sensory-transducer BlaR1 and acts as a signal transducer in the β-lactam induction of *mecA* by its anti-repressor activity. MecI acts as a repressor of *mecA* transcription and shares homology with the β-lactamase repressor BlaI. Thus, in the absence of induction via MecR1 by a β-lactam antibiotic, *mecA* transcription is tightly repressed by *mecI*. Additionally, on the basis of the similarities between *mecA* and *blaZ* regulatory sequences, it is reasonable to assume that each regulon may modulate the

transcriptional expression of *mecA*. Firstly, previous studies have provided some evidences that *mecA* transcription and PBP2a production can be affected by BlaI. Secondly, Hackbarth and colleagues have also reported that PBP2a production was converted from unregulated and constitutive to inducibly repressed by the introduction of a plasmid containing *blaR1* and *blaI*.[94] In this context, the transcription of *mecA* gene is under a dual control and its regulation seems to depend on the cross-talk between inducers and signal transmission to the repressors.[32,91,208] Of particular interest also, all *mecA* clones are not resistant to β-lactams, suggesting that the overall resistance levels depend on the efficient production of PBP2a. This explains therefore why MRSA resistance levels range from phenotypically susceptible to highly resistant strains.[33]

1.1.2.2.3. Biochemical basis of the methicillin resistance

1.1.2.2.3.1. Overall structure of PBP2a

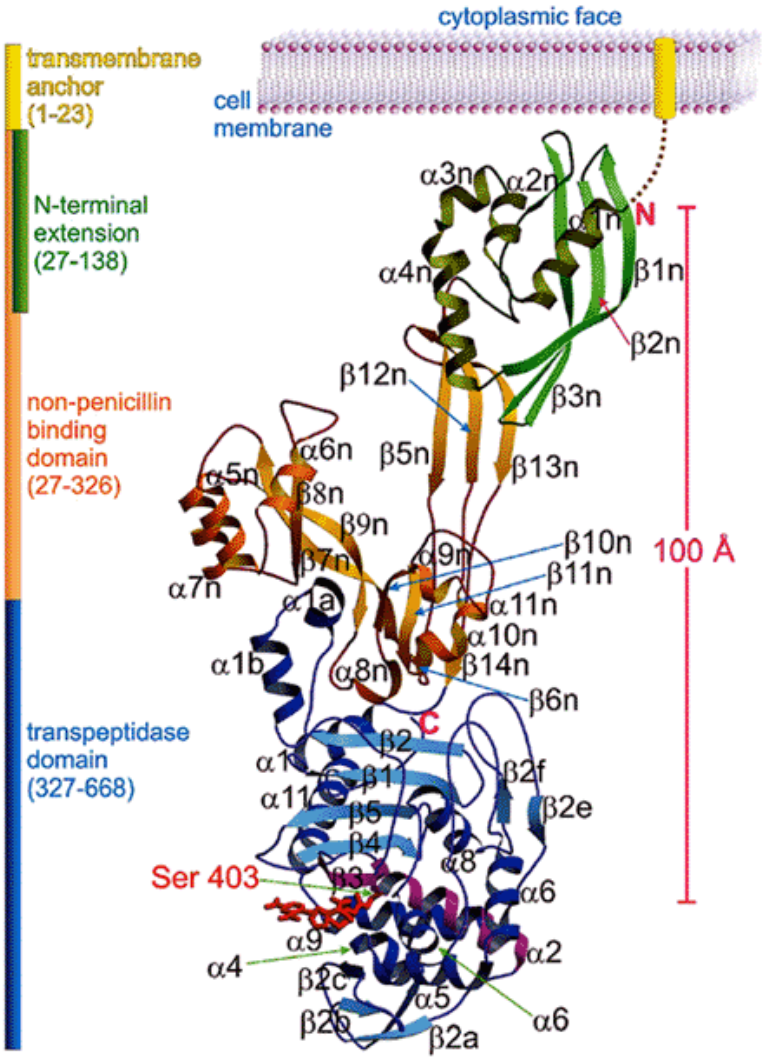
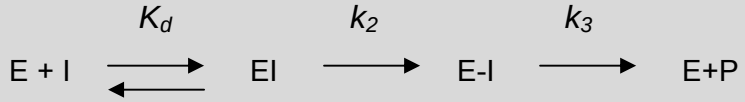


Fig.4.5. Structure of *S. aureus* PBP2a (Lim and Strynadka, 2002[138])

To the present day, many reviews have described the precise role that PBP2a confers to MRSA to circumvent the antibacterial effect of β-lactams. [11,13,82,138,141] Using soluble derivatives of this protein[81,144,246,284], it has been shown that PBP2a (overall dimension: ~ 130 x 60 x 58 Å) consists of (i) a N-terminal extension, (ii) a centralized, bilobal non penicillin-binding (nPB) domain of unknown function, and (iii) a C-terminal transpeptidase domain (Fig.4.5) that contain the active-site serine (Ser-403). Of particular interest, the transpeptidase domain of PBP2a shares a similar overall folding pattern with other transpeptidases and serine β-lactamases[149,179]. Nonetheless, structure-based alignments of the PBP2a transpeptidase domain have revealed low sequence identities (~ 10 – 20 %) and significant structural modifications from other β-lactam sensitive PBPs (see for review [138]). The prominent difference is the active site motif of the nucleophilic serine (Ser^{403}-Thr-Gln-Lys_{406}) that is located on an α-helix sequestered within an extended narrow groove. Unlike typical PBPs, the groove impairs the accessibility of the active site serine to approaching β-lactams (closed conformation).

1.1.2.2.3.2. Kinetic properties of PBP2a

The kinetic mechanism of PBP2a is described by the same three-step pathway that characterizes the others PBPs (See equation 4.1). The interaction of PBP2a with a β-lactam inhibitor begins with the rapid, reversible formation of a non-covalent Michaelis complex (described by the dissociation constant K_d), followed by the nucleophilic attack by the active-site serine Ser-403 on the β-lactam ring (to give a relatively stable covalent acyl-enzyme complex; described by the rate constant k_2) and, finally, by the hydrolytic deacylation step (described by the rate constant k_3) releasing the inactivated β-lactam and regenerating the active enzyme. [82,138]



Equation 4.1. Kinetic scheme for the interactions of PBP2a with a β-lactam agent.
Abbrev. : E, Enzyme ; EI, non-covalent pre-acylation complex; E-I, covalent acyl-enzyme complex; P, product of hydrolysis; K_d, dissociation constant Kd; k_2, acylation rate constants ; k_3, deacylation rate constants.

Comparison of the kinetic parameters of PBP2a with β-lactam sensitive-PBP (R6 PBP2x) showed that β-lactam resistance is primarily due to the inefficient formation of the acyl-enzyme intermediate (slow k_2) and does not result from a more rapid breakdown of the acyl-PBP intermediate (unchanged k_3).[143,144] Recent investigations (using cephalosporins

[nitrocefin, cefepime and ceftazidime], penicillins [ampicillin and oxacillin] and carbapenem [imipenem])[81] have reported that manifestation of resistance is also associated to a lower affinity of PBP2a for conventional β-lactams (K_d effect). In this context, improving k_2 and K_d is an effective strategy to obtain more potent anti-MRSA agent.

1.1.2.2.4. Factors affecting methicillin resistance

Numerous genes influencing methicillin-resistance phenotype have been identified. Transposon insertional mutagenesis producing susceptible mutants from MRSA strains has led to the identification of chromosomal genes, physically distinct from *mec*, that are necessary for the full expression of resistance. In 1983, the concept of *fem* factors (factors essential for methicillin resistance) was introduced by Bercher-Bächi and collegues.[31] Until now, the experimental screening for loss of methicillin resistance has led to the discovery of more than 20 genes, most of them being involved in cell-wall biosynthesis and turnover.

Additionally, external factors may also modify significantly methicillin resistance. The addition of NaCl or sucrose to the culture medium, or incubation at 30°C, enhances the expression of methicillin resistance while this latter is suppressed by incubation at 43°C or exposition at pH 5.2 (Table 4.5)[50].

TABLE 4.5. Effect of external factors on the level of methicillin resistance

Effect on methicillin resistance	External factors	Ref.
Enhancement of methicillin resistance	high salt concentration Incubation at 30°C Passage in culture medium containing β-lactam antibiotics	209 9
Decrease of methicillin resistance	Incubation at 43°C Low pH (pH 5.2)	9 210

1.2. Glycopeptides antibiotics

Since the emergence of MRSA strains in 1961, options for the treatment of *S. aureus* infections have been significantly limited. Efficacy of vancomycin, considered for many years as the last resort agent for such infections, is associated with several limitations, including relatively slow bactericidal effects, fluctuating MICs, poor pharmacokinetic properties, potential for serious toxicity and finally, the development of resistance and associated therapeutic failures.[137] Because vancomycin remains the cornerstone of treatment for all MRSA infections, the Center for Disease Control (CDC) has published guidelines (focusing on the judicious use of vancomycin, the use of effective laboratory screening methods, and

the acquisition of investigational antimicrobial drugs when clinically appropriate) regarding the prevention and control of the infections caused by vancomycin-resistant *S. aureus.*

1.2.1. Mode of action

Glycopeptides owes their antibacterial activity to interference with peptidoglycan biosynthesis. The antibacterial action of glycopeptides depends on their ability to bind with high affinity to the D-Ala-D-Ala C-terminus of the pentapeptide, blocking therefore the addition of late precursors by transglycosylation to the nascent peptidoglycan chain and preventing subsequent cross-linking by transpeptidation (Fig.4.1). Because β-lactams inhibit cell-wall biosynthesis in the later phase of peptidoglycan synthesis, there is no cross-resistance and no competition for binding sites between these agents.

1.2.2. Glycopeptides-resistant *Staphylococcus aureus*

The Antimicrobial Susceptibility Testing (AST) subcommittees of the Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS) defines Staphylocci requiring concentration of vancomycin of ≤ 2 mg/L for growth inhibition as “susceptible”, those requiring 4-8 mg/L as “intermediate”, and those requiring concentrations of vancomycin ≥ 16 mg/L as “resistant”. [242]

1.2.2.1. Vancomycin-intermediate *S. aureus* (VISA)

MRSA isolates with reduced susceptibility to vancomycin have emerged since 1996 in patients receiving prolonged courses of vancomycin therapy. [12,14,194] The VISA phenotype is somewhat unique because it appears to be the results of global metabolic changes affecting overall structure, cell-wall synthesis and autolysis.[203,231] The strains are notable for their additional quantities of synthesized peptidoglycan that confer irregularly shaped, thickened cell-wall (Fig.4.6), which presumably bind vancomycin extracellularly before reaching their targets at the cytoplasmic membrane.[97] HPLC provides further proof of this novel resistance mechanism by showing that large quantities of vancomycin become trapped in the abnormal peptidoglycan.[230] The molecular mechanism underlying these alterations in peptidoglycan biosynthesis remains, however, elusive.

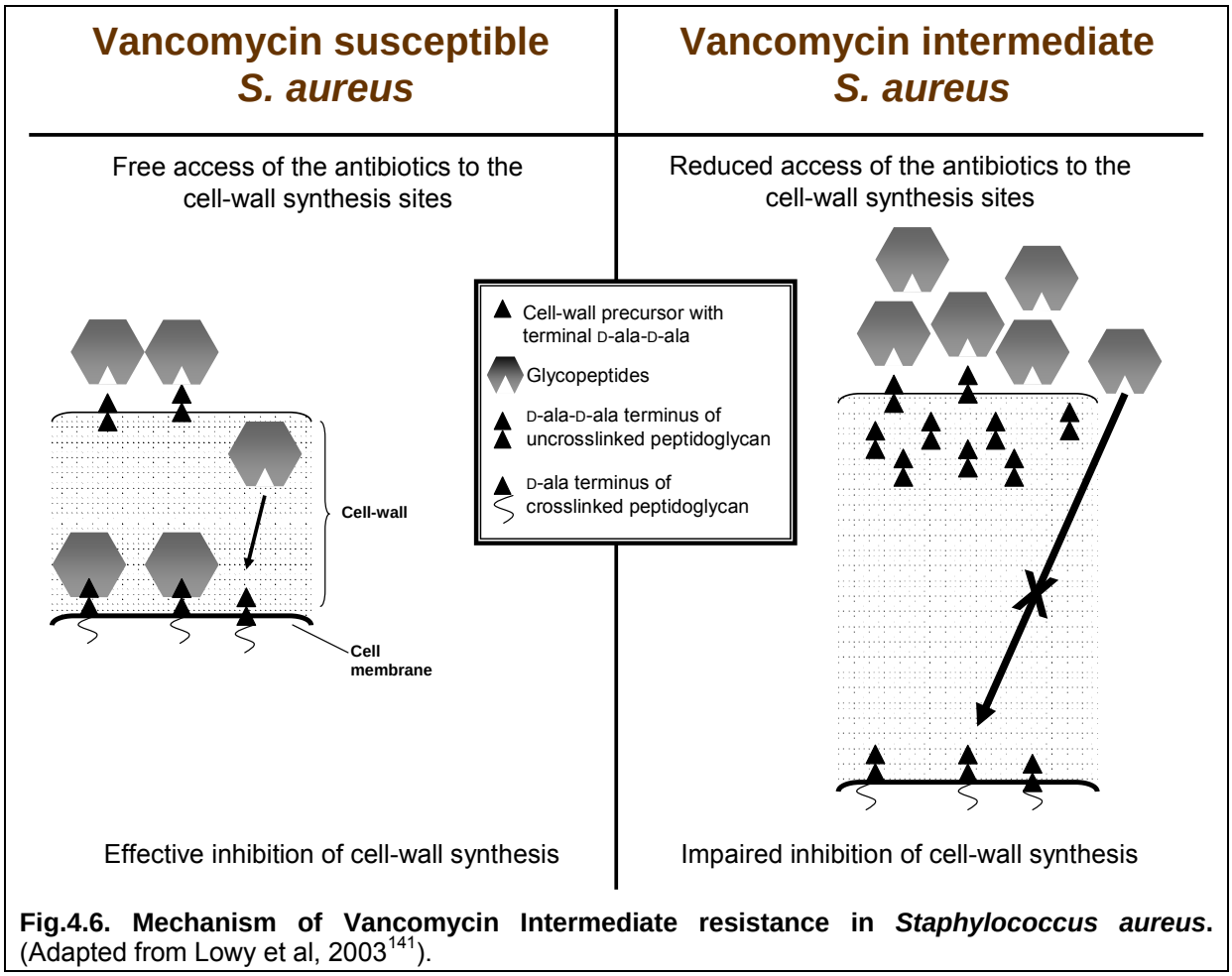


Fig.4.6. Mechanism of Vancomycin Intermediate resistance in *Staphylococcus aureus*. (Adapted from Lowy et al, 2003[141]).

Additionaly, as initially described by Jevitt et al, VISA isolates has also been associated with reduced susceptibility to daptomycin.[58] In this context, it is conceivable that the changes mediated in the cell-wall of the VISA strains may also prevent the access of daptomycin to its target.

1.2.2.2. Vancomycin-resistant *S. aureus* (VRSA)

Vancomycin resistance was first reported for enterococci in 1988 and the first *vanA*-carrying MRSA was isolated fourteen years later from a dialysis patient in Michigan (MIC, 1,024 mg/L). Since 2002, six VRSA strains have been isolated in the United States but, as far as we know, have not been reported elsewhere, except from India.[244] This second form of vancomycin resistance has resulted from the probable conjugal transfer of the *vanA* gene cluster from a vancomycin-resistant *Enterococcus faecalis.*[229] This operon encodes a dehydrogenase (VanH), which reduces pyruvate to D-Lac, and the Van-A ligase, which catalyzes the formation of an ester bond between D-Ala and D-Lac. The resulting D-Ala-D-Lac depsipeptide replaces the D-Ala-D-Ala dipeptide in peptidoglycan synthesis, decreasing

therefore markedly the affinity for glycopeptides. Synthesis of D-Ala-D-Lac occurs only with exposure to low concentrations of vancomycin. [87]

2. Inhibitors of nucleic acid synthesis

Table 4.6. List of anti-staphylococcal agents that target the synthesis of nucleic acid

Target	Pharmacochemical class	Static or cidal effect	Bacterial target
DNA synthesis	**Quinolones**	Cidal	Bacterial DNA gyrase and/or topoisomerase IV
	Ansamycins	Cidal	Bacterial RNA polymerase
	Didrofolate reductase inhibitors (trimethoprim, iclaprim)	Static	Bacterial dihydrofolate reductase
	Dihydropteroate synthase inhibitors (sulfonamides)	Static	Bacterial dihydropteroate synthase

2.1. Quinolones

Quinolones have been broadly used for the treatment of many infections due to their excellent pharmacokinetic profiles, high antimicrobial activities and low incidence of side-effects. Currently, fluoroquinolones are known to form ternary complexes in bacterial cells with (i) DNA gyrase, an enzyme introducing negative super-helical twists into DNA, and/or (ii) topoisomerase IV, an enzyme enable the separation of interlinked daughter chromosomes (Table 4.6). In this context, inhibition of these enzymes by fluoroquinolones result in bacterial cell death.[109]

Quinolone resistance among *S. aureus* (Table 4.8) has emerged quickly, more prominently among methicillin-resistant *S. aureus* strains. Resistance to quinolones results from (i) stepwise acquisition of chromosomal mutations occurring in the target of antibiotics, or by (ii) the introduction of multi-drug efflux transporters (NorA). In *Staphylococcus aureus*, increased expression of this transporter is associated with low-level resistance to hydrophilic quinolones (including ciprofloxacin, norfloxacin, …).[113,167,286]

2.2. Ansamycins

The biological action of ansamycins (e.g. rifampicin) relies on the potent inhibition of DNA-dependent RNA polymerase at the β-subunit (Table 4.6). Clinical resistance (Table 4.8) occurs rapidly under monotherapy and is associated with gene mutations altering the RNA polymerase β-subunit (*rpoB*). These mutations may occur at different sites in the RNA polymerase and lead to various degree of resistance to rifampicin.[278,279]

2.3. Inhibitors of the dihydrofolate reductase and the dyhydropteroate synthetase

Trimethoprim is a bacteriostatic antibiotic belonging to the class of dihydrofolate reductase inhibitors. By interfering with bacterial dihydrofolate reductase, this agent inhibits the synthesis of tetrahydrofolic acid, an essential precursor of *de novo* synthesis of DNA nucleotide thymidine (Table 4.6). Trimethoprim is commonly used in combination with sulfamethoxazole, a sulfonamide antibiotic, which inhibits an earlier step in the folate synthesis pathway. Clinical resistance (Table 4.8) to these agents results from structural change in dihydrofolate reductase or dydropteroate synthetase producing enzymes with lowered affinity for theses antibiotics.

3. Inhibitors of the protein synthesis

Table 4.7. List of anti-staphylococcal agent that target the synthesis of proteins

Target	Pharmacochemical class	Static or cidal effect	Bacterial target
Protein synthesis	**Aminoglycosides**	Cidal	30S ribosomal subunit
	Macrolides	Static	50S ribosomal subunit
	Lincosamides	Static	50S ribosomal subunit
	Chloramphenicol	Static	50S ribosomal subunit
	Tetracyclines	Static	30S ribosomal subunit
	Streptogramins	Static alone; cidal effect by combination	50S ribosomal subunit
	Fusidic acid	Static	Bacterial ribosomal translocase, termed as Elongation factor G (EF-G)
	Glycylcyclines	Static	30S ribosomal subunit
	Oxazolidinones	Static	50S ribosomal subunit

3.1. Aminoglycosides

Since 1940s, aminoglycosides have been an important part of the antibacterial drug arsenal. Their mechanism of action relies on efficient uptake process, and is dependent of the electrochemical gradient. In this context, transport of aminoglycoside across the bacterial membrane is considerably reduced in anaerobic environments, or during growth in high-osmolar culture media or low external pH.[150] Moreover, since these agents are polycationic, they show high binding affinity for the bacterial ribosome, allowing therefore the inhibition of protein synthesis (Table 4.7).[98,130] Clinical resistance to aminoglycosides (Table 4.8) results from (i) alteration in aminoglycosides uptake (i.e. SCVs variants)[271] or (ii) synthesis of modifying enzymes impairing the activity of these antimicrobial agents.

3.2. Tetracyclines

Tetracyclines are a family of antibiotics that appear to enter bacteria by an energy-dependent process. Once within the bacteria, these agents inhibit protein synthesis (Table 4.7) by preventing the attachment of aminoacyl-tRNA to the bacterial ribosome. Tetracycline resistance (Table 4.8) has quickly emerged due to the acquisition of *tet* genes coding for efflux transporters (*tetK, tetL)* or proteins altering ribosomal conformation (*tetM, tetO*).[54,182,247]

3.3. Chloramphenicol

Chloramphenicol appears to enter bacteria by an energy-dependent process. Once within the bacteria, the biological action of this agent relies on the inhibition of protein synthesis by reversibly binding to the 50S subunit of the ribosome (Table 4.7). Clinical resistance (Table 4.8) results from the acquisition of a chloramphenicol acetyltransferase (CAT) that acetylates the antibiotic to an inactive diacetyl derivative.[46]

3.4. Macrolides

Macrolides (e.g. erythromycin, azithromycin) inhibit RNA-dependent protein synthesis due to their affinity to the 50S ribosomal subunit (Table 4.7). Resistance to macrolides (Table 4.8) may result from a number of causes:

- Alterations of the 50S ribosomal subunit by methylation (MLS_B phenotype; resistance to all macrolides and cross-resistance to Lincosamides [lincomycin and clindamycin] and type-B Streptogramins)[65]
- Ribosomal mutations (L4 or L22 ribosomal protein)[200,201]
- Enzymatic inactivation of erythromycin and some others 14- and 15- membered ring macrolides (azithromycin) by staphylococcal esterases (*ereA* or *ereB);*[281]
- Active efflux transporters (*msrA* and *msrB* genes*).*[281]

3.5. Lincosamides

Lincosamide agents (e.g. lincomycin and clindamycin) inhibit protein synthesis (Table 4.7) in the early chain elongation by interference with the transpeptidation reaction. Clinical resistance to lincosamides results from the alteration in the 50S ribosomal subunit by methylation (MLS_B phenotype; Table 4.8).

3.6. Fusidic acid

Fusidic acid belongs to a group of antibiotics which inhibit the growth of Gram-positive bacteria, specifically by preventing the effective translocation of the elongation factor

G (EF-G) from the ribosome (Table 4.7). This agent proves active against *S. aureus*, including isolates that are methicillin-resistant. Resistance to fusidic acid (Table 4.8) may occur through a variety of mechanisms, such as chromosomal mutations altering the target site.[172]

4. Alternatives to vancomycin for the treatment of MRSA infections

4.1. Antibiotics that target the cell-wall

The bacterial membrane is an appealing target, given that most structural elements are conserved and resistance to membrane-damaging agents should require major structural changes which should be incompatible for bacterial survival.

4.1.1. Lipopeptides

Daptomycin, a cyclic lipopeptide discovered more than fifty years ago, possess bactericidal activity against MSSA and MRSA isolates. The mechanism(s) by which daptomycin kill *S. aureus* has not been certainly established, but it appears as through its target is the cytoplasmic membrane. Through previously published studies, it is understood that daptomycin initially attacks bacteria by inserting in a calcium-dependent manner its tail into the bacterial cell membrane. In this context, this agent rapidly dissipates the transmembrane electrical potential gradient (by triggering a dose-dependent efflux of potassium), which is essential for cell viability.[99,120,234] Of particular interest, daptomycin is active at all growth phases, which may be particularly useful for the treatment of indolent, deep-seated infections (endocarditis, osteomyelitis) in which bacteria reached stationary phase.[148]

Clinical development of daptomycin, which was stopped by its discoverers in the early nineties (because of unsuccessful phase II trial and muscular toxicity), was re-initiated in 1997. Owing to a better understanding of the pharmacodynamic property of this agent, it was shown that both safety and efficacy could be improved by once-daily administration. Daptomycin is now approved worldwide for the treatment of complicated skin and skin structures infections caused by susceptible *S. aureus*, as well as for *S. aureus*-induced bacteraemia and right-sided endocarditis in the United States.[95] Of note, daptomycin should not be use in pneumonia, due to its inactivation by pulmonary surfactant (related to the insertion of daptomycin within surfactant aggregates).[233] Conversely, novel investigational lipopeptides (MX-2401) show promising interest, offering over daptomycin the advantage of not being inactivated by pulmonary surfactant.

Resistance to daptomycin has already been described.[30,119,147] Increasingly frequent reports of clinical failures (particularly in the settings of deep-seated infections) have documented a loss of daptomycin susceptibility for these difficult-to-threat isolates. Although genetic mutations has been discovered (such as in the *mrpF* gene, which encodes an lysylphosphatidylglycerol synthase involved in the biosynthesis of phosphatidylglycerol [see Table 4.8])[79,119], Jones and colleagues have recently described that, compared to their parental (sensitive) clones, daptomycin-resistant *S. aureus* are associated with (i) membrane phospholipid asymmetry (increased translocation of the positively-charged phospholipid [LPG, lysine-phosphatidyl-glycerol] to the outer membrane leaflet) and (ii) alterations in surface charge (increased positive charge in the bacterial surface) and drug binding.[118]

4.1.2. Ceragenins

Ceragenins, synthetic cationic steroid molecules that mimic endogenous antimicrobial peptides, adopts an amphipathic structure in the presence of lipids.[73] Owing to their potent ability to cause membrane depolarization and permeabilization[62], these agents combine a series of advantage, namely (i) a potent *in vitro* activity against *S. aureus*, including resistant isolates *(*MRSA, VISA and VRSA isolates)[53] ; (ii) an extensive bactericidal potency[53], (iii) and a low propensity to select for resistance (see for review [260]).

Despite of these apparent advantages, the specificity of ceragenins towards bacteria seems suboptimal, since they were shown to mediate a fast and extensive membrane-damaging effect in THP-1 macrophages (Lemaire et al, 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, 2006, Poster A-0633). This extended cellular toxicity may severely limit their potential clinical use to topical indications.

4.1.3. Novel β-lactams

Within the class of β-lactams, ceftobiprole, ceftaroline and the carbapenem CS-023/RO-4908463 are specifically designed to keep activity against MRSA as a result of their increased affinity for PBP2a, the penicillin-resistant target ofMRSA.[1,45,117,170]. Ceftobiprole (see for review : [45,169,180,181]), the first broad spectrum cephalosporin to be assessed in late-stage clinical trials, seems to be a promising agent because of (i) its low MIC values against *S. aureus* (including multi-resistant VRSA isolates); and (ii) its low potential to select for resistance in preclinical, multi-passage resistance selection studies.[40]

4.1.4. Novel anti-MRSA glycopeptides

New glycopeptides have been designed to keep to keep activity against vancomycin-resistant *S. aureus*. On the first hand, dalbavancin is a second-generation semi-synthetic lipoglycopeptide that inhibits cell-wall synthesis and has *in vitro* activity against MRSA. This new agent binds to the terminal D-alanyl-D-alanine moiety of peptidoglycan precursors, blocking therefore the final stages of peptidoglycan synthesis and cell-wall formation. Due to its unique once-weekly dosing (extended half-life, 6 to 10 days) and greater tissue penetration, dalbavancin may offer advantages in the treatment of MRSA infections, such as alleviating the need for the continued presence of indwelling catheters in patients.[154,214]

On the second hand, telavancin and oritavancin are semi-synthetic glycopeptides with dual mechanism of action, including inhibition of cell-wall synthesis and membrane disruption.[106,121] Their capacity to interact and to destabilize the bacterial membrane confers to these agents a highly bactericidal potential towards *S. aureus*, including multi-resistant strains. These investigational agents are currently under investigation for the treatment of skin and soft-structures infections (SSTIs), nosocomial pneumonia, and uncomplicated bacteriemia due to *S. aureus.* (see for review : [132,254,255,261,275]).

4.2. Antibiotic targeting the synthesis of nucleic acid

4.2.1. Novel inhibitor of the dihydrofolate reductase

The diaminopyrimidine iclaprim is a novel, selective inhibitor of the dihydrofolate reductase which has shown potent *in vitro* activity against resistant *S. aureus* (MRSA, VISA, VRSA, and macrolide-, quinolone-, and trimethoprim-resistant clones). Promising results for the treatment of complicated skin and skin structures caused by MRSA isolates has been shown in clinical trials (phase II and III).[129,220]

4.3. Antibiotics targeting the synthesis of protein

4.3.1. Oxazolidinones

Oxazolidinones are a novel class of synthetic antimicrobial agents discovered in 1987. Linezolid, which is currently approved for the treatment of infections caused by *S. aureus* (complicated skin and soft-tissues infections, and nosocomial pneumonia), binds to the 23S ribosomal RNA (rRNA) of the 50S ribosomal subunit and blocks reversibly the formation of protein synthesis initiation complexes (Table 4.7).[241] Binding is, however, competitively inhibited by chloramphenicol and lincomycin, which suggests either shared or overlapping binding sites.

Because this mechanism of action is thought to be different from all other class of antibiotics, linezolid has the same *in vitro* activity against many antibiotic-sensitive and antibiotic-resistant *S. aureus* (MRSA, VISA and VRSA isolates).[20,43,99] Despite these apparent advantages, concerns about safety often limit its use (association of linezolid with serotonin toxicity and thrombocytopenia).[35,178] Additionally, clinical resistance to linezolid (Table 4.8) has developed slowly and may result from (i) target site mutations (domain V of the 23S ribosomal RNA of the 50S ribosomal subunit)[287] or (ii) ribosomal protection (*cfr* gene).[245]

4.3.2. Glycylcyclines

Compared to minocycline, tigecycline (a new agent that has been recently approved for the treatment of skin and soft-tissues infections due to MRSA, and complicated intra-abdominal infections caused by MSSA clones) harbors a modified side-chain that (i) enhances its binding to the 30S ribosomal subunit, inhibiting therefore protein synthesis and bacterial growth (Table 4.7)[173,192,206], and (ii) circumvents resistance mechanisms that plague conventional tetracyclines (Table 4.8).[42] Its main property to overcome many common resistance mechanisms allows therefore the use of tigecycline for many serious and life-threatening infections for which the use of other antibiotics is no longer appropriate.

The *in vitro* selection of mutants with reduced susceptibility to tigecycline was evaluated by McAleese and colleagues over a period of 16 days. Transcriptional profiling revealed that tigecycline mutants exhibited over 100-fold increased expression of a gene cluster, *mepRAB* (multidrug export protein), encoding (i) a MarR-like transcriptional regulator (*mepR*), (ii) a novel MATE family efflux pump (*mepA*), and (iii) a hypothetical protein of unknown function (*mepB*).[151]

4.3.3. Streptogramins

Streptogramins, which were discovered in the 1960s, consists of mixtures of two structurally distinct cyclic peptide agents (pristinamycin IA and IIB in the case of quinupristin-dalfpristin) that are separately bacteriostatic, but bactericidal in appropriate ratios. Quinupristin-dalfopristin owe its antibacterial activity by acting synergistically on the bacterial 50S ribosomal subunit, which inhibit the synthesis of proteins (Table 4.7) in drug-resistant *S. aureus*, including MRSA, VISA and VRSA isolates.[93,99] Clinical resistance (Table 4.8) has only developed at quite modest levels, through the following mechanisms: (i) plasmid-mediated target modification (MLS_B phenotype; resistance to quinupristin without affecting the susceptibility of dalfopristin); (ii) drug-modifying enzymes (*vatA*, *vatB* and *vatC* genes :

dalfopristin resistance; *vgbA* and *vgbB* genes : quinupristin resistance); or (iii) active efflux (*mrsA* and *mrsB* genes: quinupristin resistance; *vgaA* and *vgaB* genes: dalfopristin resistance).[99]

The clinical utilization of quinupristin-dalfopristin, which has been approved for the treatment of *S. aureus* infections (complicated skin and skin-structures infections caused by MSSA), is often limited by its toxicity profile (peripheral venous intolerance, arthralgias and myalgias, increases levels in conjugated bilirubin, interactions with drugs metabolized by the cytochrome P450 3A4 isoenzyme or physico-chemical incompatibilities). Nevertheless, multiple studies have reported that the safety and tolerability of this combination of antibiotics are generally favorable, providing clear benefits to ill patients with severe, refractory infections caused by Gram-positive infections.[6,207]

TABLE 4.8. Resistance mechanisms

Pharmacochemical class	Mechanism	Function	Gene
β-lactams	**Drug inactivation**	Secretion of β-lactamases	*blaZ*
	Target modification	Acquired Penicillin Binding Protein (termed PBP2a) with reduced affinity for β-lactam agents	*mecA*
		Conventional PBPs with reduced β-lactam affinity	Unknown
Glycopeptides	**Target modification**	Thickened cell-wall by secretion of additional peptidoglycan layers (Vancomycin-intermediate *S. aureus* [VISA])	Unknown
		Acquisition of the vanA operon; synthesis of cell-wall precursor ending with D-ala-D-lac depsipeptide rather than D-ala-D-ala (Vancomycin-resistant *S. aureus* [VRSA])	*vanA*
Fluoroquinolones	**Target modification**	Genes coding for subunits of DNA gyrase (gyrA or gyrB) or topoisomerase IV (grlA); Most common sites of mutations	*gyrA, gyrB, grlA*
	Efflux	Overexpression of *the protein* NorA by mutations in the transcriptional control region confers resistance to hydrophilic quinolones (norfloxacin, ciprofloxacin) and antiseptics	*norA*
		Overexpression of *norB* confers resistance to hydrophilic quinolones, ethidium bromide and cetrimide	*norB*
		Overexpression of norC confers two-fold increase of MICs for ciprofloxacin, four-fold increase of MICs for moxifloxacin, norfloxacin and garenoxacin but no change for garenoxacin	*norC*
Ansamycins (rifampicin)	**Target modification**	Gene coding for RNA polymerase β-subunit; Most common sites of mutations resulting in reduced-affinity RNA polymerase	*rpoB*
Trimethoprim	**Target modification**	Production of a dihydrofolate reductase that is resistant to binding by trimethoprim	*dhfr*
Sulfonamides	**Target modification**	Production of a dihydropteroate synthetase that is resistant to binding by sulfonamides	*dhps*
Aminoglycosides	**Drug inactivation**	Protein (AAC[6']-APH[2'']) with acetyl-transferase activity in the amino-terminal domain and phosphotransferase activity in the carboxy-terminal domain; Resistance to gentamycin, tobramycin and kanamycin	aacA3-aphD
		Protein (ANT[3'']][9]) with aminoglycoside adenylyl-transferase activity; Resistance to streptomycin and spectinomycin	aadA
		Protein (ANT[6']) with aminoglycoside adenylyltransferase activity; Resistance to streptomycin	aadE
		Protein (ANT[4'][4'']) with aminoglycoside adenylyl-transferase activity; Resistance to neomycin, kanamycin, paromomycin, tobramycin and amikacin	aadD
		Protein (APH[3']III) with aminoglycoside phospho-transferase activity; Resistance to neomycin and kanamycin	aphA
		Protein (APH[3'']) with aminoglycoside phosphotransferase activity; Resistance to streptomycin	aphC
		Protein (ANT[4']) with aminoglycoside nucleotidyltransferase activity;	*aad*

		Resistance to amikacin, kanamycin and tobramycin	
Tetracyclines	**Target modification**	Ribosomal protection; resistance to tetracycline and minocycline	*tetM, tetO*
	Efflux	Decrease tetracycline accumulation within bacteria; Resistance to tetracycline	*tetK, tetL*
		Overexpression of *tet38* confers resistance to tetracycline (but not to minocycline)	*tet38*
Glycylcycline	**Efflux**	Overexpression of mecpRAB gene cluster (encoding mepR, mepA and mepB) confers resistance to tigecycline	*mepRAB*
Chloramphenicol	**Drug inactivation**	Secretion of a specific protein with chloramphenicol acetyl-transferase activity (acetylation of the antibiotic to an inactive diacetyl derivative)	*cat*
Macrolides	**Drug inactivation**	Enzymatic inactivation by esterases; Resistance to erythromycin, azithromycin and clarithromycin	*ereA, ereB*
	Target modification	Methylation of the 23S ribosomal RNA of the 50S ribosomal subunit; Cross-resistance to lincosamides and type-B streptogramins (MLS_B)	*ermA, ermB*
	Efflux	Resistance to macrolides and type-B streptogramins	*msrA, msrB*
Lincosamides	**Target modification**	Methylation of the 23S ribosomal RNA of the 50S ribosomal subunit; Cross-resistance to lincosamides and type-B streptogramins (MLS_B)	*ermA, ermB*
Streptogramins	**Drug inactivation**	Lyase-mediated hydrolysis of type-B streptogramins	*vgbA, vgbB*
		Acquired-protein with acetyl-transferase activity; Resistance to type-A streptogramins	*vat(A,B,C)*
	Target modification	Methylation of the 23S ribosomal RNA of the 50S ribosomal subunit; Cross-resistance to lincosamides and type-B streptogramins (MLS_B)	*ermA, ermB*
		Common site of mutations associated with resistance to quinupristin-dalfopristin	*L22* gene
	Efflux	Active efflux transporters; Resistance to macrolides and type-B streptogramins	*msrA, msrB*
		Active efflux transporters; Resistance to type-A streptogramins	*vgaA, vgaB*
Fusidic acid	**Target modification**	Gene coding for the prokaryotic ribosomal translocase termed as Elongation Factor G (EF-G); most common sites of mutations	*fusA*
Oxazolidinones	**Target modification**	Gene encoding the central loop of domain V of the 23S ribosomal RNA; most common sites of mutations	*23S rRNA*
		Gene coding a methyl-transferase protein; Methylation of an adenosine at position 2503 in the 23S ribosomal RNA lead to resistance to linezolid	*cfr*
Lipopeptides	**Target modification**	Gene coding for a lysylphosphatidylglycerol synthetase; point mutations in *mprF* sequences is commonly associated with loss of daptomycin susceptibilities	*mprF*
		Gene coding for a histidine kinase; nucleotide insertion in *yycG* sequences is commonly associated with loss of daptomycin susceptibilities	*yycG*

V – PHARMACOKINETICS AND PHARMACODYNAMICS OF INTRACELLULAR ANTIBIOTICS

The intracellular location of pathogens is recognized as a critical factor in the failure of antimicrobial agents. *In vitro* and *in vivo* studies have provided experimental data demonstrating the importance of understanding (i) whether and to what extent antibiotics may or not act against intracellular pathogens, (ii) which are the pharmacokinetics and pharmacodynamics governing their activity, and (iii) how chemotherapy can be improved on that basis.[48]

1. Cellular pharmacokinetics of antibiotics : uptake and disposition of antibiotics

While conventional pharmacokinetics described drug absorption, distribution, metabolism and elimination within the body, cellular pharmacokinetics relates to the drug penetration, distribution, degradation and efflux in individual cells.[257] Table 5.1 summarizes the current knowledge of antibiotics pharmacokinetics in eukaryotic cells.

1.1. β-lactam antibiotics

β-lactams are thought to cross cell membrane by passive diffusion, achieving an apparent intracellular concentration lower than the extracellular one at equilibrium (Table 5.1).[48,49,115,133] Owing to their weak acid moieties, these agents are largely excluded from lysosomes and related acidic vacuoles. However, masking the free carboxyl group by a basic moiety is associated with substantial accumulation of the corresponding derivative[51,204].

1.2. Glycopeptides

Only a few studies have dealt with intracellular glycopeptides. These agents, which are voluminous molecules, enter cells via the endocytic pathway. Vancomycin, which is supposed to accumulate within lysosomes[26], shows slow uptake and modest accumulation in macrophages.[18] Conversely, teicoplanin, telavancin and oritavancin, which are more lipophilic agents, achieve higher cellular-to-extracellular ratio[145,257,258].

1.3. Quinolones

Quinolones have long been recognized to accumulate within eukaryotic cells.[49,83,186] These agents accumulate at moderate level (Cc/Ce : 4 to 10) and are found in the cytosolic fraction, suggesting that (i) efflux from a specific subcellular compartment is fast or (ii) that

these agents are genuinely localized within the cytosol but are able to diffuse to various subcellular compartments.[48] Efflux of fluoroquinolones is faster than uptake and seems to be mediated through MRP (multi-drug resistance associated-protein) efflux transporters.[156-158]

1.4. Ansamycins

Ansamycins have long been recognized to accumulate within eukaryotic cells.[77,162] However, it appears that the extent of cellular accumulation is quite variable among the different compounds (apparent cellular-to-extracellular ratio, 2 to 5 for rifampicin; ~ 25 for rifapentine). In addition, rifampicin has been identified as an efflux transporters substrates (P-gp and MRP)[103] and as MDR-1 or MRP2 inducer in ascites tumor cells or HepG2 cells, respectively.[90,221]

1.5. Aminoglycosides

Aminoglycosides gain access within cells (kidney and ear) through a dual process of adsorptive and receptor-mediated endocytosis. These highly polar agents bind to the negatively charged phospholipids[160] and the endocytic receptor megalin[165], accumulating therefore quickly and to a large extent. Conversely, studies with macrophages[41] and fibroblasts[250] have demonstrated that these cells accumulate only slowly aminoglycosides to an apparent cellular-to-extracellular ratio of 2 to 4 at equilibrium. Intracellular aminoglycosides are almost exclusively found within lysosomes and acidic vacuoles.[75,250]

1.6. Macrolides

Since macrolides are lipophilic weak organic bases, they achieve a marked intracellular accumulation in all types of cells. Uptake and efflux are generally rapid and reversible, with the notable exception of azithromycin,[86] for which binding to cellular structures could play a critical role in its prolonged intracellular residence. Using fractionation studies, Carlier and colleagues have reported that 1/3 of macrolides are distributed in the cytosol and 2/3 in the lysosomes (Table 5.1)[48]. Cellular accumulation of macrolides is also significantly influenced by (i) the stage of cell activation[47], and (ii) the cellular pH. Indeed, collapsing the gradient pH across the lysosomal and the pericellular membranes abolished all accumulation.[252]

1.7. Lincosamides

Lincosamide agents have been shown to accumulate within phagocytes. Among lincosamides, clindamycin has been notorious for its large cellular accumulation, which has been ascribed to (i) its basic character and (ii) the potential use of nucleoside transporter to

enter within cells.[68,100,251] This agent is not only localized in the cytosolic fraction, but also partly in the lysosomes.[251]

1.8. Tetracyclines and glycylcyclines

Much less is known about the cellular pharmacokinetics of tetracyclines and glycylcyclines. Only a few studies[174,285] has been published so far, in which minocycline accumulates within gingival fibroblasts. The same observation has been done for tigecycline, for which Ong and colleagues reported a rapid intracellular accumulation within neutrophils.[174] The mechanism of these accumulations, as well as their subcellular distributions, remain however elusive.

1.9. Oxazolidinones

Only a few studies have considered the intracellular accumulation of oxazolidinones, in which linezolid was shown to reach intracellular concentrations only slightly above the extracellular one in neutrophils, McCoy cells and macrophages.[18,184] Uptake and efflux are very fast (maximal concentration reached within 5 minutes) and 90 % of the drug being released in less than two minutes during transfer to fresh medium.

1.10. Streptogramins

Bébéar and colleagues have reported that quinupristin and dalfopristin are actively taken by murine macrophages, reaching an apparent cellular-to-extracellular ratio of 50 and 30, respectively[28,61]. After removal of these antibiotics, quinupristin and dalfopristin are rapidly released from the cells (with 45 % of the original extracellular concentration remaining in the cells after 120 min). The mechanism of this accumulation, as well as its subcellular distribution, remain however unknown.

1.11. Other antibiotics

Much less is known about the cellular pharmacokinetics of other pharmacological classes, such as daptomycin (which is supposed to be found in lysosomes[27]), co-trimoxazole, phenicoles and fusidane.

Table 5.1. Cellular pharmacokinetics of the main anti-staphylococcal agents within eukaryotic cells (Adapted from Van Bambeke et al, Curr Opin Drug Discov Devel, 2006; and Carryn et al, Infect Dis Clin N Am, 2003).

Pharmacoch. class	Antibiotics	Accumulation level at equilibrium	Active efflux	Predominant subcellular localization	Ref.
β-lactams	All	< 1	P-gp, MRP, anion/cation transporters	Cytosol	49,240
Glycopeptides	Vancomycin	8 (24 h)	Unknown	Lysosomes (kidney)	18,26
	Teicoplanin	60		Unknown	
	Oritavancin	150 to 300 (24 h)		Lysosomes	258
	Telavancin	50 (after 24 h)		Lysosomes	257
Quinolones	Ciprofloxacin, Levofloxacin,	4 to 10	MRP, P-gp, anion/cation transporters	Cytosol	158,257
	Moxifloxacin, Garenoxacin, Gemifloxacin	10 to 20	Marginal or unknown		156
Ansamycins	Rifampicin	2 to 5	P-gp, MRP	Unknown	162
	Rifapentine	~ 25			
Co-trimoxazole	Trimethoprim Sulfamethoxazole	Unknown	P-gp Unknown	Unknown	240
Aminoglycosides	All	2 to 4 (several days)	Unknown	Lysosomes	250
Phenicoles	Chloramphenicol	3 to 4	Unknown	Unknown	100
Macrolides	Erythromycin	4 to 10	P-gp	2/3 lysosomes 1/3 cytosol	77,257
	Azithromycin	40 to 300			
Lincosamides	Clindamycin	5 to 20	Unknown	Cytosol; partly in lysosomes	68,100
Streptogramins	Quinupristin-dalfopristin	50 30	Unknown	Unknown	28,61
Fusidane	Fusidic acid	7 to 10	Unknown	Unknown	77
Tetracyclines	Probably all	10-60	P-gp	Unknown	77,159,285
Oxazolidinone	Linezolid	~ 1	Unknown	Unknown	
Glycylcycline	Tigecycline	Unknown	Unknown	Unknown	174
Lipopeptide	Daptomycin	Unknown	Unknown	Lysosomes (kidney)	27

2. Cellular pharmacodynamics of antibiotics against *Staphylococcus aureus*

A large number of studies have assessed the intracellular activities of antibiotics against fully sensitive *S. aureus* in various cellular models[18,21,185,186,225]. Of particular interest, Barcia-Macay and colleagues have systematically compared the activity of various classes of antibiotics against both the extracellular and intracellular forms of *S. aureus*. As shown in Fig.5.1., all antibiotics are less active intracellularly compared to extracellularly.[18] This general low intracellular activity could result from: (i) the poor bioavailability of cellular antibiotic, making pharmacokinetic predictions incorrect; (ii) a shift of MICs towards higher values in the cellular milieu; or (iii) an altered bacterial responsiveness within eukaryotic cells.

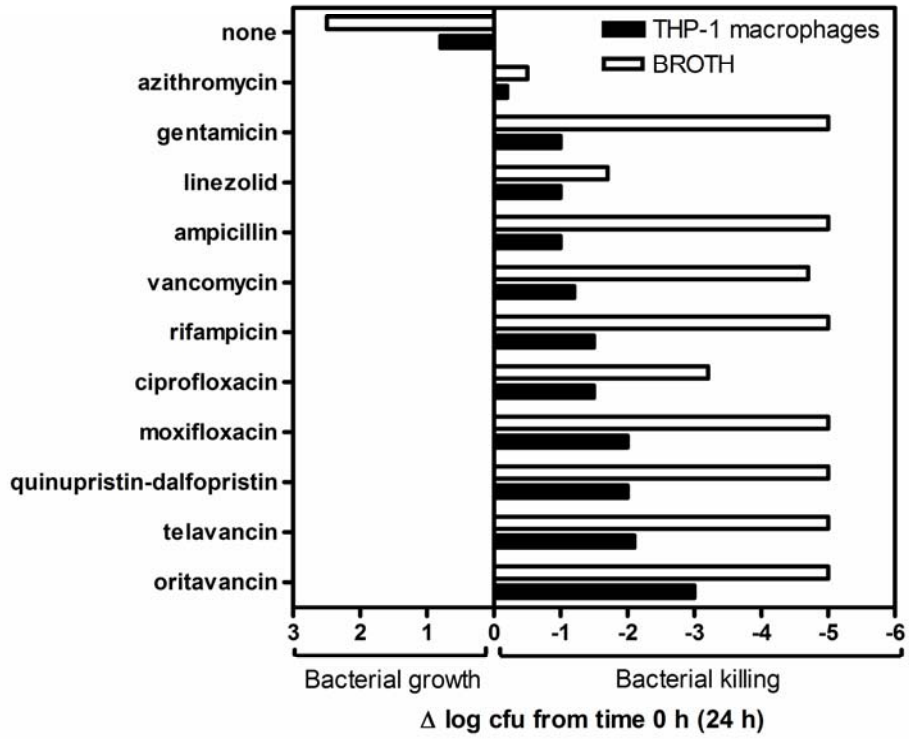


Fig.5.1. Activities of antibiotics against extracellular and intracellular *S. aureus*. The graphs show the change in the number of CFU (Δ log CFU) per ml of culture medium (extracellular) or in THP-1 macrophages (intracellular) per mg of cell protein. Each antibiotic was added at concentrations corresponding to its C_{max} in humans, a clinically meaningful concentration of antibiotics. (Adapted from Barcia-Macay et al, 2006[17,18]).

2.1. β-lactams

Despite of the lack of cellular accumulation of β-lactams in various cell types, these agents are active against intracellular pathogens such as *Listeria monocytogenes*.[48] Even if neither ampicillin or meropenem accumulate within macrophages, they prove however larger activity against the intracellular forms of *L. monocytogenes* compared to extracellular ones (-

2 log vs. -1 log CFU decrease after 24 h incubation using a concentration of antibiotic equivalent to the C_{max} [as defined by the maximal concentration of drug achieving the serum of patients receiving conventional dosing]). Michelet and colleagues have also reported that these agents will only become bactericidal intracellularly if sustained concentrations of drug are maintained over prolonged time of incubation, which is consistent with the time-dependent feature of β-lactams.[155]

2.2. Glycopeptides

Only few studies have assessed the intracellular activities of glycopeptides against *S. aureus* (Fig.5.1). While vancomycin and teicoplanin[3,18,190] achieve only static effect (~1 log CFU decrease after 24 h at C_{max}), telavancin and oritavancin prove higher efficacy against intracellular forms of *S. aureus,* achieving respectively approximatively ~ 2 and ~ 3 log decrease of CFU after 24 h in infected macrophages.[17,18] The rate and extent of killing are, however, lower intracellular compared to extracellularly (Fig.5.1).

2.3. Quinolones

Intracellular fluoroquinolones becomes effective against *S. aureus* in a time- and concentration-dependent fashion.[18,168,223,225] Against a fully susceptible isolate of *S. aureus*, the intracellular activity of quinolones varies between the compounds (log CFU decrease at Cmax [~ 4 mg/L] after 24 h: ciprofloxacin, ~ 1.4 log vs. moxifloxacin, ~ 2 log [see Fig.5.1]). The rate and extent of intracellular killings are also lower compared to broth (against extracellular pathogens), as described previously for other pharmacological classes.

2.4. Aminoglycosides

Aminoglycosides, which are mainly sequestered within acidic vacuoles, show only bacteriostatic effects (~ 1 log CFU decrease after 24 h from the original, post-phagocytosis inoculum [see Fig.5.1]) against intraphagocytic forms of *S. aureus* in a concentration-dependent fashion.[18,21,225]

2.5. Ansamycins

Rifampicin, which is highly bactericidal towards extracellular forms of *S. aureus*, demonstrates an intracellular activity against susceptible *S. aureus* (~ 1 log CFU decrease after 3 h at Cmax [4 mg/L]). In spite of this, and in sharp contrast to what is observed with extracellular bacteria, rifampicin never yields a truly intracellular bactericidal effect (~1.5 log CFU decrease [see Fig.5.1), since its E_{max} (Efficacy maximal) remains lower than 3 log CFU decrease (CLSI criteria).[18]

2.6. Macrolides

The huge accumulation of azithromycin within cells, and its co-localization within *S. aureus* in the phagolysosomes, would make many to predict a large activity of this agent. However, several studies have shown that, despite these apparent advantages, azithromycin is only bacteriostatic against intracellular forms of *S. aureus* (Fig.5.1). The reasons for these contrasting and apparently paradoxical behaviors are that azithromycin is an agent for which the local environment (and especially the acidic pH) is highly unfavourable to its activity.

2.7. Streptogramins

The combination of quinupristin and dalfopristin demonstrate significant intracellular killing of *S. aureus*[61] (17th European Congress of Clinical Microbiology and Infectious Diseases [ECCMID], Munich, Germany, Poster 2058), achieving intracellularly approx. 2 log CFU decrease at 24 h from the original, post-phagocytosis inoculum.

2.8. Lincosamides, Tetracyclines, Glycylcyclines and Oxazolidinones

Both pharmacological classes are intrinsically bacteriostatic against extracellular forms of *S. aureus* and did not reached higher maximal efficacy against the intracellular ones (Fig.5.1).[18,174,187]

3. Factors influencing the intracellular activity of antibiotics against *S. aureus*

Figure 5.3 summarizes the main parameters that may critically influence the activity of antibiotics against intracellular *S. aureus*, such as the recognition of antibiotics by active efflux transporters, the drug metabolism and binding to subcellular components, the physical conditions prevailing in the site of infection, the state of bacterial responsiveness, and the degree of cooperation with the host cell defences.

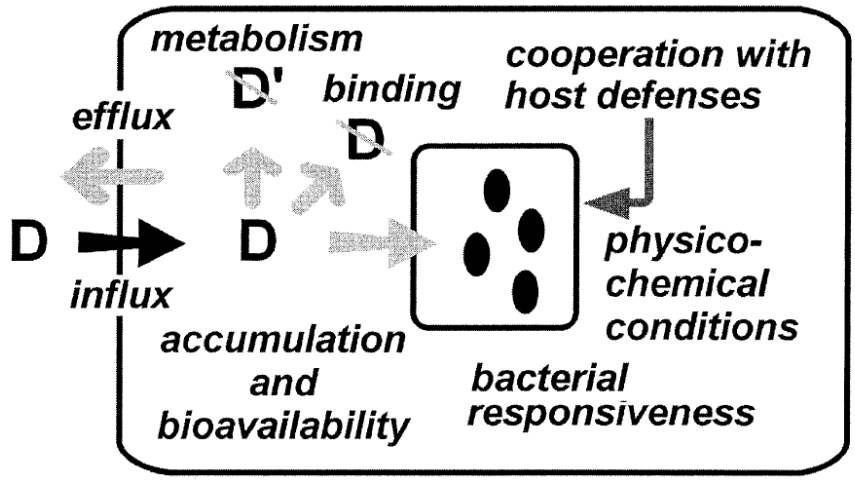


Fig.5.3. Factors affecting the accumulation (balance between drug influx and efflux, drug metabolism and binding to cellular components) and the intracellular activity of antibiotics (the physicochemical conditions prevailing at the site of infection, the state of bacterial responsiveness, and the degree of cooperation with the host defences) (Carryn et al, 2003[48]).

3.2. Accumulation levels

3.2.1. Role of efflux transporters

Active efflux of antibiotics is now recognized as a key element in the distribution and expression of drug activity. Efflux transporters are expressed at the surface of eukaryotic cells (Fig.5.4) and involved in the extrusion of various antimicrobial agents, including compounds with unrelated pharmacological properties (see for review[219,256,257,259]). Based on mechanisms and energetics, membrane transporters can be categorized into two broad classes: passive transporters (ions channels,…) and active transporters. Among these latters, multidrug transporters of the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily (including the multidrug-resistance protein P-glycoprotein [P-gp or MDR transporters] and the multiple drug-resistance proteins [MRPs]) are found ubiquitous, while organic cation or anion transporters are mainly found in the surface of epithelia.

In general, transporters act as a general means for cells to protect themselves from the undesirable invasion by amphiphilic compounds. They may also serve to facilitate the transmembrane transport of endogenous molecules, such as phospholipids (by acting as flippases), cytokines, metabolic intermediates or nutrients. When expressed in cells, multidrug transporters may reduce the cellular concentration of antibiotics (see Table 5.1), consequently affecting their pharmacological activity towards intracellular targets. In this context, the understanding of the biology of transporters is of particular pharmaceutical relevance. Structural modification and specific transporter targeting are considered as promising strategies for the design of drug with improved cellular pharmacokinetics and pharmacodynamics. This prompted us to explore some approaches to enhance the

therapeutic efficacy of intracellular antibiotics by pharmacological modulation of transporter functions. The strategy of using P-gp (verapamil, GF120918) or MRP inhibitors (gemfibrozil) has been found to significantly increase the cellular content and intracellular activity of macrolides and quinolones, respectively[157,158,224].

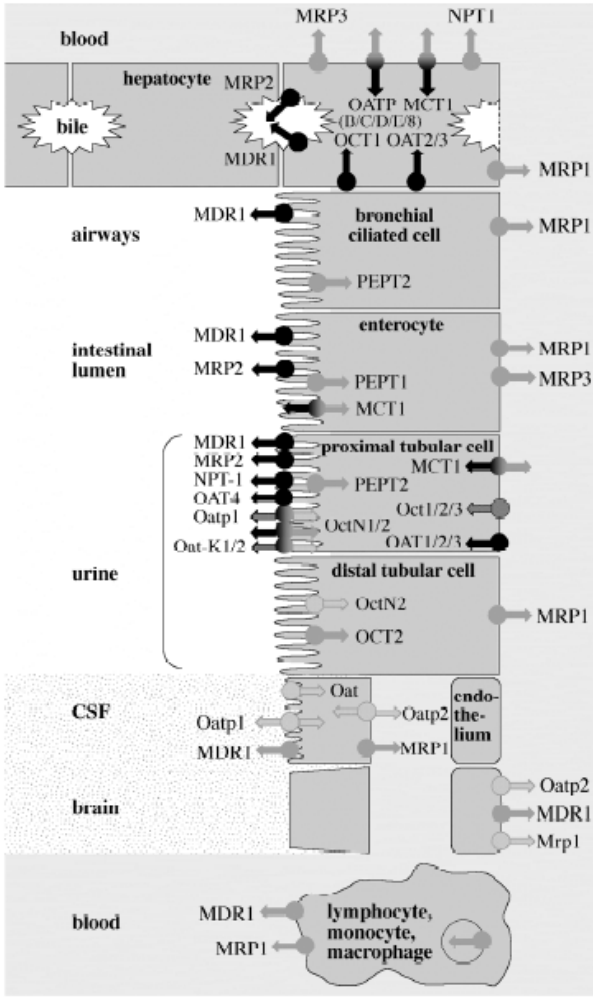


Fig.5.4. Schematic representation of the main transporters potentially involved in antibiotic movement at the level of epithelial cells (liver, bronchial tree, intestine, kidney), the blood-brain barrier or leukocytes. The level of expression of each transporter may differ between species. (Van Bambeke et al, 2003[259]).

3.2.2. ABC transporters

Approximatively 50 different ABC transporters have been identified in the human genome, divided into seven different classes (A to G) based on sequence similarities (See for review: [7,256,259]). The MDR efflux protein was the first transporter of ABC family to be described.[52] This protein, which consists of two homologous halves (each containing six transmembrane domains and an ATP-binding site) linked by a flexible region[7,112], is mainly localised on plasma membranes, but also on early endosomes and lysosomes.[80,131] Drug belonging to diverse chemical classes such as Vinca alkaloids (vinblastine, vincristine),

anthracyclines (doxorubicin, daunorubicin) or antibiotics (see Table 5.1) may interact with P-gp. These compounds are mainly lipophilic, either uncharged or cationic agents. MRP-1 transporters have similar transport specificity to MDR1, except that MRP proteins mainly transport anionic compounds such as fluoroquinolones (Table 5.1).

3.3. Intracellular expression of antibiotic activity

The activity of antibiotics may be partially defeated by the physical conditions prevailing in the infection site. In particular, local pH can affect not only antibiotic action, but also the bacterial response to antibiotics.

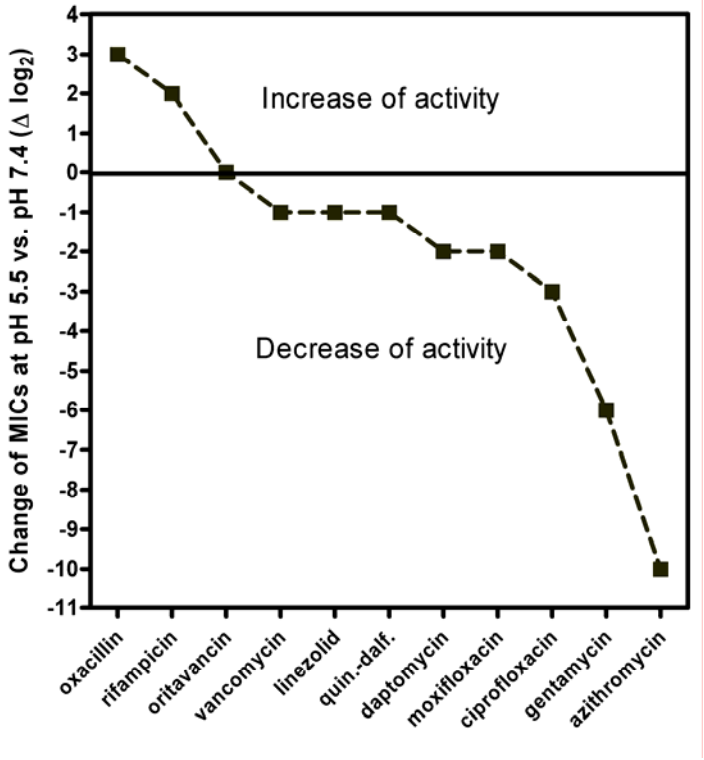


Fig.5.5. Comparative Minimal Inhibitory Concentrations (MICs) of antibiotics against *S. aureus* ATCC 25923 at pH 7.4 and 5.5. MICs were determined by micro-dilution method and read after 24 h incubation (Adapted from Baudoux et al, 2007[21]; and Barcia-Macay et al, 2006[18]).

Environmental effects on antibiotic expression of activity can be partly taken into consideration by considering the minimal inhibitory concentrations (MICs) at pH 5.5. As shown in figure 5.5, acidic milieu negatively affects the activity of gentamicin, azithromycin and, to a lesser extent, quinolones and daptomycin, but enhances that of rifampicin and β-lactams. The activity of glycopeptides, quinupristin-dalfopristin and linezolid is not significantly altered by acidic pH (< 2 $\log_2$ difference of MICs).

The cell defence mechanisms represent one another factor specific to the intracellular milieu that can either cooperate with or antagonize antibiotic action. For example, inhibiting

the oxidative burst in macrophages reduces significantly the intracellular activity of quinolones against *L. monocytogenes*, suggesting therefore that oxidant species reinforce the activity of this class of antibiotic[177]. The effects of cytokines or oxidative burst inhibition are still lacking for intraphagocytic *S. aureus*.

3.4. Intracellular bacterial responsiveness to antibiotics

When *S. aureus* enters cells, its growth rate is markedly reduced compared to extracellularly. This delay in growth may contribute to the impairment of antibiotic activity in the intracellular milieu, since many classes of antibiotics act upon bacteria in the active stage of multiplication. Moreover, using a stable thymidine-dependent SCV variant, we showed recently that the bacterial growth rate of these mutants is exceptionally impaired compared to a wild-type, dividing isolates of *S. aureus* (Fig.5.6). It is therefore not a surprise that the activity of antibiotics against these intracellular clones is significantly defeated.

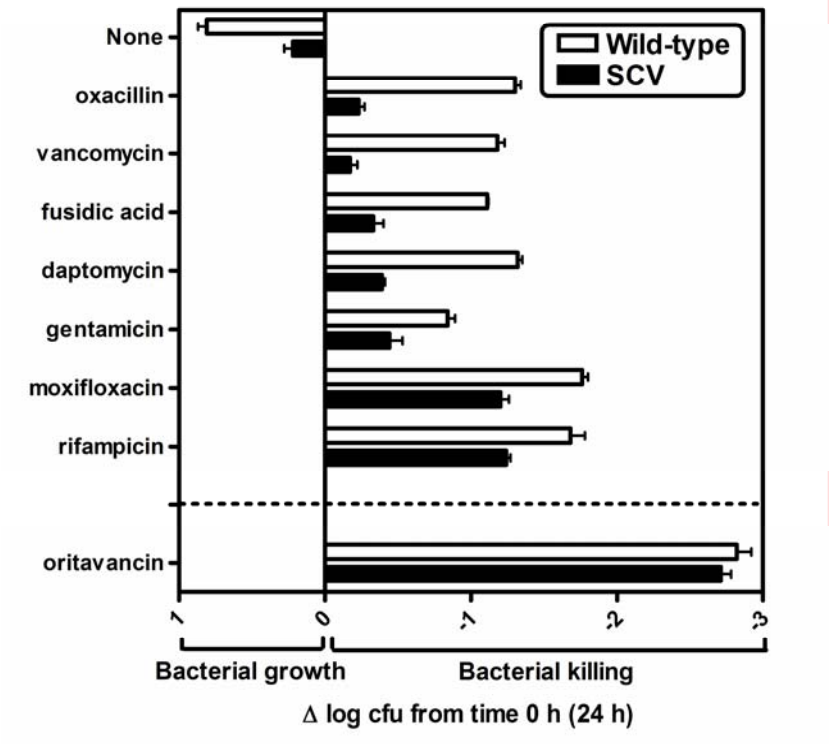


Fig.5.6. Intracellular activity of most commonly used antimicrobial agents vs. investigational molecules against intraphagocytic forms of a stable, thymidine-dependent SCV variant and its normal phenotype isolate counterpart from a cystic fibrosis patient. Activities were measured in infected THP-1 macrophages after 24 h exposition to a drug concentration equivalent to its human Cmax described in the literature (Adapted from Nguyen et al, 27th Réunion Interdisciplinaire de Chimiothérapie Anti-infectieuse [RICAI], Paris, 2007).

One another puzzling observation, when dealing with intracellular bacteria, is the lack of bacterial eradication for all the antibiotics tested so far, even when concentrations were brought to more than 250-fold multiples of MICs[18,21]. Hence, this suggests that part of the bacterial inoculum is inaccessible or metabolically insensitive to all antibiotics. This has been observed not only with *S. aureus* but also with *L. monocytogenes*[49], and does not seem to be linked with the selection of resistant mutants. It's therefore tempting to speculate that this lack of intracellular forms eradication could be one of the reasons for antibiotic failure in severe *S. aureus* infections, which are characterized by the maintenance of a residual inoculum inaccessible to the action of antimicrobial agents and from which reinfection are likely.

AIM OF THE THESIS

As described in the introduction, *Staphylococcus aureus* is an aggressive pathogen creating significant public health threat. The intracellular survival of this organism, combined to the emergence of multi-resistant isolates, is often considered as an important determinant in the recurrent and relapsing character of staphylococcal infections. In this context, selecting an optimal treatment to eradicate the intracellular forms of *S. aureus* remains challenging, since routine evaluation of antibiotic activity is usually performed against extracellular bacteria only. Yet, the intracellular activity of antibiotics of most anti-staphylococcal agents is markedly lower compared to what is observed extracellularly.

In this context, this thesis aimed at investigating the pharmacodynamic properties of antibiotics against *Staphylococcus aureus*, by:

(i) Developing appropriate models of cellular infections that might represent more likely the clinical situation (phagocytic cells vs. non-phagocytic cells)

(ii) Comparing systematically the activity of antibiotics against isolates of current and epidemiological interest following a pharmacological approach, namely the use of time- and dose-response studies

(iii) Unravelling the influence of the cellular milieu on the intracellular activity of antibiotic

RESULTS

My contributions to this study are the following:

1) **The demonstration of the concentration- and time-dependent activity of β-lactam antibiotics against intraphagocytic *S. aureus.***

   (Publication #1)

2) **The evaluation of the role of acidic pH in the restored susceptibility of Methicillin-Resistant *Staphylococcus aureus* isolates (MRSA) to β-lactam antibiotics**

   (Publication #2, #3, and #4)

3) **The evalution of the modulation of the cellular accumulation and the intracellular activity of daptomycin by the P-glycoprotein (MDR-1) efflux transporter.**

   (Publication #5)

4) **The assessment of the extracellular and the intracellular activities of antibiotics against clinical isolates of *S. aureus* recovered from a patient with persistent bacteraemia and endocarditis.**

   (Publication #6)

I - Concentration- and time-dependent activity of β-lactam antibiotics against intraphagocytic *S. aureus*

Le temps révèle tout: c'est un bavard
qui parle sans être interoggé
Euripide

Publication #1: Activity of three β-lactams (ertapenem, meropenem and ampicillin) against intraphagocytic *Listeria monocytogenes* and *Staphylococcus aureus*.

Sandrine Lemaire, Françoise Van Bambeke, Marie-Paule Mingeot-Leclercq and Paul M. Tulkens

The present study, which reports preliminary results that are further elaborated in the following publications, is a pharmacological comparison of the activity of three β-lactams (ampicillin, meropenem and ertapenem [a carbapenem with prolonged half-life]) against extracellular and intracellular *S. aureus* (phagolysosomial bacterium) and *L. monocytogenes* (cytosolic bacterium).

For a long time, β-lactam antibiotics have long been recognized to do not accumulate within cells, and it has often been concluded that these agents were unable to penetrate cells. The present study shows that this is probably incorrect because ampicillin, meropenem and ertapenem are able to efficiently penetrate into THP-1 macrophages, achieving an apparent cellular concentration lower than the extracellular one at equilibrium (Cc/Ce < 1). Despite of their poor accumulation within cells, both β-lactams are actually significantly active against intracellular forms of *S. aureus* (strain ATCC 25923, a fully susceptible isolate). The intracellular activity of β-lactams is critically dependent upon their extracellular concentrations and the duration of cell exposure to antibiotics. In this context, it must be emphasized that a significant reduction of the intracellular inoculum may be obtained when (i) the extracellular concentration markedly exceeded the MIC of the organism (which is actually clinically pertinent owing to the important Cmax observed upon administration in healthy volunteers [e.g. meropenem, 50 mg/L; ertapenem, 155 mg/L), and (ii) the incubation time was brought to 24 h. Therefore, this suggests that large extracellular concentrations and prolonged exposure may compensate for the lack of cellular accumulation.

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Activity of three β-lactams (ertapenem, meropenem and ampicillin) against intraphagocytic *Listeria monocytogenes* and *Staphylococcus aureus*

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***Objectives*: Assessment of the activity of three β-lactams [ertapenem (a carbapenem with a prolonged half-life), meropenem and ampicillin] against intraphagocytic *Listeria monocytogenes* and *Staphylococcus aureus*.**

***Methods*: Quantitative measurements of cfu changes in broth and in THP-1 macrophages (post-phagocytosis) over time (5 and 24 h) at concentrations spanning from sub-MICs to C_{max} (maximal concentration typically observed in patients' serum upon administration of conventional doses); morphological studies using an electron microscope; evaluation of drug stability (HPLC), protein binding (equilibrium dialysis) and measurement of drug cellular accumulation (microbiological assay).**

***Results*: Ertapenem was unable to control *L. monocytogenes* growth in THP-1 macrophages at all concentrations and times tested, even under conditions where ampicillin and meropenem were bactericidal. This behaviour could not be ascribed to drug instability, protein binding or lack of cell accumulation in comparison with ampicillin or meropenem. Ertapenem, ampicillin and meropenem were equally effective at reducing the post-phagocytosis inoculum of *S. aureus* (~1 log cfu), and caused conspicuous changes in the morphology of intracellular bacteria consistent with their lysis. These effects were obtained, however, only at large multiples (100-fold or more) of the MIC maintained over 24 h. Because of the high intrinsic antimicrobial potency of the β-lactams studied, these concentrations were below the C_{max}.**

***Conclusions*: Ertapenem will probably be ineffective against intraphagocytic forms of *L. monocytogenes* for reasons that remain to be discovered. Conversely, ertapenem could be an alternative to ampicillin and meropenem against intraphagocytic *S. aureus* since its longer half-life may allow high concentrations to be maintained for more prolonged times.**

Keywords: *L. monocytogenes*, *S. aureus*, THP-1 macrophages, ertapenem, ampicillin, meropenem

Introduction

Treatment of intracellular infections remains a medical challenge, mainly due to the inability of many antibiotics to penetrate and act in the intracellular milieu.[1,2] Infections caused by *Listeria monocytogenes* and *Staphylococcus aureus* are typical in this context since these organisms are difficult to eradicate even after sustained antibiotic therapy, probably because of the persistence of intracellular forms of these bacteria in both phagocytic and non-phagocytic cells.[3,4] Treatment of listeriosis is commonly undertaken with a combination of ampicillin and gentamicin,[5] but meropenem in monotherapy has been found active in experimental meningitis caused by *Listeria*.[6] Treatment of staphylococcal infections mainly relies on a β-lactam resistant to β-lactamase (at least for methicillin-susceptible organisms). In both types of infection, prolonged therapies are often needed to prevent recurrences and/or relapses in case of complicated infections, or in cancer or immunosuppressed patients.[7,8] Ertapenem is a β-lactamase-resistant β-lactam that shares many of the antimicrobial properties of meropenem with respect to

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Gram-positive organisms, but is characterized by a prolonged half-life allowing for a once-daily administration.[9,10] Ertapenem may therefore present a significant clinical advantage over meropenem and most other β-lactams for long-term therapies. As a consequence, we investigated the potential of ertapenem to act upon intraphagocytic *L. monocytogenes* and *S. aureus*. For this purpose, we used THP-1 cells, a model of human macrophages that has been validated for the study of *Listeria* intracellular infection,[11 13] and which we recently adapted for evaluation of the activity of antibiotics against intracellular *S. aureus*. Ertapenem was systematically compared with ampicillin and meropenem, which have both been found active against intracellular *L. monocytogenes* in these cells.[13]

Materials and methods

Bacterial strains, determination of MIC and MBC, and time and dose–kill studies in acellular media

L. monocytogenes. We used a haemolysin-producing strain EGD and followed exactly the methods described previously,[12] except that determination of MBCs used a 10^6 cfu/mL inoculum.
S. aureus. We used a non-β-lactamase-producing strain (ATCC 25923) following the methods described previously.[14]

Cells, cell infection and assessment of intracellular activities of antibiotics

THP-1 myelomonocytic cells[15] were used throughout our experiments. For *L. monocytogenes*, we followed a method described previously[12] with addition of gentamicin (1 mg/L; $\sim 1 \times$ MIC) for control cultures (no β-lactam added) if maintained for more than 5 h.[13] For *S. aureus*, we used an adaptation of the method described previously for J774 macrophages[14] taking into account that THP-1 cells spontaneously grow in suspension. Opsonization was performed with non-decomplemented, freshly thawed human serum diluted 1:10 in serum-free culture medium (RPMI 1640). Phagocytosis was performed at a 4:1 bacteria/macrophage ratio. Elimination of non-phagocytosed bacteria, and collection of cells at the end of the experiment were, therefore, made by centrifugation at room temperature [1300 rpm; 8 min; Eppendorf 5810R Centrifuge equipped with a A-4-62 rotor (Eppendorf Gerätgebau GmbH, Engeldorf, Germany)].

Determination of cellular carbapenem accumulation

Cells were collected and washed free from culture medium by three successive centrifugations in ice-cold phosphate-buffered saline (PBS), resuspended in distilled water and subjected to sonication (10 s at 50 watts; Labsonic L, Braun Biotech International GmbH, Melsungen, Germany). Ertapenem was then assayed by a microbiological method (to avoid the necessity of extraction, and thereby obtaining the needed sensitivity), using *Escherichia coli* as test organism and following the general procedure described previously[16] [lowest limit of detection; 0.25 mg/L for ertapenem and 1 mg/L for meropenem; typical linearity: up to 60–70 mg/L ($R^2 = 0.998$ for ertapenem and 0.989 for meropenem)]. Cell proteins were measured in parallel using the Folin–Ciocalteu/biuret method,[17] and the results used to compute the apparent cellular concentration of antibiotics based on a volume ratio of 5 μL of cell volume per mg protein, as in our previous publications dealing with THP-1 cells.[12,18]

Electron microscopy

Cells were infected as described above, except that the initial inoculum was increased to 20 bacteria per macrophage. This larger inoculum did not modify the intracellular activity of the β-lactams studied. Sample handling was then performed as described previously.[18]

Stability studies of carbapenems

Stability in culture media was evaluated by HPLC to provide us with positive identification of the molecules studied (in comparison with genuine standards) and higher reproducibility compared with bioassays. Assay was based on a published method,[19] but extraction was performed three times in succession with pooling of the upper phases in order to improve drug recovery [typical values: 82.3 ± 0.5% ($n = 3$) for ertapenem; 100.0 ± 0.1% for meropenem; a single extraction of ertapenem yielded only 16.6 ± 0.9% ($n = 3$) recovery]. Chromatography was made through a Lichrosphere 100 RP-18 column (25 cm × 4 mm, 5 μm; Merck AG, Darmstadt, Germany). Elution was made with acetonitrile/25 mM phosphate buffer pH 6.5 (v/v, 7:93) for both ertapenem and meropenem, with typical retention times of 8 and 5 min, respectively [linearity (for standards): 0.09–200 mg/L ($R^2 = 0.999$) and 0.19–200 mg/L ($R^2 = 0.999$)].

Protein binding studies

We used the equilibrium dialysis technique (cut-off, 6000–8000 mol. wt; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) with membranes soaked successively in three baths of water, and three baths of PBS for 15 min each. Preliminary studies showed that equilibration required a minimum of 32 h at 37°C under constant rotation at 8 rpm and without serum. The concentration of antibiotic in the serum-free compartment was measured by HPLC (see method above) at the end of the experiment and compared with the initial concentration to calculate the percentage of bound drug: 100−[(concentration in serum-free cell × 2)/(initial concentration)].

Materials

Ertapenem, meropenem and gentamicin were obtained as Invanz®, Meronem® and Geomycine®, respectively (i.e. the registered commercial products for parenteral administration in Belgium), and supplied by Merck Sharp & Dohme BV (Haarlem, The Netherlands), Astra Pharmaceutical (Brussels, Belgium) and GlaxoSmithKline s.a. [Rixensart, Belgium; on behalf of Schering-Plough Belgium (Brussels, Belgium)]. Ampicillin was purchased as the sodium salt from Sigma–Aldrich (St Louis, MO, USA), and cell culture media and serum were obtained from Gibco Biocult (Paisley, UK). All other reagents were obtained from Merck AG or from Sigma–Aldrich.

Statistical analyses

Curve fitting analyses were made with GraphPad Prism® software (version 4.0) and group comparisons (Student's *t*-test, one-way analyses of variance) with Instat Prism® software (version 3.01), both from GraphPad Prism® software, San Diego, CA, USA.

Table 1. MICs and MBCs of antibiotics for the strains of *L. monocytogenes* and *S. aureus* used in this study

Organism	Antibiotic	pH	MIC (mg/L)[a]	MBC (mg/L)[b]
L. monocytogenes	ampicillin	7.4	0.32 ± 0.10	>64
	meropenem	7.4	0.05 ± 0.00	>64
	ertapenem	7.4	0.48 ± 0.03	>64
S. aureus	ampicillin	5.5	0.03 ± 0.01	
		7.4	0.07 ± 0.01	0.125
	meropenem	5.5	0.06 ± 0.02	
		7.4	0.15 ± 0.01	0.25
	ertapenem	5.5	0.06 ± 0.00	
		7.4	0.11 ± 0.01	0.25

[a]Arithmetic dilutions ($n = 3$).
[b]Geometric dilutions (typical results).

Results

MICs and MBCs

Table 1 shows the MICs and MBCs observed in the present study. With respect to *L. monocytogenes*, ertapenem showed an MIC similar to ampicillin and was thus ~10-fold less potent than meropenem. All three β-lactams were bacteriostatic towards *L. monocytogenes*. For *S. aureus*, MICs were determined both at pH 7.4 and pH 5.5 to mimic the conditions prevailing in the extracellular milieu and in phagolysosomes, respectively. All three β-lactams had low MICs with no significant difference between them, and with slightly lower values at pH 5.5. MBCs were about two-fold larger than the MICs demonstrating the bactericidal activity of these β-lactams against the strain of *S. aureus* used.

Time–kill studies at increasing concentrations

In the first series of experiments, the influence of the incubation time on the activity of antibiotics was evaluated at increasing fractional concentrations observed in the serum of patients receiving conventional doses of the antibiotics tested. The concentrations were chosen as guided by the C_{max} reported for the respective antibiotics in the literature (50, 50 and 155 mg/L for ampicillin, meropenem and ertapenem, respectively[9,20,21]) and using two incubation time periods (5 and 24 h). Results are shown in Figure 1 in a synoptic fashion comparing the activities seen in broth (mimicking the situation of extracellular bacteria) with those in cells (phagocytosed bacteria) for all three antibiotics and for both types of bacteria. Considering the results globally, two main aspects were striking. First, it appears that most of the antibacterial effects (based on the decrease in cfu) were already obtained at the lowest concentration tested [for meropenem towards phagocytosed *L. monocytogenes*, however, a gain of ~1 log cfu decrease could be obtained at 24 h when increasing the concentration from the lowest to the highest value tested (5–50 mg/L)]. Secondly, all effects were also time-dependent, with significant gains of activity in all conditions when incubation was prolonged from 5 to 24 h (the effect of ertapenem, however, was only to slow down bacterial growth). Concentrating now on *L. monocytogenes*, we see that the extracellular activity of all three β-lactams was essentially bacteriostatic at 5 h, and that only a modest decrease in cfu (0.5–0.7 log units) was obtained at 24 h at all concentrations tested. Examination of the results obtained for intracellular activity revealed: (i) that intracellular bacterial growth was essentially similar to that in broth; (ii) that meropenem and ampicillin were bacteriostatic at 5 h at all concentrations tested, but achieved a bactericidal effect at 24 h, as previously described;[13] and (iii) that, in sharp contrast, ertapenem was unable to decrease the post-phagocytosis inoculum, with bacteria actually growing at a multiplication rate about half of that of controls at all concentrations tested. Moving now to *S. aureus*, we first see that all three β-lactams exerted a marked bactericidal effect towards bacteria in broth (~5 log decrease in 24 h) without significant differences between them. Intracellular growth of *S. aureus* in control cells was first delayed, but eventually proceeded at a rate that allowed to reach in 24 h an increase over the original inoculum of about two-thirds of what was seen in broth (as already reported for J774 macrophages[14]). All three β-lactams were able to significantly reduce the number of cell-associated cfu, but this reduction (0.7–1.2 log units) was much less pronounced than for bacteria in broth. No marked difference was seen between the three drugs.

Morphological studies

Because the results shown in Figure 1 suggested that ertapenem was unable to block the intracellular growth of *L. monocytogenes* while being active against intracellular *S. aureus*, we performed electron microscopic studies to directly examine the morphology of the intracellular bacteria after 24 h of incubation in control cells and in cells incubated with this antibiotic. Figure 2 (a–d) shows that both control cells and ertapenem-treated cells contained an abundance of intact *L. monocytogenes* in various stages of phagocytosis (Figure 2a), division (Figure 2b), and clearly present in the cytosol while being surrounded with actin (Figure 2b–d). No apparent differences were seen between control and ertapenem-treated cells. In contrast, *L. monocytogenes*-infected cells that had been incubated with ampicillin or meropenem for 24 h contained no recognizable *Listeria* apart from a few structures that looked like highly damaged bacterial cells (not shown). Cells infected with *S. aureus* and left without antibiotic showed clearly recognizable bacteria, sometimes in a process of division, in what appeared like phagocytic vacuoles (Figure 2e). In contrast to what had been seen with *L. monocytogenes*, the morphology of *S. aureus* was clearly altered in ertapenem-treated cells (Figure 2f–h), where most sections showed bacterial ghosts (Figure 2f and h), bacterial bodies with evidence of partial loss of electron-dense material (Figure 2g), together with apparently intact bacteria (Figure 2f). Similar images were obtained for meropenem- and ampicillin-treated cells (not shown).

Concentration–kill studies

In order to get more insight into the contrasting behaviour of ertapenem towards intracellular *L. monocytogenes* and *S. aureus* on the one hand, and to better analyse the differences in activity seen with ampicillin and meropenem on the other hand, we systematically compared intracellular and extracellular activities

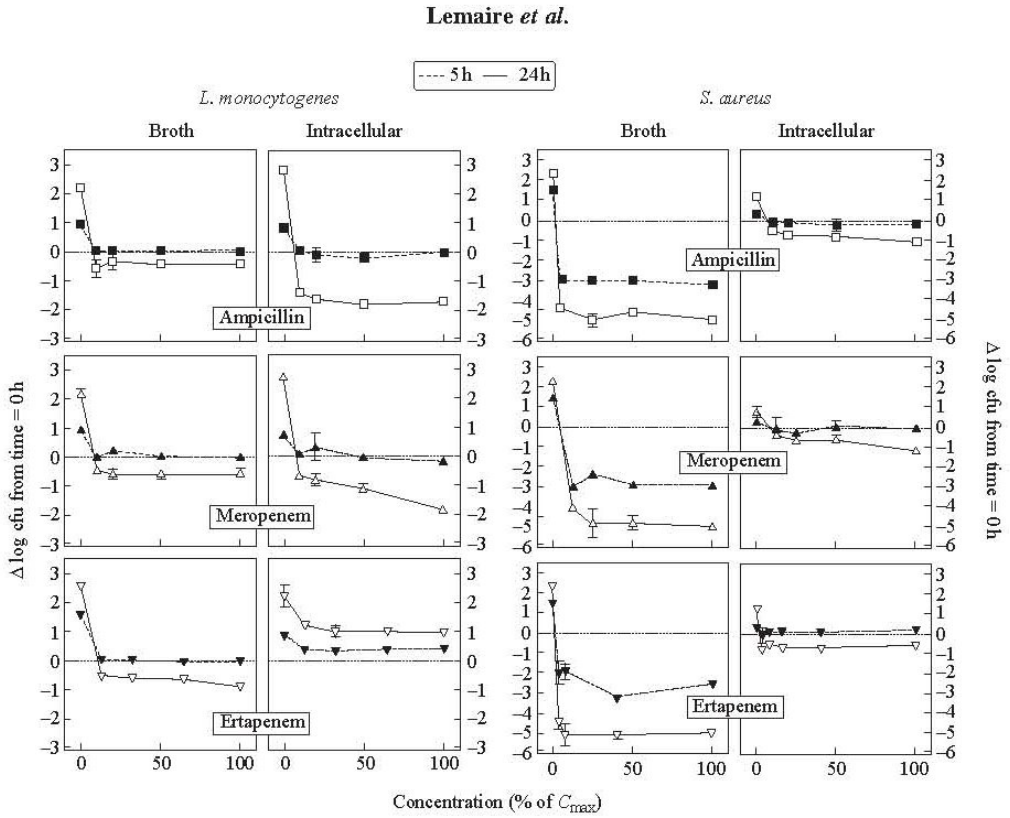

Figure 1. Concentration–killing effects of ampicillin (squares), meropenem (triangles) and ertapenem (inverted triangles) towards *L. monocytogenes* (left-hand panels) and *S. aureus* (right-hand panels) in broth (first and third columns of panels, taken from left to right), or after phagocytosis by THP-1 macrophages (second and fourth columns of panels, taken from left to right). Incubation with antibiotics was for 5 h (closed symbols; broken lines) or 24 h (open symbols, continuous lines) at the concentrations indicated in the abscissa, which are expressed as fractions of the maximal serum concentration (C_{max}) observed in humans after conventional administration of these drugs.[9,20,21] Note that the lowest concentrations tested are always far above the MIC for the corresponding organism (for *L. monocytogenes*, MICs of ampicillin, meropenem and ertapenem are 0.64, 0.1 and 0.31% of C_{max}, respectively; for *S. aureus*, MICs of ampicillin, meropenem and ertapenem are 0.06, 0.12 and 0.04% of C_{max}). All values are means ± SD of three independent determinations (SD bars that are not visible are smaller than the size of the symbols).

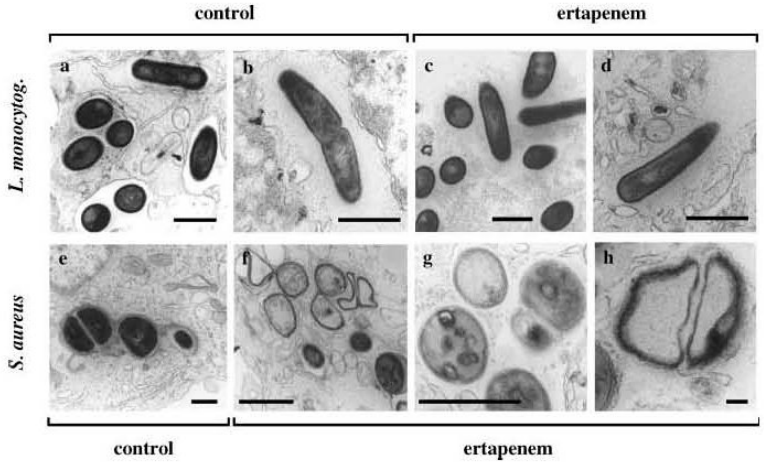

Figure 2. Morphological appearance of *L. monocytogenes* (a–d) and *S. aureus* (e–h) in cells 24 h after infection. Control (a, b and e): cells were incubated with gentamicin (1 × MIC) to prevent the extracellular growth of bacteria and the ensuing cell death due to acidification of the medium; this did not impair the intracellular growth of bacteria.[13,14] Ertapenem (c, d and f–h): cells were incubated in the presence of ertapenem (155 mg/L). Scale bars are 0.5 μm.

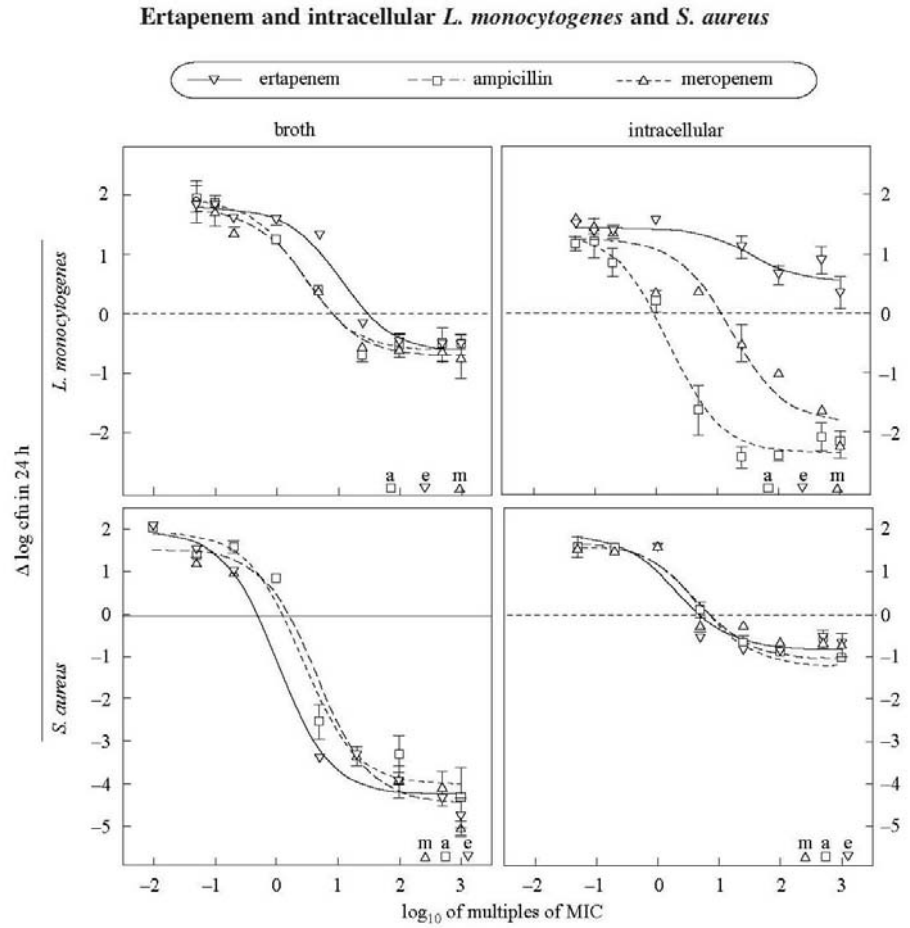

Figure 3. Concentration–killing curves of ampicillin (squares), meropenem (triangles) and ertapenem (inverted triangles) towards *L. monocytogenes* (upper panels) and *S. aureus* (lower panels) in broth (left-hand panels) or in THP-1 macrophages (right-hand panels). The abscissas show the initial concentrations of the antibiotics in multiples of their MIC in broth (measured at pH 7.4 for *L. monocytogenes*; for *S. aureus*, we used the values measured at pH 5.5 for the left-hand panel, and those measured at pH 7.4 for the right-hand panel; see Table 1 for details). Note that the concentration range spans from sub-MIC to concentrations exceeding the MIC by several orders of magnitude. The symbols and letters (e, ertapenem; a, ampicillin; m, meropenem) close to the abscissas in each panel are located at, and indicate the multiples of MIC corresponding to the maximal concentration tested chosen as guided by the C_{max} reported for the respective antibiotics in the literature (50, 50 and 155 mg/L for ampicillin, meropenem and ertapenem, respectively[9,20,21]). The ordinates show the changes in cfu ($\log_{10}$) per mL of broth (left-hand panels) or per mg of cell protein (right-hand panels) as observed after 24 h of incubation in comparison with the original inocula (horizontal broken lines). All values are means ± SD ($n = 3$). Sigmoidal functions were fitted to the data after logarithmic transformation [goodness of fits (R^2): *L. monocytogenes* in broth: ertapenem 0.980, ampicillin 0.982, meropenem 0.987; *L. monocytogenes* in cells: ertapenem 0.858, ampicillin 0.985, meropenem 0.928; *S. aureus* in broth: ertapenem 0986, ampicillin 0.970, meropenem 0.980; *S. aureus* in cells: ertapenem 0.900, ampicillin 0.889, meropenem 0.834].

over a wider range of extracellular concentrations than in the experiments reported in Figure 1. For this purpose, bacteria or cells were exposed to a range of concentrations from sub-MIC to ~1000 × MIC values, allowing for a direct pharmacological comparison between drugs. Results are shown in Figure 3 with activity expressed as a function of multiples of the MIC. This allows for direct comparisons at equipotent concentrations. Considering *L. monocytogenes* first, all three β-lactams showed a concentration-dependent activity in broth in a range of one- to 100-fold their MICs. The bacterial responses to meropenem and ampicillin were undistinguishable whereas ertapenem was about four-fold less potent in that range. For intracellular bacteria, both meropenem and ampicillin showed a concentration-dependent activity that reached a bactericidal effect (~2 log decrease in cfu) at high concentrations. Ampicillin was systematically ~10-fold more potent than meropenem in the 1–100 × MIC range. In contrast, bacterial growth was seen for ertapenem at all concentrations tested, and the function that could be fitted to the data suggested that ertapenem would be unable to achieve even a bacteriostatic effect whatever its extracellular concentration. Moving now to *S. aureus*, the data show: (i) that a bactericidal effect was obtained against bacteria in broth from low multiples of MIC with four-fold higher potency for ertapenem compared with the two other β-lactams; (ii) that intracellular

activity was, like in broth, concentration-dependent; (iii) but that the reduction of the inoculum was quite limited (~1 log only) in all cases, with no meaningful difference in bacterial response between the three antibiotics.

Stability and protein binding of carbapenems and influence of variation of the serum concentration on activity of ertapenem against L. monocytogenes

The surprising results observed with ertapenem towards intracellular *L. monocytogenes* prompted us to run a series of controls to check for an artefactual cause. We first examined whether the drug would remain sufficiently stable in the culture medium, based on previous experience with meropenem and imipenem that showed a fast degradation of both drugs when incubated as concentrated solutions in aqueous media at 37°C.[22] During incubation in complete culture medium, ertapenem degradation (original concentration 155 mg/L) proceeded according to zero-order kinetics, with ~62% of drug remaining intact after 24 h. For meropenem (original concentration 50 mg/L), degradation reached ~50% over the same period. We also tested whether the high protein binding of ertapenem reported in human serum[23] would play a critical role here. Equilibrium dialysis experiments, showed that, indeed, only 32.1 ± 0.6% (n = 3) of total ertapenem was free in complete culture medium (10% fetal calf serum). Reducing the serum concentration to 2% increased this value to 42.6 ± 2.5% (n = 3). However, this did not improve the activity against intracellular *L. monocytogenes* [changes in post-phagocytosis inoculum (log cfu) of 0.63 ± 0.09 versus 0.70 ± 0.09 at 5 h and of 1.83 ± 0.12 versus 1.77 ± 0.19 at 24 h for cells incubated with 2% and 10% serum, respectively].

Cellular accumulation of ertapenem

L. monocytogenes-infected and non-infected cells were collected after 5 h and 24 h of incubation with ertapenem (155 mg/L) and subjected to bioassay. The apparent cell antibiotic concentrations (in mg per litre of cell volume) were 83.1 ± 7.1 and 68.6 ± 1.5 at 5 h, and 27.2 ± 9.3 and 37.1 ± 12.2 at 24 h for infected and uninfected cells, respectively.

Discussion

The present study is a pharmacological comparison of the activity of three β-lactams against two kinds of extracellular and intracellular bacteria with different susceptibilities in broth and distinct subcellular localizations in macrophages. Three main limitations prevent extrapolating its results to the *in vivo* situation without caution, namely that: (i) THP-1 cells display only poor intrinsic defences against intracellular infection; (ii) other cells than macrophages may be invaded by the organisms studied; and (iii) only laboratory strains were used. Given these caveats, we, nevertheless, may draw a series of general and specific conclusions as far as antibiotic activity per se is concerned towards both extracellular and intracellular organisms.

Upon testing for activity over a wide range of concentrations covering values below and above the MIC (i.e. when using a pharmacologically oriented design), all three antibiotics showed clear-cut concentration-related effects towards both extracellular and intracellular bacteria. This may come as a surprise, since β-lactams have for long been ranked as concentration-independent antibiotics.[24] However, we show here that the antibacterial concentration effect actually follows a sigmoidal function. Because the MICs of the β-lactams studied here are quite low with respect to the organisms tested, almost maximal effects can be observed at antibiotic concentrations corresponding to low (4–10%) fractional amounts of the maximal concentration tested (chosen based on an estimation of the C_{max} in patients). The clinical implication could be that β-lactam therapy will be optimal, with respect to both extracellular and intracellular bacteria, only if the serum concentration remains at values exceeding the MIC for the whole period of observation (time becoming then the predominant parameter governing activity). Conversely, β-lactams will be expected to show concentration-dependent effects if their concentration falls closer to the MIC, which will be the case *in vivo* upon under-dosing or with organisms with high MIC values.

Quite surprisingly, ertapenem, which shares many of the properties of meropenem against Gram-positive organisms,[9] was inefficient against the intraphagocytic forms of *L. monocytogenes* under conditions in which ampicillin and meropenem are active (as demonstrated here and in our previous publications[12,13]). This could not be ascribed to drug instability (in comparison with meropenem) or lack of cell penetration [the concentrations of ertapenem being higher than those of meropenem or of ampicillin (at extracellular concentrations of 50 mg/L as used here)[12]]. Ertapenem is known to be highly protein bound.[10] While binding to serum proteins present in the culture fluid is probably unimportant in our model (decreasing the protein concentration did not improve the intracellular activity of ertapenem), the situation may be different within the cell where phagocytosed *L. monocytogenes* becomes surrounded by a thick layer of actin.[18]

In contrast, all three β-lactams were active against intracellular *S. aureus*. The morphological studies are of particular interest, since they revealed images of bacterial ghosts quite similar to those seen with extracellular *S. aureus* exposed to penicillin[25] or faropenem,[26] which are directly related to the binding of the β-lactams to their targets. β-Lactams are not expected to accumulate in cells, owing to the presence of a free carboxyl function on all these molecules.[1,2,27] Yet, we observe here that the cellular concentration of ertapenem can reach values far above the MIC for *S. aureus*. When phagocytosed by macrophages, this bacterium is, however, not free in the cytosol but is primarily located in phagolysosomes and related vacuoles,[28] which are acidic,[29] and where the concentration of β-lactams is expected to be much lower.[27] This may explain why many reports have pointed to the apparent inability of β-lactams to act against intraphagosomal organisms in general,[1,2,30] and *S. aureus* in particular.[31,32] In this context, it must be emphasized that a significant reduction in the intracellular inoculum was obtained here only when (i) the extracellular concentration markedly exceeded the MIC for the offending organism (and was actually close to the maximal concentration tested), and (ii) the incubation time was brought to 24 h. This suggests that large extracellular concentrations and prolonged exposure may compensate for the lack of cellular accumulation, as previously proposed based on studies with methicillin,[33] especially since acidic pH does not adversely affect activity. We also cannot exclude the possibility that the activity of β-lactams towards phagosomal organisms is enhanced by cellular factors, as previously suggested.[34,35] However, we have to stress the fact that

eradication of *S. aureus* remained quite limited in our model, as was also noted for fluoroquinolones in similar models.[14,36] Translated to the *in vivo* situation, this may have important consequences in terms of recurrence of the infection and emergence of resistance. These points will need to be specifically addressed in future studies, the aim of which should be to establish what is the true impact or the limitations of the present findings.

Acknowledgements

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II - Role of acidic pH in the restored susceptibility of Methicillin-Resistant *Staphylococcus aureus* (MRSA) to β-lactam antibiotics

Publication #2: Role of acidic pH in the susceptibility of intraphagocytic Methicillin-Resistant *Staphylococcus aureus* strains to meropenem and cloxacillin

Sandrine Lemaire, Françoise Van Bambeke, Marie-Paule Mingeot-Leclercq, Youri Glupczynski and Paul M. Tulkens

Restoration of the susceptibility of MRSA to β-lactams by acidic pH was described in the early 1970s by Sabath and colleagues[210] but was not considered of clinical importance by its discoverers. The present study confirms these observations and extends it to the phagolysosomes of infected macrophages in which both MSSA and MRSA isolates survives and thrives, giving it a broader significance that originally appreciated. These observations were gained from independent approaches, namely (i) the similar pharmacodynamic responses of cloxacillin and meropenem towards intraphagocytic MSSA and MRSA; and (ii) the use of ammonium chloride (a lysosomotropic agent neutralizing the pH of lysosomal and phagolysosomial compartments), which make intraphagocytic MRSA insensitive to the action of cloxacillin and meropenem.

In the early 1980's, Hartman and Tomasz have first suggested that the effects of acidic pH on methicillin resistance were related to a decreased bacterial content in PBP2a[104]. However, we show that growing bacteria at acidic pH (i) does not alter neither the expression of the PBP2a-encoding gene (*mecA*) nor that of its regulatory genes; and (ii) does not decrease the amount of immunodetectable PBP2a in whole cells preparations. We found also that cell-wall preparations isolated from MRSA grown and exposed to radio-labelled penicillin G at acidic pH show a larger amount of radioactive amount than if bacteria had been grown at neutral pH (this amount being actually brought to a level similar to what is observed with MSSA grown and exposed to radio-labelled penicillin G at neutral pH).

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Role of Acidic pH in the Susceptibility of Intraphagocytic Methicillin-Resistant *Staphylococcus aureus* Strains to Meropenem and Cloxacillin[▽]

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Early studies showed that methicillin-resistant *Staphylococcus aureus* (MRSA) strains are susceptible to β-lactams when they are exposed to pH ≤ 5.5 in broth. Because *S. aureus* survives in the phagolysosomes of macrophages, where the pH may be acidic, we have examined the susceptibility of MRSA ATCC 33591 phagocytized by human THP-1 macrophages to meropenem (MEM) and cloxacillin (CLX). Using a pharmacodynamic model assessing key pharmacological (50% effective concentration and maximal efficacy) and microbiological (static concentration) descriptors of antibiotic activity, we show that intraphagocytic MRSA strains are as sensitive to MEM and CLX as methicillin-susceptible *S. aureus* (MSSA; ATCC 25923). This observation was replicated in broth if the pH was brought to 5.5 and was confirmed with clinical strains. Electron microscopy showed that both the MRSA and the MSSA strains localized and multiplied in membrane-bounded structures (phagolysosomes) in the absence of β-lactams. Incubation of the infected macrophages with ammonium chloride (to raise the phagolysosomal pH) made MRSA insensitive to MEM and CLX. No difference was seen in *mec*, *mecA*, *mecI*, *mecR1*, *femA*, and *femB* expression (reversed transcription-PCR) or in PBP 2a content (immunodetection) in MRSA grown in broth at pH 5.5 compared with that in MRSA grown in broth at 7.4. The level of [^{14}C]benzylpenicillin binding to cell walls prepared from a non-β-lactamase-producing MRSA clinical isolate was two times lower than that to cell walls prepared from MSSA ATCC 25923 at pH 7.4, but the levels increased to similar values for both strains at pH 5.5. These data suggest that the restoration of susceptibility of intraphagocytic of MRSA to MEM and CLX is due to the acidic pH prevailing in phagolysosomes and is mediated by an enhanced binding to penicillin-binding proteins.

Staphylococcus aureus causes a wide range of severe and often life-threatening infections, such as endocarditis, osteomyelitis, and complicated skin and skin structure infections. Often considered an extracellular organism, *S. aureus* is capable of surviving within phagocytic and nonphagocytic cells (4, 11), which is probably an important determinant in the recurrent and relapsing character of these infections. There is substantial evidence that the intracellular milieu may modulate both the pharmacological properties of the antibiotics and the response of the bacteria (20). Yet, this factor is not taken into account in the assessment of bacterial susceptibility to drugs in the routine clinical microbiology. In the course of a study on the activities of β-lactams against *S. aureus* phagocytosed by human THP-1 macrophages (10), we noted that methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) strains showed similar susceptibilities to meropenem (10a), suggesting a restoration of susceptibility of MRSA to β-lactams in the intracellular milieu. We present here a detailed account of this finding and extend it to cloxacillin, taken as a typical β-lactamase-resistant penicillin. Our studies suggest that the restoration of the susceptibilities to β-lactams is due to the acidic pH prevailing in the vacuoles where *S. aureus* sojourns and thrives.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. All key experiments were performed with one MSSA strain (MSSA strain ATCC 25923; American Type Culture Collection [ATCC], Manassas, VA) and one MRSA β-lactamase-producing strain (MRSA strain ATCC 33591). Additional surveys were made with recent Belgian MRSA isolates (three hospital-acquired strains [obtained during this study by Y.G.] and three community-acquired strains [two strains obtained during this study by Y.G. and one strain (NRS 192) obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus*, Focus Technologies, Inc., Herndon, VA]).

For [^{14}C]benzylpenicillin binding studies, we used a non-penicillinase-producing MRSA strain (strain 459, clinical isolate, which was checked for the absence of production of penicillinase with the BBL Dryslide Nitrocefin reagent [Becton, Dickinson and Co., Cockeysville, MD] and for the presence of *mecA* by PCR amplification [see below]). All bacteria were grown in Mueller-Hinton broth supplemented with 2% NaCl (wt/vol) for the MRSA strains.

Cell cultures. All experiments were performed with THP-1 cells (ATCC TIB-202), a human myelomonocytic cell line displaying macrophage-like activity, exactly as described previously (10).

Susceptibility testing and 24-h dose-response curve studies in broth. Bacteria in exponential phase of growth were harvested and resuspended at a final density of 10^6 CFU/ml in broth adjusted to pH 7.4, 6.0, or 5.5. MIC determinations and 24-h dose-response curve studies were performed as described previously (10). We checked that the final pH of the broths at the end of the 24-h incubation period was close to the original one at all antibiotic concentrations equal to the MIC or above.

Cell infection, assessment of intracellular activities of antibiotics, and morphological studies. Infection of THP-1 cells, assessment of intracellular activity, and electron microscopy studies were performed as described earlier for *S.*

aureus ATCC 25923 (10), except that the concentration of gentamicin added to the culture medium of the controls (to which no β-lactam was added) to prevent the extracellular growth of bacteria was reduced to 0.5× MIC to better minimize its influence on the intracellular bacterial growth (see reference 1 for a full description of the gentamicin concentration effects). As reported previously (1), small colony variants were only very infrequently observed under the conditions of our experiments.

RT-PCR studies. Reverse transcription-PCR (RT-PCR) was used for the semiquantitative detection of *mecA*, *mecI*, *mecR1*, *femA*, and *femB* by using 16S rRNA as the housekeeping gene and by following a previously established procedure (9) and published primers (7, 14, 21), but with the following modifications. The bacteria were grown in broth adjusted to pH 7.4 or 5.5, collected in mid-exponential phase of growth (optical density at 600, ~0.3), and lysed with lysostaphin (100 mg/liter) and lysozyme (3 g/liter). Total RNA was extracted with an RNeasy Mini kit (QIAGEN, Hilden, Germany), the contaminating DNA was eliminated with RQ1 RNase-free DNase I (Promega Corporation, Madison, WI), and the RNA was further extracted with RNeasy mini columns. The PCRs were carried out in a Gene Cyder thermal cyder (Bio-Rad Laboratories, Hercules, CA) with 30 cycles to ensure amplification in the exponential range.

Immunoassay of PBP 2a. We used the MRSA latex screening test (Oxoid Ltd., Hants, United Kingdom), according to the method of Zhao et al. (23), and bacteria grown at pH 7.4 or 5.5; but the bacteria were tested at the pH (pH 7.0) of the commercial kit. Pilot experiments showed that the time for agglutination (20 to 180 s; detected with the naked eye) was inversely proportional ($R^2 \geq 0.912$) to the $\log_{10}$ of the bacterial density for both MRSA ATCC 33591 and MRSA strain 459 (5×10^9 to 2.5×10^8 CFU/ml) for bacteria grown at pH 7.4 or 5.5.

Whole-cell-wall binding of [^{14}C]benzylpenicillin. The assay for the whole-cell-wall binding of [^{14}C]benzylpenicillin was performed with strain 459 by a previously published procedure (2), with the following modifications. The bacteria were grown at pH 7.4 or 5.5 for 5 h and, thereafter, were exposed to [^{14}C]benzylpenicillin (50 mg/liter) for 30 min either at pH 5.5 or at pH 7.4, which generated four different conditions: pH 7.4 (culture) and pH 7.4 (binding), pH 7.4 (culture) and pH 5.5 (binding), pH 5.5 (culture) and pH 7.4 (binding), and pH 5.5 (culture) and pH 5.5 (binding). The bacteria were collected by centrifugation, washed four times with phosphate-buffered saline, and lysed by three successive freeze-thaw cycles (5 min at −80°C, followed by 5 min at 37°C.). The amount of bound radioactivity was measured by scintillation counting and was expressed by reference to the protein content in the sample.

Antibiotics and main reagents. Penicillin G, oxacillin (potency, 93%), and cloxacillin (potency, 88.4%) were purchased from Sigma-Aldrich (St. Louis, MO); and [^{14}C]benzylpenicillin (specific activity, 59 mCi/mmol) was purchased from Amersham Biosciences, Little Chalfont, United Kingdom (now GE Healthcare UK Ltd.). Meropenem, gentamicin, and ertapenem were obtained as the corresponding branded products distributed for clinical use in Belgium: meropenem was obtained as Meronem from AstraZeneca Pharmaceuticals (Brussels, Belgium); gentamicin was obtained as Geomycine from GlaxoSmithKline s.a. (Rixensart, Belgium), and ertapenem was made available to us as Invanz by Merck Sharp & Dohme Ltd. (Hoddesdon, United Kingdom). Cell culture or microbiology media were from Invitrogen (Life Science Technologies, Paisley, United Kingdom) or Becton Dickinson. Unless stated otherwise, all other reagents were obtained from Merck KgaA (Darmstadt, Germany) or Sigma-Aldrich.

Statistical analyses. Curve-fitting analyses were performed by using GraphPad Prism software (version 4.02) for Windows (GraphPad Prism Software, San Diego, CA). Analysis of variance (ANOVA) was performed with GraphPad Instat software, version 3.06 (GraphPad Prism software); and analysis of covariance was performed with XLStat (version 7.5.2; Addinsoft SARL, Paris, France).

RESULTS

Intracellular susceptibilities of MSSA and MRSA to meropenem and cloxacillin. In a first series of experiments, we compared the susceptibilities of intraphagocytic MSSA ATCC 25923 and MRSA ATCC 33591 to meropenem and cloxacillin after 24 h of incubation over a wide range of extracellular concentrations to obtain a pharmacological description of the bacterial response to these antibiotics (1). As shown in Fig. 1, we observed clear-cut, concentration-dependent effects on

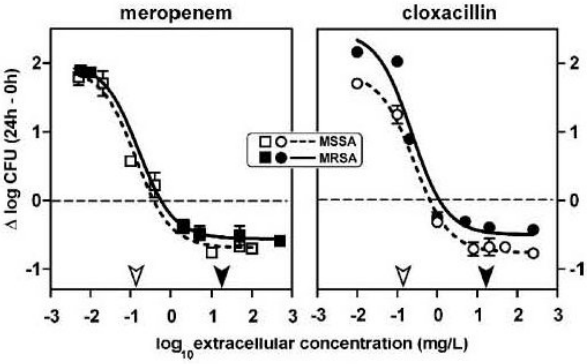

FIG. 1. Concentration killing effects of meropenem (squares; left panel) and cloxacillin (circles; right panel) toward MSSA strain ATCC 25923 (open symbols and dotted line) and MRSA strain ATCC 33591 (closed symbols and continuous line) after phagocytosis by THP-1 macrophages. Cells were incubated with the antibiotics for 24 h at the concentrations (total drug) indicated on the abscissa. All values are the means ± standard deviations of three independent determinations (standard deviation bars that are not visible are smaller than the size of the symbols). The arrows along the abscissa point to the MIC of the organisms determined in broth at pH 7.4 (open arrows, MSSA strain ATCC 25923; closed arrows, MRSA ATCC 33591).

which a model based on the Hill equation could be fitted by nonlinear regression. This allowed determining the typical pharmacological descriptors of relative potency (50% effective concentration [EC_{50}]) and apparent maximal efficacy (E_{max}) of each drug for each condition, together with an estimation of their corresponding apparent static concentrations (Table 1). This analysis showed that there was no significant difference in the responses of intracellular MSSA ATCC 25923 and MRSA ATCC 33591 to these two antibiotics, whatever criterion was used, even though there was, as anticipated, a large difference in susceptibility when the MICs were measured in broth at pH 7.4 (values shown by arrows in the graphs of Fig. 1). This suggested that MSSA ATCC 25923 and MRSA ATCC 33591 had very similar susceptibilities to meropenem and cloxacillin in the intracellular milieu.

Morphological studies. Previous studies have shown that MSSA strain ATCC 25923 phagocytosed by THP-1 cells sojourns and multiplies in membrane-bounded vacuoles belonging to the phagolysosomal apparatus (1, 10). Electron microscopy was therefore used to compare the intracellular localization of MRSA ATCC 33591 to that of MSSA ATCC 25923. In both cases, intracellular bacteria were systematically seen enclosed in membrane-bound structures (Fig. 2), consistent with a phagolysosomal localization.

Studies of MSSA and MRSA susceptibility to meropenem and cloxacillin at acidic pH in broth. Because phagolysosomes are known to be acidic (12), we examined the extent to which the acidic pH would restore the susceptibility of MRSA ATCC 33591 to β-lactams in broth. Figure 3 shows that while acidic pH only modestly decreased the MICs of meropenem and cloxacillin against MSSA ATCC 25923, it drastically reduced those of the same antibiotics against MRSA ATCC 33591, bringing them to values close to the MICs of MSSA ATCC 25923 at pH 5 to 5.5. In parallel, we examined the effect of pH on bacterial growth and observed that pH 5.5 allowed reliable

TABLE 1. Pertinent regression parameters,[a] statistical analysis, and calculation of the static concentrations from the dose-response curves shown in Fig. 1 and 3

Condition	Antibiotic[b]	Strain[c]	Regression parameter values (95% confidence intervals)		Statistical analysis[d]		Static concn[e] (mg/liter)[f]
			E_{max}[g] (mg/liter)[f]	EC_{50}[h] (mg/liter)[f]	R^2	All data	
THP-1 macrophages	MEM	MSSA	−0.69 (−0.95 to −0.42) a-A	0.14 (0.01 to 0.08) a-A	0.980	a-A	0.38
	MEM	MRSA	−0.56 (−1.21 to −0.18) a-A	0.16 (0.05 to 0.49) a-A	0.999	a-A	0.56
	CLX	MSSA	−0.76 (−0.94 to −0.58) a-A	0.27 (0.03 to 0.14) a-A	0.995	a-A	0.67
	CLX	MRSA	−0.50 (−0.95 to 0.08) a-A	0.23 (0.01 to 0.14) a-A	0.953	a-A	0.80
Broth pH 7.4	MEM	MSSA	−3.91 (−4.90 to −2.93) a-B	0.29 (0.07 to 1.28) a-A	0.984	a-B	0.18
	MEM	MRSA	NF[i]	NF	0.990		22.9
	CLX	MSSA	−2.70 (−3.48 to −1.93) b-C	0.35 (0.13 to 0.90) a-A	0.969	b-B	0.35
	CLX	MRSA	−0.39 (−1.09 to 0.30) c-A	16.9 (8.19 to 34.92) b-B	0.968	c-B	86.12
Broth pH 5.5	MEM	MSSA	−4.02 (−4.51 to −3.54) a-B	0.20 (0.08 to 0.48) a-A	0.988	a-B	0.10
	MEM	MRSA	−3.60 (−4.15 to −3.04) a,b-B,C	0.33 (0.17 to 0.63) a-A	0.980	a-B	0.25
	CLX	MSSA	−2.99 (−4.02 to −1.97) b,c-C,D	0.22 (0.08 to 0.57) a-A	0.950	b-B	0.18
	CLX	MRSA	−2.57 (−2.93 to −2.19) c-D	0.07 (0.03 to 0.14) a-A	0.983	b-C	0.10

[a] The regression parameters are based on the Hill equation (by using a slope factor of 1).
[b] MEM, meropenem; CLX, cloxacillin.
[c] MSSA, strain ATCC 25923; MRSA, strain ATCC 33591.
[d] Statistical analysis is as follows: values (E_{max} or EC_{50}) or rows (all data) with different letters are significantly different from each other within the pertinent comparison group ($P < 0.05$; lowercase letters, comparisons within each condition [THP-1 macrophages, broth pH 7.4, or broth pH 5.5]; uppercase letters, comparison throughout all conditions). E_{max} and EC_{50}, one-way ANOVA (with Tukey's test for multiple comparisons) for values of the corresponding parameters; all data, analysis of covariance (with Tukey's test for multiple comparisons) for all experimental data.
[e] Concentration (mg/liter) resulting in no apparent bacterial growth (the number of CFU was identical to that in the original inoculum), as determined by graphical interpolation.
[f] Total drug.
[g] CFU decrease (in $\log_{10}$ units) at 24 h from the corresponding original inoculum, as extrapolated for antibiotic concentration at infinity; the counts for samples yielding less than 5 counts were considered below the detection level.
[h] Concentration (mg/liter) causing a reduction of the inoculum halfway between the initial (E_0) and the maximal (E_{max}) values, as obtained from the Hill equation (by using a slope factor of 1).
[i] NF, the Hill equation could not be fitted to the data.

reproducible growth without a marked difference with that at neutral pH (24-h $\log_{10}$ CFU increases at pH 5.5 versus pH 7.4, 2.49 ± 0.01 and 2.85 ± 0.03, respectively, for MSSA ATCC 25923 and 2.78 ± 0.05 and 3.13 ± 0.02, respectively, for MRSA ATCC 33591).

We therefore examined the full dose-response curves of both strains for meropenem and cloxacillin in broth at pH 5.5 versus those at pH 7.4, and the results are shown in Fig. 4, with the pertinent pharmacological descriptors and the apparent static concentrations for each condition presented in Table 1. As anticipated, meropenem and cloxacillin were considerably less active against MRSA ATCC 33591 than against MSSA ATCC 25923 at pH 7.4 (based on the determination of the E_{max} and EC_{50} values and of the apparent static concentrations). Yet, there was no significant difference between the responses of the two strains (based on the same criteria) when the assays were performed at pH 5.5 (cloxacillin, however, showed a slightly weaker E_{max} compared to that of meropenem under all conditions).

Observations with additional strains of clinical interest. Three hospital-acquired MRSA isolates and three community-acquired MRSA isolates were tested for their susceptibilities to meropenem and cloxacillin (i) at acidic pH in broth and (ii) after phagocytosis by THP-1 macrophages. The results were very similar to those described for MRSA ATCC 33591 in Fig. 1 and 2.

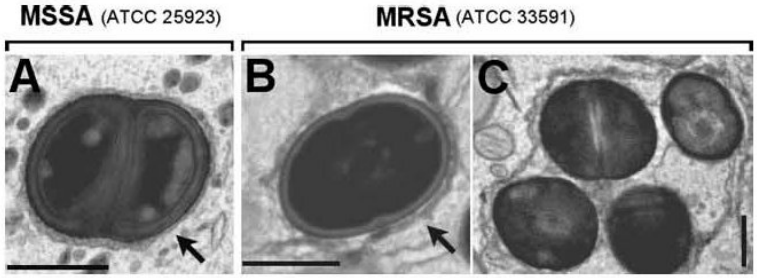

FIG. 2. Morphology of *S. aureus* in THP-1 macrophages. Cells were allowed to phagocytize the bacteria for 1 h and thereafter were incubated for 24 h (in the presence of gentamicin at 0.5× MIC) to prevent the extracellular growth of bacteria and ensuing cell death due to acidification of the medium). (A and B) Arrows point to the membrane surrounding the bacterial profiles; (C) evidence of multiplication of MRSA in a membrane-bounded structure. Bars, 0.5 μm.

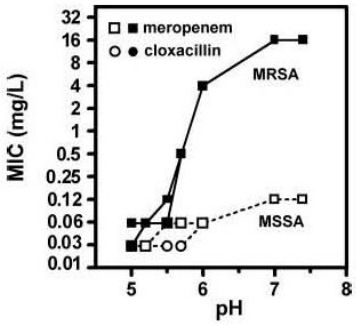

FIG. 3. Influence of pH on the MICs of meropenem and cloxacillin for MRSA ATCC 25923 and MRSA ATCC 33591 as determined in broth. Each datum point corresponds to three determinations with identical results. Symbols for one antibiotic that are not visible are overlapped by the corresponding symbols of the other antibiotic.

Influence of ammonium chloride on the intracellular activities of meropenem and cloxacillin. Ammonium chloride raises the pH of phagolysosomes of macrophages because of its proton-shuttling properties (17). Since the previous experiments identified acidic pH as a potential determinant for restoring the susceptibility of intraphagocytic MRSA ATCC 33591 to meropenem and cloxacillin, we compared the activities of both antibiotics against this strain in THP-1 macrophages in the absence and in the presence of 10 mM ammonium chloride,

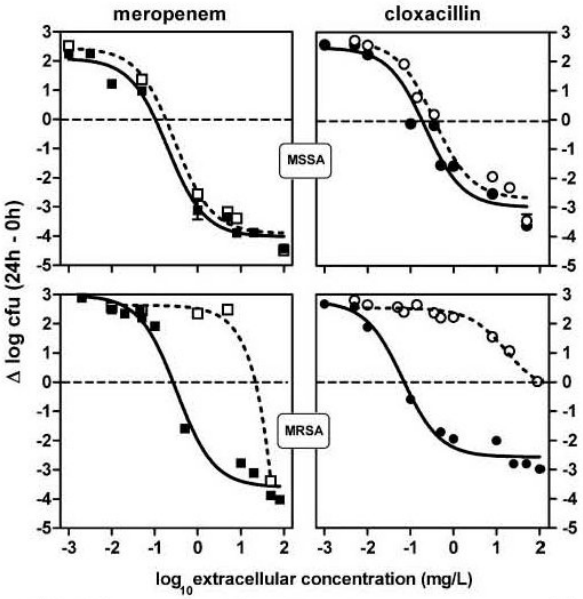

FIG. 4. Concentration killing effects of meropenem (squares; left panels) and cloxacillin (circles; right panels) toward MSSA strain ATCC 25923 (upper panels) and MRSA strain ATCC 33591 (lower panels) in broth at an initial pH of 7.4 (open symbols and dotted line) or 5.5 (closed symbols and continuous line). The bacteria were incubated with the antibiotics for 24 h at the concentrations (total drug) indicated on the abscissa. All values are the means ± standard deviations of three independent determinations (standard deviation bars that are not visible are smaller than the size of the symbols).

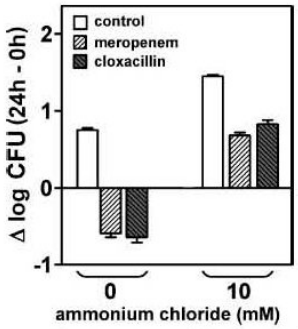

FIG. 5. Influence of ammonium chloride on the intracellular growth of methicillin-resistant *S. aureus* (ATCC 33591) and on the activities of meropenem and cloxacillin in THP-1 macrophages. Ammonium chloride was added after phagocytosis simultaneously with the antibiotics, and the cells were further incubated for 24 h before collection. Open bars, control (cells incubated for 24 h with gentamicin at 0.5× MIC to prevent the extracellular growth of bacteria and the ensuing cell death due to acidification of the medium); striped white bars, meropenem (50 mg/liter [total drug; corresponding to the human total drug C_{max}]); striped gray bars, cloxacillin (8 mg/liter [total drug; corresponding to the human total drug C_{max}]). All values are the means ± standard deviations of three independent determinations.

using fixed concentrations (meropenem, 50 mg/liter; cloxacillin, 8 mg/liter) that ensured maximal efficacy (based on the data in Fig. 1) and that are clinically meaningful (corresponding to the maximum concentration in the serum [C_{max}; the concentration of total drug] of humans). Figure 5 shows that ammonium chloride (i) caused an increased intracellular growth of MRSA ATCC 33591 in control cultures (incubated with gentamicin (0.5× MIC) only) and (ii) made meropenem and cloxacillin essentially inactive.

Influence of acidic pH on the expression of *mecA* and its regulatory and accessory genes and PBP 2a immunodetection in MRSA. RT-PCR failed to reveal significant differences in the expression of *mecA* in MRSA ATCC 33591 grown in broth at pH 7.4 or 5.5, in the absence or the presence of ertapenem (used as an inducer; ratios of *mecA*/16S rRNA, 0.21 ± 0.04 at pH 7.4 and 0.19 ± 0.05 at pH 5.5 without ertapenem; 0.79 ± 0.04 at pH 7.4 and 0.67 ± 0.08 at pH 5.5 with ertapenem [0.5× MIC; 5 h]). No influence of pH was noted for the expression of *mecR1*, *femA*, and *femB* in bacteria grown at pH 5.5 or 7.4 with or without inducer. The expression of *mecI* could not be detected with RNA at amounts of up to 200 ng and 35 amplifications cycles. Finally, there was no difference in the time of agglutination of anti-PBP 2a monoclonal antibody-coated latex particles (tested at different bacterial densities to check for the consistency of the results) between MRSA ATCC 33491 and MRSA strain 459 when they were grown at pH 7.4 or 5.5 in the presence of meropenem as an inducer (0.5× MIC; 24 h).

Influence of pH on [^{14}C]benzylpenicillin binding to MSSA ATCC 25923 and MRSA. Experiments for determination of the influence of pH on [^{14}C]benzylpenicillin binding were performed with a clinical isolate (MRSA strain 459) rather than with MRSA ATCC 33591 because the latter produces an active penicillinase and no radiolabeled β-lactamase-resistant β-lactam was available to us. We first checked that the susceptibility of MRSA strain 459 to β-lactams was significantly increased

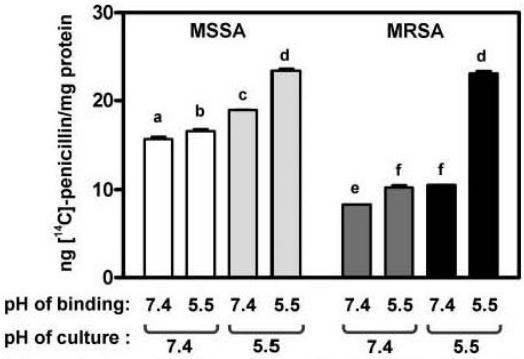

FIG. 6. Binding of [^{14}C]benzylpenicillin to MSSA strain ATCC 25923 (open and light gray bars) and MRSA strain 459 (β-lactamase negative; dark gray and closed bars) after 5 h of growth in broth at an initial pH of 7.4 (open or dark gray bars) or 5.5 (light gray or closed bars). For each culture condition, binding was made at pH 7.4 or 5.5, as indicated on the abscissa. All values are the means ± standard deviations of three independent determinations (standard deviation bars that are not visible are smaller than the size of the frame of the corresponding bar). Statistical analysis (ANOVA), bars with different letters are significantly different from all others ($P < 0.01$).

when it was tested in broth at pH 5.5 compared with that in broth at pH 7.4 (MICs, 0.03 mg/liter and 0.25 mg/liter, respectively, for penicillin G; 0.03 mg/liter and 0.125 mg/liter, respectively, for cloxacillin; and 0.03 mg/liter and 1 to 2 mg/liter, respectively, for oxacillin). Figure 6 shows that binding of [^{14}C]benzylpenicillin for MRSA strain 459 was only half of the level observed for MSSA ATCC 25923 when the bacteria had been grown and tested at pH 7.4; for MSSA, binding increased in a stepwise fashion if (i) the bacteria were grown at pH 7.4 but binding was performed at pH 5.5, (ii) the bacteria were grown at pH 5.5 and binding was performed at pH 7.4, and (iii) the bacteria were grown at pH 5.5 and binding was performed at pH 5.5. For MRSA, a small increase in binding was seen for bacteria grown at pH 7.4 and tested at pH 5.5 or grown at pH 5.5 and tested at pH 5.5, but a twofold increase in binding was seen if the bacteria were grown and tested at pH 5.5. The level of binding was then not significantly different from what was observed for MSSA grown and tested under the same conditions.

DISCUSSION

Restoration of the susceptibility of MRSA to β-lactams by acidic pH was described for a large number of laboratory and clinical isolates in the early 1970s (18) but was not considered of clinical importance by its discoverers. Indeed, they saw this effect in a reproducible fashion only at pHs lower than 5.5 (by using agar dilution methods) and were "not aware of this degree of acidity occurring frequently in infected foci" (18). The present study confirms this original observation and extends it to recent hospital- as well as community-acquired MRSA isolates. The present study also gives the observation a larger significance by showing that (i) full restoration of susceptibility can be obtained in a reproducible fashion at pH 5.5 or lower when assays are performed in pH-adjusted broths and (ii) the vacuoles in which *S. aureus* survives and thrives in macrophages may represent foci of infection with a degree of acidity sufficient to cause such a restoration of activity. We base the latter conclusion on four complementary pieces of evidence.

First, whereas MRSA ATCC 33591 and MSSA ATCC 25923 showed the expected differences in susceptibility to meropenem and cloxacillin when they were tested in broth at pH 7.4, they could not be distinguished from one another when they were exposed to these antibiotics at pH 5.5 in broth, based on both pharmacological criteria (shape of dose-response curves, E_{max}, EC_{50}) and microbiological criteria (static concentration, MIC). Second, MRSA ATCC 33591 and MSSA ATCC 25923 also could not be distinguished by their responses to meropenem or cloxacillin when they were challenged with these antibiotics after phagocytosis by THP-1 macrophages and by using the same pharmacological and microbiological criteria used for broth. Third, both strains clearly develop in phagolysosomes after phagocytosis by THP-1 macrophages (based on electron microscopic studies), and we know that the pH of phagolysosomes of macrophages is about 5 (12). Fourth, addition of ammonium chloride, which is known to raise the pH of phagolysosomes of macrophages (17), makes intraphagocytic MRSA ATCC 33591 insensitive to meropenem and cloxacillin.

Twelve years after its original discovery, the restoration of susceptibility of MRSA grown at acidic pH to β-lactams was ascribed by Hartman and Tomasz to the absence of expression of PBP 2a, based on its lack of detection with ^{3}H-labeled benzylpenicillin, even at high concentrations (8). We show here that the growth of bacteria at pH 5.5 rather than at pH 7.4 (i) does not alter the level of expression of the gene encoding the gene corresponding to PBP 2a (*mecA*) or of its regulatory genes when expression is examined by RT-PCR (a similar conclusion was drawn for the MRSA COL strain by using microarray analysis [22]) and (ii) does not modify the bacterial content in immunodetectable PBP 2a. The observation of Hartman and Tomasz must, therefore, be reinterpreted as indicating not the absence of PBP 2a but the inability of PBP 2a to bind to penicillin when it is expressed in bacteria growing at acidic pH. We could also see that cell walls from MRSA strain 459 bound about half the amount of ^{14}C-labeled benzylpenicillin bound by cell walls from MSSA ATCC 25923 at pH 7.4 but that cell walls from both strains bound a larger and not significantly different amount at pH 5.5. A tentative, global interpretation of these data is, therefore, that acidic pH (i) makes PBP 2a unable to bind to β-lactams but improves the binding of penicillin to other targets (explaining the decrease in the MIC for MSSA at acidic pH) and (ii) also makes PBP 2a unable to compensate for the inactivation of the other PBPs, resulting in the similar susceptibilities of MRSA and MRSA to β-lactams. Thus, PBP 2a exposed to acidic pH may actually be an inactive enzyme. At pH 7.4, PBP 2a already shows very weak binding and a low acylation rate when it is exposed to oxacillin but maintains efficient peptidoglycan synthesis activity. Substantial conformational changes in PBP 2a are, however, required for these reactions to occur (6), and acidic pH may make such changes impossible or much too slow (recent data indicate that cephalosporins showing activity against MRSA act by facilitating this conformational change [5]). We also know that the full expression of methicillin resistance

requires (i) the transglycosylase function of PBP 2 to be maintained, together with the transpeptidase function of PBP 2a (15), and (ii) the capacity of PBP 2a to properly localize the other components necessary for cell wall synthesis, including PBP 2, at the site of division of *S. aureus* (16). It is therefore also possible that acidic pH prevents PBP 2 from functioning in conjunction with PBP 2a and/or perturbs its recruitment. These nonmutually exclusive hypotheses may now need to be tested by the use of appropriate biochemical and morphological approaches.

Pending more detailed mechanistic investigations, the present data may already be of significance for the present therapy of MRSA infections and for an improved evaluation of presently available as well as novel antistaphylococcal agents. First, our observations with MRSA ATCC 33591 can probably be generalized to most clinical strains, since the restoration of susceptibility to methicillin by acidic pH was considered to be a general property of all MRSA isolates in its original description (8) and has been confirmed here with recent isolates. Second, our observations may trigger further in vitro and animal studies to delineate the therapeutic interest of including a β-lactam in the treatment of MRSA infections in situations where intracellular forms are suspected to play an important role the intracellular persistence of *S. aureus*, causing relapses and recurrences (19, 20). While it is not certain that intracellular *S. aureus* will always be confined within acidic phagolysosomes, restoration of the susceptibility of MRSA to β-lactams could also take place in other environments, such as the skin surface (3, 13), the vagina, or the urinary tract, which are all habitats where the pH may reach a sufficiently low value. Finally, the present data show that the study of the actual susceptibility of intracellular bacteria to antibiotics is critical to obtaining a comprehensive view of their therapeutic potential, since this susceptibility cannot simply be deduced from the cellular accumulation and disposition properties of the drug.

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Publication #3: Restoration of susceptibility of Methicillin-Resistant *S. aureus* (MRSA) to beta-lactams after phagocytosis by eukaryotic cells: comparison between strains, cells and antibiotics.

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In the present study, we wishes to address three critical questions to further delineate the pertinence of our recent observations demonstrating that MRSA isolates recover susceptibility to β-lactam antibiotics when phagocytosed by THP-1 macrophages, namely whether it can be extended to:

(i) Other cells than macrophages, such as non-professional phagocytes
(ii) Other molecules selected among the three currently registered anti-staphylococcal β-lactams
(iii) MRSA isolates of current clinical and epidemiological interest (including the multi-drug resistant Vancomycin-Intermediate *S. aureus* and a recent Belgian MRSA isolate of animal origin).

Our data show that the suppression of methicillin resistance after phagocytosis is neither strain nor cell-specific. Comparison between β-lactams, however, suggests that this effect is preferentially seen with penicillins and carbapenems as opposed to cephalosporins.

ABSTRACT

S. aureus invades eukaryotic cells. MRSA ATCC 33591 phagocytized by human THP-1 macrophages shows complete restoration of susceptibility to cloxacillin and meropenem, becoming indistinguishable from MSSA ATCC 25923 due to the acid pH prevailing in phagolysosomes (Lemaire *et al.*, Antimicrob. Agents Chemother. 51:1627-32, 2007). We have examined whether this observation can be extended to (i) strains of current clinical and epidemiological interest (3 HA-MRSA; 2 CA-MRSA; 2 HA-MRSA with VISA phenotype; 1 HA-MRSA with VRSA phenotype; 1 animal [porcine] MRSA); (ii) activated THP-1 cells and non-professional phagocytes (keratinocytes, CALU-3 bronchial epithelial cells); (iii) other β-lactams (imipenem, oxacillin, cefuroxime, cefepime). All strains showed (i) a marked reduction of MIC in broth at pH 5.5 vs. pH 7.4; (ii) sigmoidal dose-response curves to cloxacillin (0.01 to 100 MIC; 24 h incubation) after phagocytosis by THP-1 macrophages, indistinguishable from each other and from that of MSSA ATCC 25923 (relative potency [E_{50}], 6.09 x MIC; CI: 4.50 to 8.25); relative efficacy [E_{max}] -0. 69 log cfu; CI: -0.79 to -0.58). Similar dose-response curves to cloxacillin were also observed for MSSA ATCC 25923 and MRSA ATCC 33591 after phagocytosis by activated THP-1 macrophages, keratinocytes, and Calu-3 cells. By contrast, there was a lesser restoration of susceptibility of MRSA ATCC 33591 to cefuroxime and cefepime after phagocytosis by THP-1 macrophages, even when data were normalized for differences in MICs. We conclude that the restoration of MRSA susceptibility to β-lactams after phagocytosis is independent of the strain and the type of cells, but varies between β-lactams.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is becoming an increasingly problematic pathogen. While long confined to the hospital setting or closely-related environments such as nursing homes, this phenotype is now emerging in the community as well as in food and pet animals and their human contacts ([1,2,8,11,16,18,20,31,40,42,43]). MRSA has thus become a true epidemiological scourge with serious therapeutic implications as well as a potential cause of large economical losses. Beyond creating immediate local and general damages (by the expression of various virulence factors), staphylococcal infections show a high degree of recurrence and relapses, sometimes even at years interval. This has been ascribed, at least in part, to the capacity of *S. aureus* to infect and survive in various cell types ([10,19,25,26,33,37]), a situation which could be aggravated in case of MRSA ([7]). A recent study has shown that *S. aureus* modulates its gene expression at early times after phagocytosis to promote intracellular survival ([13]). There is also now substantial evidence that the intracellular milieu is unfavorable to the action of many currently registered antistaphylococcal antibiotics ([4,34]), including vancomycin ([3,44]), explaining why intracellular forms of *S. aureus* are difficult to eradicate.

In the course of systematic studies aimed at measuring the intracellular activity of various anti-staphylococcal antibiotics, we made the unanticipated observation that MRSA strains phagocytized by human THP-1 macrophages show complete restoration of their susceptibility to cloxacillin and meropenem, making them virtually indistinguishable from MSSA in our model ([21]). This phenomenon could be ascribed to the acidic pH (5-5.5) to which intracellular *S. aureus* becomes exposed once phagocytized and transfered to phagolysosomes ([21]). This allowed linkage of the current observation to the well known suppression of intrinsic resistance of MRSA to methicillin and other penicillins at acidic pH described in the early 1970's ([35]). Recent studies have now shown that β-lactams are able to bind and acylate PBP 2a at pH 5.5, probably thanks to an acid pH-favored conformational change of the transpeptidase catalytic site of this protein from a closed to open state (Lemaire *et al.*, submitted for publication). In the present study, we wished to address three critical questions to further delineate the pertinence and potential applications of our observation concerning the intraphagocytic MRSA, namely whether it can it be extended (i) to strains of current clinical and epidemiological interest (within the context of the present MRSA threat); (ii) to other cell types than macrophages, including non-professional phagocytes; (iii) to other molecules selected among the three main classes of currently registered anti-staphylococcal β-lactams. Our current data show that the suppression of methicillin resistance after phagocytosis is neither strain nor cell-specific. Comparison between β-lactams, however, suggests that this effect is preferentially seen with penicillins and carbapenems as opposed to cephalosporins.

MATERIALS AND METHODS

Antibiotics and main reagents. Oxacillin, cloxacillin, dicloxacillin, nafcillin, cefotaxime, cephalexin were purchased from Sigma-Aldrich, St Louis, MO. Faropenem and cefadroxil were obtained as microbiological standard from Shenzhen Haibin Pharmaceuticals, Shenzhen, Guangdong, China, and from Bristol-Myers/Squibb, Princeton, NJ., respectively. The other antibiotics were obtained as the clinical products for intravenous administration to humans and complying with the European Pharmacopoeia (Gentamicin as GEOMYCIN [distributed in Belgium by Glaxo-SmithKline SA, Genval]; cefepime as MAXIPIME [Bristol-Myers/Squibb, Brussels]; cefuroxime as CEFURIM [Teva Pharma Belgium, Wilrijk]; meropenem as MERONEM [AstraZeneca, Brussels]; imipenem as TIENAM [Merck Sharp and Dohme, Brussels]); ertapenem as INVANZ [Merck Sharp and Dohme GmbH, Haar, Germany]). Cell culture media and serum were from Invitrogen (Life science Technologies, Paisley, UK) or Becton Dickinson. Unless stated otherwise, all other reagents were obtained from Merck AG (Darmstadt, Germany) or Sigma-Aldrich.

Cells lines. Experiments were performed with (i) THP-1 cells (ATCC TIB-202; American Tissue Culture Collection, Manassas, VA), a human myelomonocytic cell line displaying macrophage-like activity, and maintained in our laboratory as previously described ([6]); (ii) activated THP-1 cells (reported to show increased phagocytic activity, HLA-DR expression, and enhanced constitutive production of interleukin 1β [[38]]) prepared from unactivated cells by a 24 h culture in the presence of 2 mg/L phorbol myristate acetate ([12]); (iii) primary cultures of human skin keratinocytes (obtained from Invitrogen SA, Merelbeke, Belgium), and (iv) Calu-3 human bronchial epithelial cell line (ATCC HTB-55) and maintained in our laboratory as previously described ([15]) but without coating of the culture flasks.

Bacterial strains and susceptibility testing. The strains used in this study, their main characteristics, and their origins are shown in Table 1. They were obtained from the American Tissue Culture Collection, the Network on Antimicrobial Resistance in *Staphylococcus aureus* [NARSA] Program (operated by Eurofins Medinet, Inc., Hendon, VA; supported under NIAID/NIH Contract No. HHSN2722007 00055C), or from the collections of two of us (PA and YG). MICs were measured by microdilutions as described earlier ([21]). The MRSA phenotype of each strain was confirmed by detection of *mecA* by Polymerase chain reaction (PCR) as described previously ([21]) and the *SCCmec* subgroup of each strains established by multiplex PCR assay ([45]).

Table 1. Strains used in this study (phenotypes and origins, SCCMEC subgroup, MIC to cloxacillin in broth (at pH 7.4 and 5.5)

Strain no.	phenotype	type of strain and origin	SCCMEC group [a]	MIC (mg/L)	
				pH 7.4	pH 5.5
ATCC25923	MSSA β-lactamase (-)	laboratory standard [b]	na	0.125	0.06
ATCC33591	HA-MRSA (inducible)	laboratory standard [b]	III	16	0.06
NRS100 (COL)	MRSA (constitutive) β-lactamase (-)	clinical (operation theater; historical) [c] ([9,29])	I	> 512	0.25
N4120032	HA-MRSA	clinical (wound infection) [d]	I	1	0.06
N4120210	HA-MRSA	clinical (urinary tract infection) [d]	nd	2	0.06
HMC546	HA-MRSA	clinical (endocarditis [aortic valve]) [e] ([17])	IVa	16	0.25
N4090440	CA-MRSA (PVL+)	clinical (wound infection) [d]	IVa	0.5-1	0.03
N4042228	CA-MRSA (PVL+)	clinical (septicemia secondary to soft tissue abscess) [d]	IVa	0.5	0.03
NRS192	CA-MRSA (PVL+)	clinical (pneumonia, septic arthritis) [c]	IVa	1	0.06
NRS18	HA-MRSA / VISA	clinical (wound, skin and soft-tissues infection) [c]	II	8	0.06
NRS126	HA-MRSA / VISA	clinical (bloodstream infection) [c]	II	16	0.06
VRS2	HA-MRSA / VRSA	clinical (wound, skin and soft-tissues infection) [c]	II	32	0.06
N7112046	animal MRSA	carriage (food-animal caregiver) [d]	nd	512	0.125

[a] detection of *mecA* by PCR and *SCCmec* by multiplex PCR
[b] from the American Tissue Culture Collection (Manassas, VA); [d] clinical collection (YG) ; [e] clinical collection (PA).
[c] from the Network on Antimicrobial Resistance in *Staphylococcus aureus* [NARSA] Program (operated by Eurofins Medinet, Inc., Hendon, VA; supported under NIAID/NIH Contract no. HHSN2722007 00055C); details on each is strain is available from

Cells, cell infection and assessment of intracellular activity of antibiotics.
Infection of unactivated THP-1 cells was performed exactly as described previously ([4,21,36]) and the same procedure was followed for activated THP-1. Infection of human skin keratinocytes and bronchial epithelial cell line was performed as for J774 macrophages ([36]), except that *S. aureus* internalization was allowed to take place for 2 h with an initial inoculum of $5x10^7$ to $1x10^8$ bacteria/mL ([26]). For both cell types, the post-phagocytosis inoculum was comprised between 1.5 to 3.0 x 10^6 CFU per mg of cell protein, a value close to that used for THP-1 macrophages.

Assay of cell-associated carbapenems (imipenem, meropenem) and isoxazoylpenicillins (oxacillin and cloxacillin). Cell lysates were prepared after 24 h incubation and used for microbiological assay with MSSA ATCC25923 following the general method previously described (see [4,5,22] for details). The cell antibiotic content was expressed as a function of the total sample protein content, and its concentration ratio over the extracellular concentration calculated based on a cell volume of 5 µl per mg protein.

Analysis of carbapenems and isoxazoylpenicillins stability at neutral and acid pH. Analyses were made by following the disappearance of the corresponding signal in HPLC upon incubation of the drugs in buffered media, using protocols previously used for testing the stability of ertapenem and meropenem in culture medium ([[22]; linear response over a 1-500 (R^2=0.999) and a 0.19-200 mg/L (R^2 = 0.999) range for imipenem and meropenem, respectively]) and with adaptations (mobile phase: 25 mM phosphate buffer pH 6.7 - acetonitrile 75:25 v/v) for oxacillin and cloxacillin (linear response over a 16-1,000 mg/L for both antibiotics [$R^2 \geq 0.999$]).

Statistical analyses. Curves-fitting analyses were made using GraphPad Prism® version 4.02 for Windows (GraphPad Prism Software, San Diego, CA. Analysis of variance (ANOVA) was made with GraphPad Instat® version 3.06 (GraphPad Prism Software).

RESULTS

Intracellular susceptibility of MRSA strains of clinical and epidemiological interest to cloxacillin. In a first series of experiments, we examined whether the suppression of methicillin resistance originally seen with the laboratory MRSA strain ATCC 33591 when growing in THP-1 macrophages could be generalized to other MRSA strains from defined epidemiological and clinical interest. For this purpose, THP-1 macrophages were infected with the different strains listed in Table 1. The intracellular growth of each strain measured after 24 h and in the absence of added cloxacillin (but in the presence of gentamicin [0.5 MIC] to prevent extracellular growth [[4]]) was essentially similar to that of ATCC 33591 (about 2 $\log_{10}$ cfu increase in 24 h; data not shown), indicating a similar level of permissivity. Experiments were then conducted in the presence of cloxacillin at increasing extracellular concentrations from about 1/100 to 100-fold its MIC (as measured in broth at pH 5.5) to analyze the full dose-response effect of the antibiotic. In all cases, typical concentration-dependent effects were seen on which a model based on the Hill equation could be fitted (this model and its pertinence to obtain a valid description of the pharmacological parameters governing intracellular antibiotic activity is discussed in ref. [[4]]). Statistical analysis of the individual dose-response curves, together with that of pertinent quantitative parameters for each strain (relative efficacy [E_{max}]; relative potency [EC_{50}]; apparent static concentration; see Supplementary Material) revealed no significant or only minor difference between strains (including MSSA ATCC 25923). All data were therefore pooled and global results, together with the corresponding Hill function are shown in Figure 1 (see caption for pertinent regression parameters). This shows that (i) a mean static effect was obtained for an external concentration of about 19-fold the MIC (measured at pH 5.5; corresponding to concentrations spanning roughly between 0.5 and 4 mg/L).; (ii) activity neared a maximum for an extracellular concentration of about 1,000-fold the MIC (30 to 250 mg/L); (iii) the maximal activity was reached about 0.7 log cfu decrease from the original inoculum whatever the strain examined (including MSSA ATCC 25923).

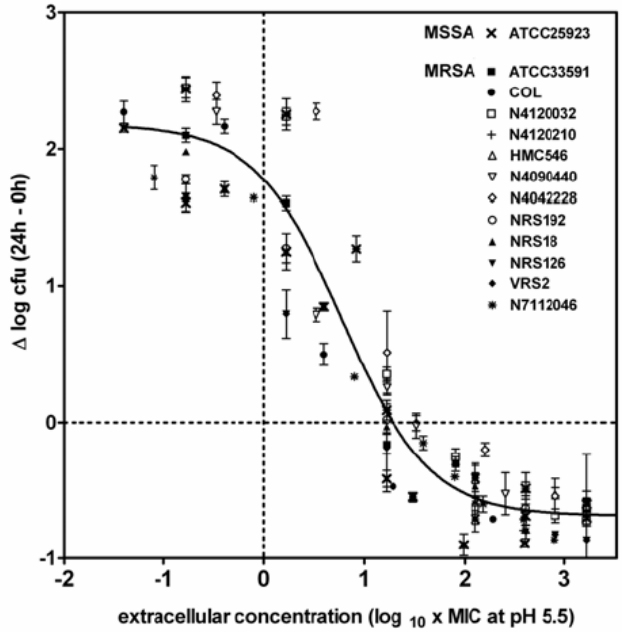

Figure 1: Global dose-response curve of cloxacillin against the intracellular forms of the 13 *S. aureus* strains described in Table 1 after phagocytosis by THP-1 macrophages (see Supplementary Material for dose responses for each strain). The ordinate shows the change in the number of cfu (means ± SD; n=3; several SD bars are smaller than the symbols) per mg of cell protein. The abscissa is the $\log_{10}$ of the cloxacillin extracellular concentration ($\log_{10}$ of the multiples of its MIC as measured towards the corresponding strain at pH 5.5; see Table 1). Data of all strains were used to fit one single sigmoidal function with an Hill coefficient of ([28]) and with the following parameters (with 95 % CI): E_{min} (change in bacterial counts over the original inoculum for an infinitely low cloxacillin concentration) = 2.18 $\log_{10}$ cfu (2.01 to 2.35); E_{max} (change in bacterial counts over the original inoculum for an infinitely large cloxacillin concentration) = -0.68 $\log_{10}$ cfu (-0.79 to -0.58); E_{50} (cloxacillin concentration in multiples of MIC measured at pH 5.5 for an change in bacterial counts half-way between E_{min} and E_{max}) = 6.09 (4.50 to 8.25); goodness of fit (R^2): 0.925. The apparent static concentration (no apparent change from the original inculum; dotted horizontal line) is ~ 19.1 x the MIC measured at pH 5.5. (see Supplementary Material for parameters observed with each strain).

Susceptibility of MRSA ATCC 33591 to cloxacillin after phagocytosis by unstimulated and stimulated macrophages, keratinocytes and bronchial epithelial cell lines.

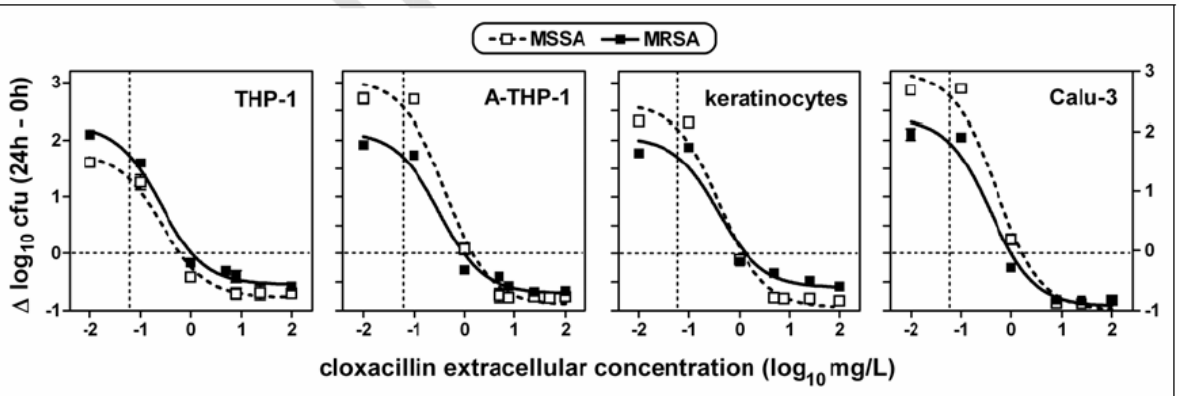

Figure 2: Dose-response curves of cloxacillin against the intracellular forms of *S. aureus* ATCC 25923 (MSSA; open symbols and dotted lines) and *S. aureus* ATCC 33591 (MRSA; closed symbols and continuous line) after phagocytosis by (from left to right) human unstimulated human THP-1 macrophages, human activated THP-1 macrophages, human skin keratinocytes, and human bronchial epithelial cells (Calu-3). The ordinate shows the change in the number of cfu per mg of cell protein (means ± SD; n=3; most SD bars are smaller than the symbols). The abscissa is the $\log_{10}$ of the cloxacillin extracellular concentration (in mg/L). The horizontal dotted line corresponds to an apparent static effect. The vertical dotted line corresponds to the MIC of cloxacillin towards the two strains used in these experiments when tested in broth at pH 5.5 (0.06 mg/L). All data were used to fit single sigmoidal functions as indicated in Figure 1. See Table 2 for goodness of fit and pertinent regression parameters.

This second series of experiments addressed the question as whether the results obtained with THP-1 macrophages were dependent on lack of activation of these cells, and whether results could be extended to other cells of interest in the context of persistent staphylococcoccal infections. Thus, we compared the susceptibility of the fully susceptible strain ATCC 25923 to that of the MRSA strain ATCC 33591 after phagocytosis by unstimulated vs. activated forms of THP-1 cells, and by human skin keratinocytes and human Calu-3 bronchial epithelial cells, using the same model (dose-response study at 24 h) as in the previous experiments. The results are shown graphically in Figure 2 with pertinent numerical and statistical data presented in Table 2.

Intracellular growth of MSSA in absence of antibiotic (as estimated by extrapolation or an infinitely low cloxacillin concentration) was more important after phagocytosis by activated THP-1 macrophages, keratinocytes and Calu-3 cells (in that order) than in unstimulated THP-1 macrophages. This was not the case for MRSA, which grew more extensively than MSSA in unstimulated THP-1 macrophages but less extensively in the 3 latter cell types. In spite of these differences in apparent growth, the pharmacological responses of both strains to cloxacillin (in terms of E_{50} and E_{max}) were nevertheless similar in the four types of cells. Of note is the fact that the static concentrations of cloxacillin never exceeded 0.6 to 1.6 mg/L, i.e. about 10 to 30-fold lower than its MIC at pH 7.4.

Table 2: Susceptibility of MSSA ATCC 25923 and MRSA ATCC 33591 to cloxacillin after phagocytosis by THP-1 macrophages, activated THP-1 macrophages (A-THP-1), keratinocytes, and Calu-3 cells. The parameters shown in the table are derived from the analysis of the data shown in Figure 2.

Conditions	MSSA [a]					MRSA [a]				
	E_{min} [b] (95 % CI)	E_{max} [c] (95 % CI)	EC_{50} [c] (95 % CI)	C_s [e]	R^2	E_{min} [b] (95 % CI)	E_{max} [c] (95 % CI)	EC_{50} [d] (95 % CI)	C_s [e]	R^2
THP-1	1.79 (1.23 to 2.35) a;A	-0.77 (-1.08 to -0.47) a;A	0.27 (0.09 to 0.82) a;A	0.6	0.987	2.27 (1.73 to 2.81) a;A*	-0.55 (-0.86 to -0.24) a;A*	0.26 (0.09 to 0.70) a;A	0.9	0.990
A-THP-1	3.05 (2.42 to 3.69) b;A	-0.90 (-1.24 to -0.56) a;A	0.43 (0.18 to 1.02) a;A	1.3	0.981	2.15 (1.52 to 2.79) a;B	-0.70 (-1.04 to -0.36) a;A*	0.32 (0.09 to 1.04) a;A	1.0	0.977
Keratino-cytes	2.64 (1.94 to 3.34) a*,b;A	-0.95 (-1.37 to -0.53) a;A	0.42 (0.14 to 1.23) a;A	1.1	0.980	2.06 (1.10 to 3.02) a;A	-0.60 (-1.22 to 0.02) a;A	0.39 (0.05 to 2.86) a;A	1.3	0.959
Calu-3	3.19 (2.28 to 4.11) b;A	-0.99 (-1.63 to -0.36) a;A	0.52 (0.15 to 1.77) a;A	1.6	0.983	2.39 (1.49 to 3.29) a;A*	-0.93 (-1.49 to -0.37) a;A	0.39 (0.08 to 1.71) a;A	1.0	0.977

[a] cloxacillin has the same MIC (0.06 mg/L) towards both strains when tested at pH 5.5 (see Table 1)
[b] cfu increase (in $\log_{10}$ units) at 24 h from the corresponding original inoculum, as extrapolated for a cloxacillin infinitely low concentration.
[c] cfu decrease (in $\log_{10}$ units) at 24 h from the corresponding original inoculum, as extrapolated for antibiotic concentration at infinitely high concentration.
[d] Concentration (mg/liter; total drug) causing a reduction of the inoculum halfway between the initial (E_0) and the maximal (E_{max}) values, as obtained from the Hill equation (by using a slope factor of 1).
[e] Concentration (mg/liter; total drug) resulting in no apparent bacterial growth (the number of CFU was identical to that of the original inoculum), as determined by graphical interpolation.
* Statistical analysis:
- global analysis of the Hill functions describing the dose-reponses (i) MSSA vs. MRSA for each cell type (unpaired t-test two-tailed; using all data points): no significant difference; comparison between cell types (one-way ANOVA with Tuckey test for multiple comparisons): no significant difference.
- analysis of parameters per column (on-way ANOVA with Tuckey test for multiple comparisons): figures with different lower case letters are significantly different from each other ($p < 0.01$; when the same letter has a $^+$, the difference between values is significant at p between 0.01 and 0.05)
- analysis of parameters per row (unpaired, two-tailed t-test between corresponding parameters for MSSA and MRSA: figures with different upper case letters are significantly different from each other ($p < 0.01$); when the same letter is with $^+$, the difference between values is significant at p between 0.01 and 0.05).

(statistical analysis of the E_{50} parameters was performed on the corresponding $\log_{10}$ values)

Susceptibility of MRSA ATCC 33591 to various β-lactams after phagocytosis by THP-1 macrophages. Our last series of experiments aimed at examining whether the restoration of susceptibility of phagocytized MRSA to cloxacilin (and meropenem [23]) could also be observed for other currently used registered anti-staphylococcal β-lactams. Figure 3 (left panel) shows changes in MIC observed for both MSSA ATCC 25923 and MRSA ATCC 33591 when tested at pH 5.5 vs. pH 7.4 against 4 β-lactamase-resistant penicillins, 5 cephalosporins, and 4 penems. Whereas only modest (about 2 dilutions) but constant decreases in MIC were noted with MSSA ATCC 25923, large reductions were observed for MRSA ATCC 33591. These, however, were much more variable between drugs. Thus, while values recorded at pH 5.5 reached 8 mg/L for cefotaxime, they were as low as 0.0156 mg/L for imipenem. Closer analysis of the results allowed us to observe that the mean specific change in susceptibility of MRSA ATCC 33591 (after discounting the effect seen for MSSA 259123 considered to a general effect unrelated the modulation of methicillin resistance [23]) was, generally speaking, lowest for cephalosporins (4.8 dilutions), intermediate for β-lactamase-resistant penicillins (6.75 dilutions), and highest for penems (8.25 dilutions).

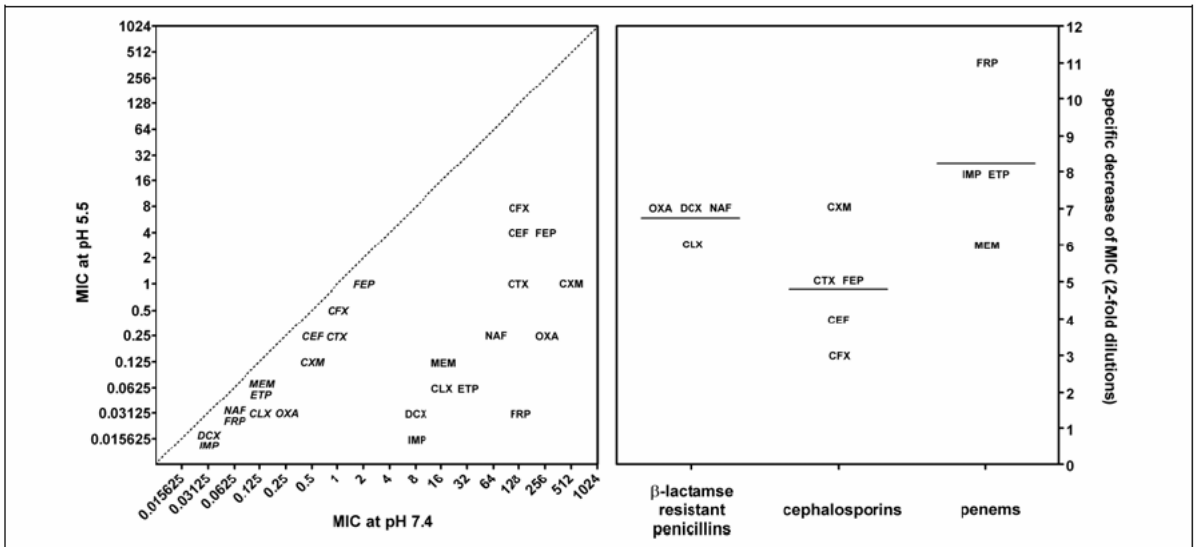

Figure 3: Influence of pH on the MICs of β-lactams for MSSA ATCC 25923 (italics) or MRSA ATCC 33591 (plain characters). OXA, oxacillin; CLX, cloxacillin; DCX, dicloxacillin; NFC, nafcillin; CFX, cephalexin; CEF, cefadroxil; CXM, cefuroxime; CTX, cefotaxime; FEP, cefepime; IMP, imipenem; MEM, meropenem; ETP, ertapenem; FRP, faropenem. Left panel: correlation between the values observed at pH7.4 and those observed at pH 5.5 for all antibiotics (the dotted line shows equivalence); when two antibiotics have the same MIC, they are placed on top of each other and aligned on their MIC at pH 7.4. Right panel: difference in MIC (expressed as Δ $\log_2$ [or 2-fold dilutions decreases]) for MRSA ATCC 33591 when tested at pH 5.5 vs. pH 7.4 after discounting the change observed for the corresponding antibiotic for MSSA 25923 upon the same change of pH. Data are broken down by antibiotic class. The horizontal bars are the corresponding means.

Based upon a similar relative level of suppression of methicillin resistance at acidic pH (reduction of MIC of about 7 $\log_2$ dilutions), 4 molecules (oxacillin, imipenem, cefuroxime, and cefepime) were selected, together with cloxacillin and meropenem, for in-depth pharmacological analysis of their activity against MRSA ATCC 33591 phagocytized by THP-1 macrophages. Results, normalized based on the MICs measured for each antibiotic at pH 5.5, are shown graphically in Figure 4. In all cases, a concentration-dependent similar to that already described was observed. Quantitative analysis showed that the dose responses obtained for MRSA with imipenem, meropenem, oxacillin, and cloxacillin were virtually indistinguishable from those obtained for the same antibiotic with MSSA. The relative potency (E_{50}) of imipenem towards both strains, however, was about 100-fold lower (in multiples of MIC) than that of meropenem (see also the differences in static concentrations). The situation was very different for cefuroxime and cefepime. First, there was a considerable separation of the dose-response curves between MSSA and MRSA (the latter being shifted towards larger concentrations), despite the fact that the data had been normalized based on MIC values measured at pH 5.5 (the difference would have been even larger if data had been expressed as a function of weight concentrations). This suggests that restoration of susceptibility of MRSA towards those two cephalosporins upon phagocytosis by THP-1 macrophages, was only partial. Second, close analysis revealed, at least for cefepime, that part of the difference was due to the fact that this antibiotic had a quite high relative potency (low EC_{50} values) towards MSSA (taking into account its MIC) compared to the other antibiotics. Third, and quite conspicuously, the relative

efficacies (E_{max}) of both cephalosporins, were significantly weaker than those of the penicillins and the penems, with cefuroxime being actually unable to achieve more than a strictly bacteriostatic effect.

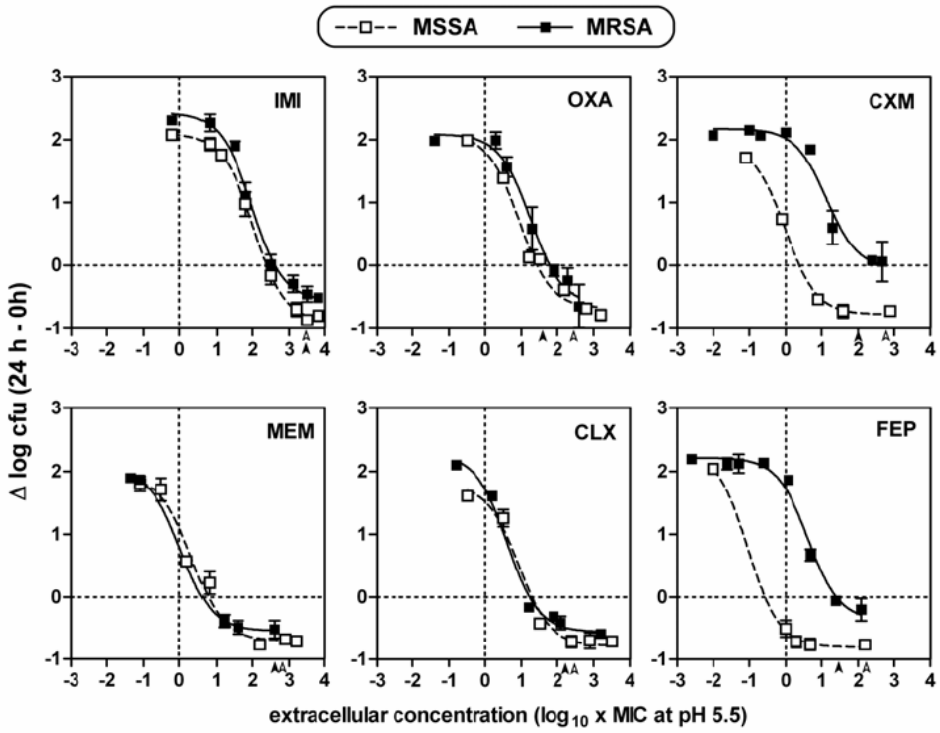

Figure 4. Dose-response curves of two penems (left panels: IMI, imipenem; MEM, meropenem;), two β-lactamase-resistant penicillins (middle panels: OXA, oxacillin; CLX, cloxacillin) and two cephalosporisn (CXM, cefuroxime;FEP, cefepime) against the intracellular forms of *S. aureus* ATCC 25923 (MSSA; open symbols and dotted lines) and *S. aureus* ATCC 33591 (MRSA; closed symbols and continuous line) after phagocytosis by human THP-1 macrophages. The ordinate shows the change in the number of cfu per mg of cell protein (means ± SD; n=3; most SD bars are smaller than the symbols). The horizontal dotted line corresponds to an apparent static effect. The abscissa is the log_{10} of the cloxacillin extracellular concentration (log_{10} of the multiples of its MIC as measured with the corresponding antibiotic and towards the corresponding strain (ARCC 25923 or ATCC 33591) at pH 5.5 [see Figure 3]). The open and closed arrows point to the concentrations corresponding t the reporterd serum C_{max} of the corresponding antibiotic when administered to humans at conventional doses (IMI, 52 mg/L; MEM, 50 mg/L; OXA, 10 mg/L; CLX, 8 mg/L; CXM, 100 mg/L; FEP, 130 mg/L). with the vertical dotted line corresponding to this MIC. All data were used to fit sigmoidal functions as described in Figure 2.

Stability and cell accumulation of carbapenems and isoxazolylpenicillins. Because the experiments shown above had disclosed significant differences in the dose-effects relationships between imipenem and meropenem, we compared the stability and cellular accumulation of these two antibiotics, using also isoxazolylpencillins as controls. Stability was assessed by measuring the amount of drug (initial concentration, 50 mg/L) remaining after 24 h incubation at 37°C in buffered media at pH 7.4 and 5.5 (mimicking the extracellular and intraphagosomal environments). The percentages of remaining imipenem were 39.3 ± 2.3 (pH 7.4) and 30.5 ± 2.1 (pH 5.5), and those of meropenem 49.3 ± 1.0 and 40.3 ± 0.9, vs. 94.2 ± 0.1 and 91.8 ± 0.1 for oxacillin and 97.8 ± 1.4 and 93.3 ± 0.2 for cloxacillin . We then measured the apparent cellular to extracellular concentration ratios of each antibiotic after 24 h incubation at an extracellular concentration of 250 mg/L (this high concentration was needed to obtain measurable cellular contents). Values were 0.05 ± 0.02, 0.20 ± 0.02, 0.20 ± 0.02, and 0.28 ± 0.01 for imipenem, meropenem, oxacillin and cloxacillin, respectively.

DISCUSSION

The present study expands on our recent observation demonstrating that MRSA recover susceptibility to β-lactam antibiotics when phagocytized by eukaryotic cells. The new findings presented here are based on an established approach aimed at obtaining a comprehensive description of the pharmacological properties of antibiotics against intracellular bacteria in terms of dose-effect relationships ([4]).

The first new finding is that the observations originally made with a laboratory strain of MRSA can be extended to several of MRSA isolates of clinical and epidemiological interest. These strains being from various, unrelated origins, we may reasonably assume that what is shown here is probably applicable to most MRSA strains, irrespective of their subgroup. Their common phenotypic character is the expression of PBP2a (also referred to in the literature as PBP 2' [[14]]). In association with the transglycosylase domain of the native PBP 2, the activity of which is not inhibited by β-lactams ([32]), PBP2a performs the critical cross-linking of the cell wall of the bacterium and, as such, serves a vital function for MRSA. We showed, using purified PBP2a, that acidic pH allows β-lactams to acylate this protein thanks to pH-induced conformational change (Lemaire *et al.*, submitted for publication). Increased interactions with other targets at acidic pH may also play an additive role ([21]). The present data provide the necessary background for further advanced studies by indentifying which β-lactams are more prone to cause such effects.

The second key finding is that the suppression of methicillin resistance in phagocytized MRSA is probably not cell-specific. This opens interesting perspectives for the assessment of such phenomenon *in vivo*, with special attention to tissues such as skin and bronchial epithelia where survival of intracellular *S. aureus* may play a critical role ([24,25]). Our conclusions, however, should be clearly limited to those cells in which *S. aureus* gains access and sojorn in acidic compartments. While this may be the case for many cells, including most professional phagocytes and epithelial cells such as those studied, this may not be the case for other cell types such as endothelial cells where *S. aureus* appears to access and thrive in the cytosol ([27]), thus remaining at a neutral pH. Studies combining subcellular localization approaches with an evaluation of the modulation of methicillin resistance would be rewarding in this context.

A third, but unanticipated finding from the present study is that the suppression of methicillin resistance in phagocytized bacteria seems not to be obtained at the same level by all β-lactams, with the two cephalosporins tested being obviously at a disadvantage in this context, even when compared after normalization for differences in MIC at acid pH. This, most likely, is related to the way cells handle these cephalosporins because pH seems to influence their activity largely to the same extent as for other β-lactams. Further studies (i) examining a larger number of molecules to obtain more comprehensive structure-effect relationship data, (ii) comparing the cell uptake and disposition properties of cephalosporins to those of penicillins and penems, (iii) and analyzing the behavior of the novel anti-MRSA cephalosporins could help clarifying the picture. The weaker relative potency (larger EC_{50}) of imipenem compared to meropenem and the isoxazolylpenicillins was also puzzling at first glance. It could not possibly be related to a difference in the restoration of susceptibility of MRSA to imipenem since the dose-response curves of MRSA and MSSA were both shifted to large value compared to meropenem or isoxazolylpenicllins, and indistinguishable from each other. A partial explanation of pharmacokinetic nature is probably the lower cellular accumulation of imipenem compared to meropenem and isoxazolylpenicillins, coupled with the slightly larger instability of imipenem compared to meropenem. The instability of carbapenems in aqueous media has already been largely documented ([39]). Our data suggest that more detailed studies aiming at defining how and to what extent intracellular degradation of carbapenems or other pharmacodynamic factors could decrease their potency against intracellular *S. aureus* are be warranted.

The biological and chemotherapeutic significance of the present observations also deserve attention. Restoration of the susceptibility of MRSA to β-lactams by acidic pH was discovered in the early 1970s ([35]) but was not considered of clinical importance by its discoverers. However, *S. aureus* show a high tolerance to pH variations allowing its survival within acidic habitats ([41]). This survival is probably a key determinant in the relapsing and recurrent character of many staphylococcal infections. It is, therefore, somewhat ironic, that making a diagnostic of MRSA will invariably lead the clinician to discontinue β-lactams in favor of vancomycin or linezolid, two antibiotics which know to be poorly effective against intracellular forms of *S. aureus* ([3,4],Lemaire *et al.*, submitted for publication).

While this could call for recommending β-lactams for treating intracellular MRSA infections, our findings, and the model used, have obvious limits. First, the intraphagocytic activity of β-lactams against *S. aureus* remains quantitatively limited in terms of maximal effect (E_{max} of about 1 $\log_{10}$ cfu at most) in comparison with what can be obtained with fluoroquinolones or the novel lipoglycopeptides (2-3 $\log_{10}$ cfu; [3,4]). Thus, β-lactams would by no means qualify as bactericidal towards intracellular *S. aureus*, according to the general definition of a bactericidal effect proposed by the US National Committee for Clinical Laboratory Standards (3 $\log_{10}$ cfu decrease [30]). Yet, what we see with β-lactams is, at least, as important as with other drugs recommended against multiresistant bacteria such as linezolid ([4]). The data, however, suggest that fairly large concentrations β-lactams, maintained for prolonged periods of time, will be needed to obtain a maximal effect. β-lactams, indeed, act only slowly on intracellular *S. aureus* ([22]). Second, our study was limited to a single 24 h time point, used constant antibiotic concentrations, and did not allow to model human protein binding effects (see discussion in [3,22]). These limitations are largely imposed by experimental constraints and will need to be addressed in future experiments. Third, conventional β-lactams will, obviously, be ineffective against the extracellular forms of MRSA. Their use to fight intracellular MRSA should, therefore, be always combined with that of other agents acting against these extracellular forms. Carefully designed *in vitro* and *in vivo* studies examining this new type of combination therapy to control MRSA infection could be useful in this context.

Acknowledgements

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Publication #4: Restoration of susceptibility of Methicillin-Resistant *Staphylococcus aureus* (MRSA) to β-lactam antibiotics by acidic pH: role of Penicillin-Binding Protein 2a (PBP2a).

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The understanding of the molecular and biochemical mechanisms underlying this restoration of susceptibility of MRSA to β-lactams is of profound significance because it would, indeed, not only explain the susceptibility of MRSA isolates within the subcellular environnements, but it might also reveal some potential new targets in the treatment of this versatile pathogen.

In this report, we have examined in greater depth the relationship between pH and the function of PBP2a (the key factor involved in methicillin resistance). We document herein that, at lower pH, (i) PBP2a bind more avidly β-lactams; (ii) the non-covalent pre-acylation complex exhibits a lower dissociation constant (K_d) and an increased rate of acyl-enzyme formation (k_2) without change in hydrolytic deacylation rate (k_3); and (iii) PBP2a undergoes a conformational change (which is a crucial step for the opening of the active site from the closed conformation) in the presence of oxacillin when exposed under acidic conditions. In terms of mechanistic consequences, these variations were quite similar than those recently reported for new cephalosporins that are capable of inhibiting more efficiently the resistant PBP2a. Hence, these findings provide new insights in the understanding of the modification of methicillin resistance by acidic pH.

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a global scourge, and treatment options are becoming limited. The MRSA phenotype reverts to that of β-lactam sensitive *S. aureus* when bacteria are grown at pH 5.0 in broth and, more importantly in a medical perspective (protracted, relapsing infections), after phagocytosis by macrophages where the bacteria thrive in the acidic environment of phagolysosomes. The central factor for the MRSA phenotype is the function of PBP 2a, a unique transpeptidase that is poorly inhibited by β-lactam antibiotics because of a closed conformation of its active site. We document herein by binding studies to the purified PBP 2a, by acylation/deacylation kinetics, and by circular dichroism spectroscopy that at lower pH (i) PBP 2a binds β-lactams more avidly; (ii) the non-covalent pre-acylation complex exhibits a lower dissociation constant and an increased rate of acyl-enzyme formation (first-order rate constant) without change in hydrolytic deacylation rate; (iii) PBP 2a undergoes a conformational change in the presence of β-lactam consistent with the opening of the active site from the closed conformation. These observations argue that PBP 2a most likely evolved for its physiological function at pH 7 or higher by adopting a closed conformation, which is not maintained at acidic pH. Our report should provide the impetus for closer examination of the properties of PBP 2a at low pH, since these may provide useful hints as how to make β-lactams acting towards MRSA in other environments, and thereby, identifying novel points of intervention in combating this problematic organism.

INTRODUCTION

Methicillin-resistant *S. aureus* (MRSA)[2] is a difficult bacterial pathogen to treat, which is presently a clinical scourge. This organism is resistant to all commercially available β-lactams antibiotics, which are currently mainstays of treatment of bacterial infections. MRSA is a global problem in hospitals, in nursing homes and in communities (1-5). It has now also been found frequent in pets (6) or food animals (7-9), becoming a potential issue for those who come in close contact with these animals (10;11). MRSA represents also a therapeutic challenge because effective therapeutic options are becoming limited. Vancomycin, the antibiotic most commonly recommended for treating infections caused by the hospital-acquired strains of MRSA, is, indeed, increasingly compromised by the spreading of variants with reduced susceptibility (VISA isolates) and, at some places, the emergence of full resistant organisms (12). Novel antibiotics with approved indications for MRSA infections such as linezolid (an oxazolidinone), daptomycin (a lipopetide), or tigecycline (a modified tetracycline) are not devoid of significant and sometimes treatment-limiting side effects (13). Furthermore, resistance to at least the first two in MRSA has already been noted as well (14).

The major mechanism of resistance of MRSA to β-lactam antibiotics is due to the acquisition of the *mecA* gene encoding an additional penicillin-binding protein (PBP 2a [15]; also referred to in the literature as PBP 2' [16]) that can function as a transpeptidase. In the face of the challenge by β-lactam antibiotics, the other staphylococcal transpeptidases are inhibited, but PBP 2a retains its activity. In association with the transglycosylase domain of the native PBP2, the activity of which is not inhibited by β-lactams (17), PBP 2a performs the critical cross-linking of the bacterium and as such serves a vital function for the organism. Understanding of the properties of PBP 2a is at the forefront of restoring susceptibility to β-lactams, which is an important aim (18). Efforts toward this goal largely encompass work in the direction of discovery of novel β-lactams that inhibit both PBP 2a and other transpeptidases (19). It is of note, however, that susceptibility of MRSA to conventional β-lactams can be almost fully restored if bacteria are grown at pH of 5.5 or lower. This phenomenon was originally described in the early 1970s for hospital-acquired MRSA growing in broth and exposed to β-lactamase-resistant penicillins or first-generation cephalosporins (20). This observation has now been confirmed for other β-lactams, including carbapenems, and applies not only to hospital- but also community-acquired MRSA (21). It has also been extended to MRSA growing intracellularly in phagolysosomes of macrophages (where pH is in the acidic range [22]), giving it a broader significance than originally appreciated. This influence of acidic pH was first ascribed to a diminished copy numbers of PBP 2a (15). We showed, however, that growing bacteria at acidic pH (i) alters neither the expression of the PBP 2a-encoding gene (*mecA*) nor that of its regulatory genes; (ii) does not decrease the amount of the immunodectectable PBP 2a in whole bacteria (21). We also found that MRSA grown and exposed to [^{3}H]penicillin G at acidic pH show a larger retention of radioactivity than if

bacteria had been grown at neutral pH (this amount being actually brought to a level similar to what is observed with MSSA grown and exposed to [^{3}H]penicillin G at neutral pH) (21).

An in-depth understanding of the molecular mechanism underlying restoration of susceptibility of MRSA to β-lactams by acidic pH is of profound significance. It would, indeed, not only explain the antibiotic susceptibility of the organism within phagolysosomes, and other habitats where pH is low, but it might also reveal avenues of intervention in treatment of this problematic bacterium. In this report, we have examined the relationship between pH and the function of PBP 2a. Our data show that acidic pH causes a significant change in conformational behavior of PBP 2a, rendering it more susceptible to β-lactams. In terms of mechanistic consequence, we assert that these observations are quite similar to those recently reported for new cephalosporins that are capable of inhibiting PBP 2a effectively, arguing for an underlying principal that governs both cases (23-25).

EXPERIMENTAL PROCEDURES

Strains and susceptibilities — A methicillin-sensitive β-lactamase-negative *S. aureus* (ATCC 25923; American Type Culture Collection, Manassas, VA), a methicillin-resistant β-lactamase-negative *S. aureus* (COL [referenced as NRS100; Network on Antimicrobial Resistance in *Staphylococcus aureus*, Focus technologies, Inc., Herndon, VA), and a methicillin-resistant β-lactamase-positive *S. aureus* (ATCC 33591) were used thorough these studies. MICs were measured by microdilution (log_2 progression) in Muller Hinton broth supplemented by 2% NaCl for MRSA (US Clinical Laboratory Standards Institute [CLSI], Wayne, PA), and adjusted to appropriate pH by careful addition of 1 N HCl (we checked in previous work (21) that the final pH of the broths at the end of the 24 h incubation period was close to the original one at all antibiotic concentrations equal to the MIC or above).

Binding of Bocillin FL to whole cells — Bocillin FL is a fluorescent derivative of penicillin V (26; see also Supplementary material) that has been validated for PBP binding studies (27). We used it here to examine the penicillin-binding properties of bacteria growing at acidic *vs*. neutral pH, following a procedure used previously to study the binding of radiolabelled penicillin G (21;28). A 10 mL portion of growing bacteria (5 h culture; OD_{660nm}: approx. 0.5) were exposed to Bocillin FL (25 µg) for 30 min at pH 7.4 or 5.5. Bacteria were then collected by centrifugation, washed 4 times with phosphate buffered saline, and lysed by three successive freeze-thawing cycles (5 min at -80 °C followed by 5 min at 37 °C). The amount of fluorescent material was measured in a Packard Fluorocount Microplate Reader instrument (Perkin Elmer Life and Analytical Science, Inc, Waltham, MA) using excitation and emission wavelengths set at 485 and 530 nm, respectively, with linearity obtained between 0.002 and 2.5 mg/L [$R^2 > 0.998$]), and expressed by reference to the sample protein content.

Cloning, purification and determination of the solubility of PBP 2a — A general procedure for protein purification was used as described earlier (29). The solubility of the protein was assessed as described (23), except that the protein was incubated for up to 72 h in 25 mM Hepes (pH 7.0) or in 25 mM phosphate buffer (pH 5.5), both supplemented with 1 M NaCl. The results for each condition of pH indicated that the protein remained fully soluble for at least up to 3 days.

Bocillin FL - PBP 2a binding assay — We adapted a previously described method (30) but using Bocillin FL as a reporter antibiotic. The purified protein was firstly diluted for 10 min in pH-adjusted buffer containing 1 M NaCl (pH 7.0, 25 mM HEPES buffer; pH 5.0 to 7.0, 25 mM phosphate buffer; we checked that the results obtained at pH 7.0 were not influenced by the buffer used) and then incubated with Bocillin FL for various time intervals. Aliquots were collected, proteins separated by SDS-PAGE electrophoresis, and the acyl-enzyme complex detected by fluorescence (Storm 840® Fluorimager, Amersham Biosciences, Little Chalfont, Buckinghamshire, England). Results were expressed as relative emission intensities. Equiloading of the gels was checked in all experiments by Coomassie blue staining.

Determination of the Kinetic Parameters for Interactions of nitrocefin with PBP 2a — Acylation and deacylation studies (using nitrocefin for acylation and Bocillin FL for deacylation as reporter molecules, respectively) were performed as described earlier (29).

Circular Dichroism — CD spectra of PBP 2a were recorded on a stopped-flow circular dichroism spectrometer (Aviv model 202 SF, Aviv Biomedical Inc., Lakewood, N.J.) with a 2-mm path length at 25°C in the absence or presence of 30 μM oxacillin (23).

Materials — Penicillin G, penicillin V, oxacillin, and nitrocefin were purchased from Sigma-Aldrich, St-Louis, MO, and Bocillin FL from Invitrogen Corp., Carlsbad, CA (the structures of these compounds are shown in the Supplemental Material). Unless stated otherwise, all other reagents were obtained from Merck AG, Darmstadt, Germany or from Sigma Aldrich.

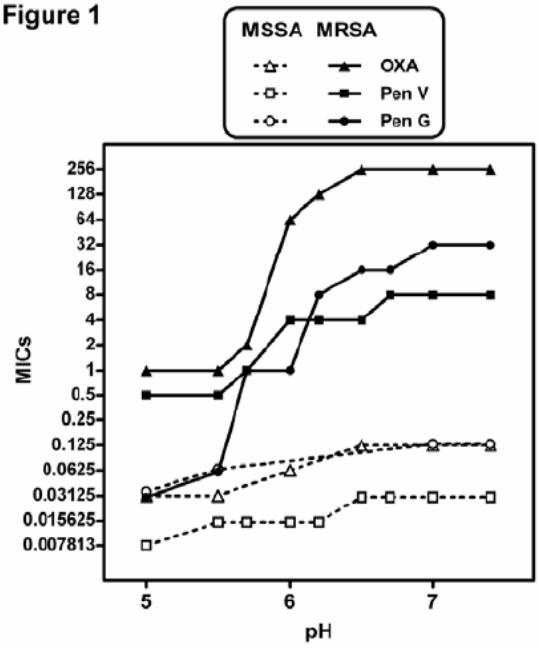
Statistical analyses — Curve-fitting analyses were made using GraphPad Prism® version 4.03 for Windows (GraphPad Prism Software, San Diego, CA). The significance of the differences between paired values was tested by Student's *t* test (Excel 2000; Microsoft, Inc., Bellevue, WA).

RESULTS

MRSA is known to produce PBP 2a in manifesting resistance to β-lactam antibiotics upon exposure to the antibiotic. The induction mechanism of PBP2a, as well as that of β-lactamase (which was the first main mechanism of resistance of *S. aureus* towards early penicillins, but was successfully coped with by the synthesis of β-lactamase-resistant penicillins and cephalosporins, and β-lactamase inhibitors) has been reviewed by Fuda *et al*. (18) and will not be discussed here in the interest of brevity.

In studying the mechanism of β-lactam resistance by MRSA, we already have reported that acidic pH markedly decreases the MIC of cloxacillin (a β-lactamase-resistant penicillin) and meropenem (a carbapenem) for the β-lactamase-positive MRSA strain ATCC 3591 (21). In the context of the present study, we repeated these experiments with the β-lactamase-negative MRSA COL strain in comparison with the β-lactamase-negative MSSA strain ATCC 25923, using penicillin G (also known as benzylpenicillin, selected as reference β-lactam), penicillin V (Bocillin FL used for our binding and kinetic studies is a fluorogenic derivative of penicillin V [26;27]), and oxacillin (a β-lactamase-resistant penicillin recommended for the treatment of infections caused by MSSA and also used by us in subsequent studies).

Figure 1



Influence of pH on the MIC of penicillin G (Pen G, circles), penicillin V (Pen V, squares) and oxacillin (OXA, triangles) towards MSSA ATCC 25923 (open symbols) and MRSA COL (closed symbols) strains.

The results presented in Figure 1 reveal that, as a general trend, decreasing the pH from 7.4 to 5.0 lowered the MICs for all three antibiotics. The results were quite dramatic for penicillin G, with MICs towards the MRSA COL strain experiencing attenuation of 11 $\log_2$ dilutions, reaching values identical to those observed for this antibiotic when tested against the MSSA ATCC 25923 strain (for which acidic pH caused only a modest decrease [1 $\log_2$ dilution] compared to neutral pH). For oxacillin, the decrease in MICs for the MRSA COL strain reached 9 $\log_2$ dilutions for the same change of pH. The decrease in MICs for penicillin V for the MRSA COL strain was less marked, but still quite significant (4 $\log_2$ dilutions). The MICs of oxacillin and penicillin V at pH 5 were similar (0.25-1 mg/L) but about 4-5 $\log_2$ dilutions higher than those of penicillin G. Thus, the restoration of absolute susceptibility of the MRSA COL strain to oxacillin and penicillin V was less marked than for penicillin G, a point that will need to be taken into account for correct interpretation of the results of our subsequent studies.

We then compared the influence of pH on total penicillin-binding properties of growing MRSA (COL strain) and MSSA (ATCC 25923 strain) by exposing them to Bocillin FL in broth, and measuring thereafter the amount of bocillin FL associated with cell extracts. Figure 2 (upper panel) shows that the binding of Bocillin FL was considerably lower for MRSA in comparison to MSSA when bacteria had been grown and binding performed at pH 7. Binding to MRSA was increased about 50-fold when the experiment was performed at pH 5.5. Most interestingly, no significant difference was noted between MSSA and MRSA for bacteria grown and tested at that pH.

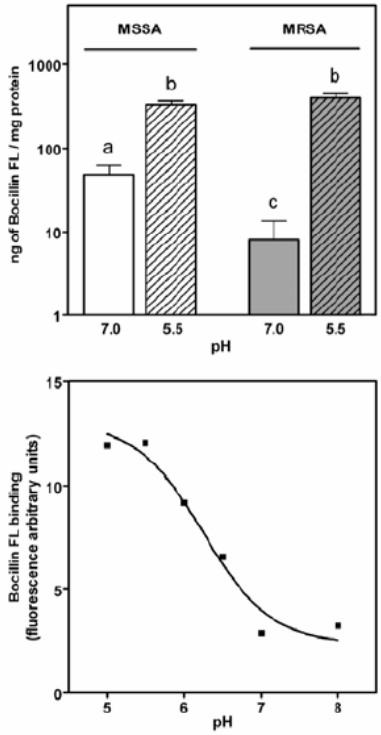


Fig. 2. Influence of pH on the binding of Bocillin FL to whole cells and to purified PBP 2a. Upper panel: growing bacteria were incubated for 30 min in broth at 37°C with Bocillin FL at the pH indicated in the abscissa, and the samples were prepared for fluorescence measurement. White bars, MSSA ATCC 25923; grey bars, MRSA COL. Values are means ± SD (n = 3). Statistical analysis, bars with different letters are significantly different from all others ($p < 0.01$). Lower panel: Bocillin FL (0.2 µg) was mixed with 3 µM purified PBP 2a in 50 mM phosphate buffers adjusted to different pH values (the value recorded in HEPES buffer at pH 7.0 was not significantly different from that shown for the corresponding phosphate buffer here).

These experiments prompted us to directly measure the binding of Bocillin FL to purified PBP 2a. Figure 2 (lower panel) shows that binding was pH dependent with values about 5-fold larger at pH 5 compared to pH 7. This indicates that the covalent chemistry that PBP 2a experiences, and, by necessity, the access to the active site by the antibiotic, is enhanced at lower pH. In subsequent experiments, the influence of pH on binding of Bocillin FL to PBP 2a was further characterized with respect to concentration and time. Results presented in Figure 3 show (i) that more Bocillin FL could

bind to PBP 2a at low concentrations at pH 5.5 compared to pH 7.0 (left panel), denoting potentially a higher affinity; and (ii) that acidic pH made this binding to take place at a faster rate and allowed the process to reach higher saturation levels, if extrapolated to an infinite time (right panel).

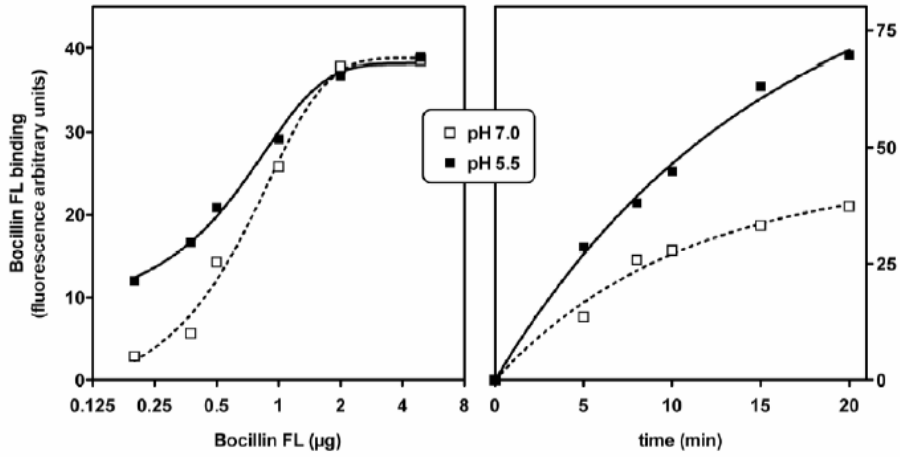


Fig. 3. Influence of pH on the binding of Bocillin FL to purified PBP 2a. Incubations were performed at room temperature in 1 M NaCl buffered at 5.5 (25 mM phosphate) or at 7.0 (25 mM HEPES) with PBP 2a (3 µM). Left panel: binding measured after 25 min in the presence of increasing amounts of Bocillin FL (regression parameters [Hill equation] for pH 5.5 and pH 7.0, respectively: R^2 = 0.999 and 0.991; EC_{50} = 0.15 and 2.64 µg). Right panel: binding measured after increasing incubation times in the presence of 0.5 µg of Bocillin FL (regression parameters [one phase exponential association] for pH 5.5 and 7.0, respectively: R^2 = 0.995 and 0.983; half-time to apparent saturation: 10.5 min [CI: 7.6-17.8] and 7.4 [CI: 4.6-18.7]; y_{max} = 96.7 and 44.6 fluorescence arbitrary units).

The kinetics of PBP 2a acylation and deacylation by β-lactams at neutral and acidic pH were then undertaken using nitrocefin and Bocillin FL as reporter molecules for acylation and deacylation, respectively. We took advantage of the distinctive change in the absorption spectrum of nitrocefin upon cleavage of the amide bond in the β-lactam ring by enzyme acylation, and the capacity of Bocillin FL to bind PBP 2a as the acyl-enzyme species experienced deacylation (see [29] for experimental design). We first investigated nitrocefin for the purpose of this work by measuring its MIC towards the MRSA COL strain at pH 5.5 vs. 7.4 and observed values of 0.25 and 2 mg/L, respectively. Subsequently, we determined certain kinetic parameters for interactions of the antibiotic with PBP 2a (Table 1). The minimal interaction scheme assumes binding of nitrocefin (N) to the enzyme in an initial non-covalent complex (EN), which proceeds to acylate the active site serine of PBP 2a, to give rise to the covalent complex E-N (Table 1). This covalent complex enjoys substantial longevity, but nonetheless ultimately experiences hydrolytic deacylation to liberate PBP 2a from inhibition. Table 1 shows the pertinent dissociation constant (K_d) of the nitrocefin-PBP 2a non-covalent complex (EN), the PBP 2a acylation rate constant (k_2) to yield the acyl-enzyme species (E-N), and the deacylation rate constant from E-N (k_3). It appears that at the acidic pH the dissociation constant for the pre-acylation complex was lowered by twofold and the rate constant for enzyme acylation was enhanced by somewhat more than the same magnitude. We hasten to add that PBP 2a does not have the catalytic machinery for the deacylation of the acyl-enzyme species and this is the reason that once the acyl-enzyme species has formed, it enjoys substantial longevity. As such, we note that the values for the deacylation rate constants at the two pH values remain unchanged within the error limits recorded for the determinations. This is exactly as would have been expected. As a result, the apparent second-order rate constant for the process between PBP 2a and nitrocefin (k_2/K_d), a measure of the overall efficiency of PBP 2a inhibition by the β-lactam, experienced an increased of 5-fold in acidic pH, in agreement with the change in MIC.

TABLE 1. Kinetic parameters for interactions of nitrocefin and PBP 2a at pH 5.0 and 7.0		
Parameter[a]	pH	
	7.0	5.5
K_d (μM)	195 ± 28	100 ± 30
k_2 (s^{-1} x 10^3)	6.0 ± 0.6	15.2 ± 1.5
k_3 (s^{-1} x 10^6)	2.5 ± 0.2	2.8 ± 0.2
k_2/K_d (s^{-1} M^{-1})	31 ± 3	150 ± 14

where E is the PBP 2a, N is nitrocefin, EN the non-covalent complex between PBP 2a and nitrocefin, E-N the covalent complex between PBP 2a and nitrocefin (acyl-enzyme), P the product of hydrolysis of nitrocefin, K_d the dissociation constant for the non-covalent complex EN, k_2 the acylation rate constant, k_3 the deacylation constant, and k_2/K_d the second-order rate constant for the bimolecular encounter between nitrocefin and PBP 2a.

The results reported above argue a general improved access by the antibiotic to the active site. This is manifested in lowering of the dissociation constant for the pre-acylation complex, which indicates a more favorable formation of the complex at the lower pH. In addition, we note a more favorable process for the acylation of the enzyme. These observations are telling in view of the available X-ray structure for PBP 2a in the native state and in complex with two antibiotics, including nitrocefin (31). The existing structural information reveals PBP 2a to exist in a state that is best described as a closed conformation for the active site for both the native and the acyl-enzyme species (29). This closed active site is the reason that the existing β-lactam antibiotic cannot inhibit the enzyme. Yet, we know that the enzyme is catalytically competent in cross-linking of the cell wall. Previous work disclosed that PBP 2a has an allosteric site for its substrate, the cell wall (32). Binding of the cell wall at this allosteric site triggers a conformational change in the protein that leads to the opening of the active site and access for the substrate to it. Fuda *et al*. have reported that for β-lactam antibiotics to gain access to the active site—given ample time and favorable establishment of equilibria—the protein has to undergo a conformational change to open the active site before the antibiotic has the ability to acylate the serine (29). Hence, conformational changes are necessary both for the physiological function of the enzyme in cross-linking of the cell wall and for its inhibition by β-lactams.

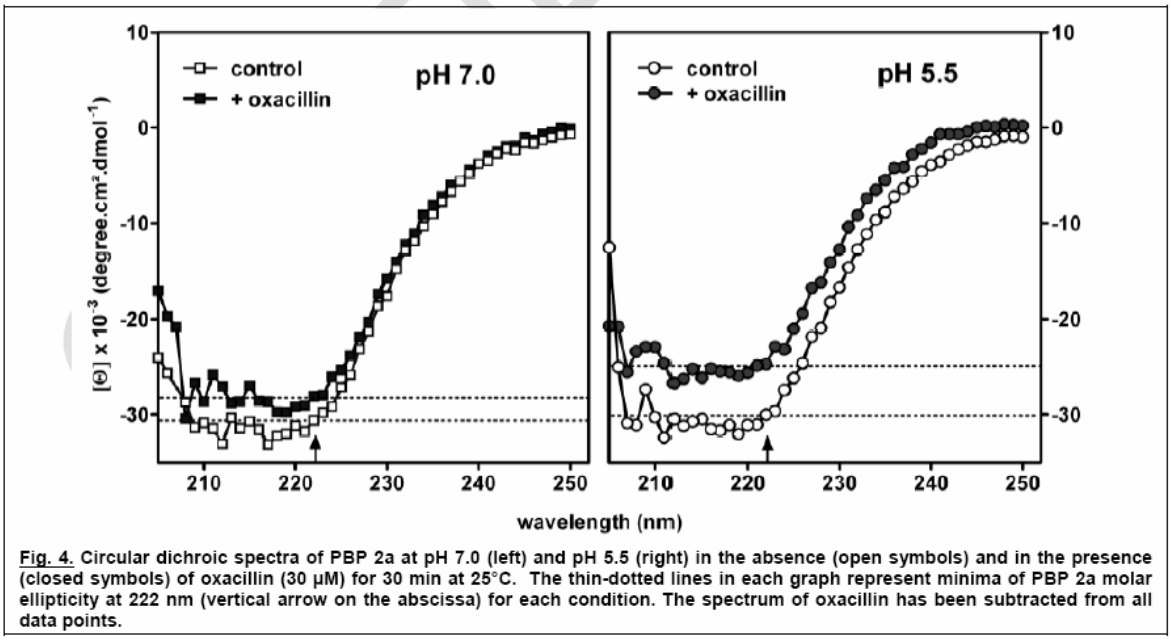


Fig. 4. Circular dichroic spectra of PBP 2a at pH 7.0 (left) and pH 5.5 (right) in the absence (open symbols) and in the presence (closed symbols) of oxacillin (30 μM) for 30 min at 25°C. The thin-dotted lines in each graph represent minima of PBP 2a molar ellipticity at 222 nm (vertical arrow on the abscissa) for each condition. The spectrum of oxacillin has been subtracted from all data points.

Edified by this information, we wondered whether the pH of the medium played a role in the conformational changes of PBP 2a in the presence of the antibiotic. This possibility was explored using circular dichroism (CD) spectroscopy of the protein in the presence and in the absence of

oxacillin. As revealed in Figure 4, the spectra of PBP 2a at pH 7.0 and 5.5 were virtually the same in the absence of antibiotic. On exposure of PBP 2a to oxacillin, however, we noted a change in the conformation of the protein, as was reported in an earlier study (29). Whereas this conformational flexibility was seen at both pH values, the magnitude of the effect was larger at pH 5.5. Moreover, Figure 5 shows that the change (as detected by the decrease in ellipticity at both 208 and 222 nm) occurred within minutes of exposure to oxacillin at pH 5.5, with the new value remaining quite stable for at least 3 h. In contrast, this change developed only slowly at pH 7.0.

Figure 5

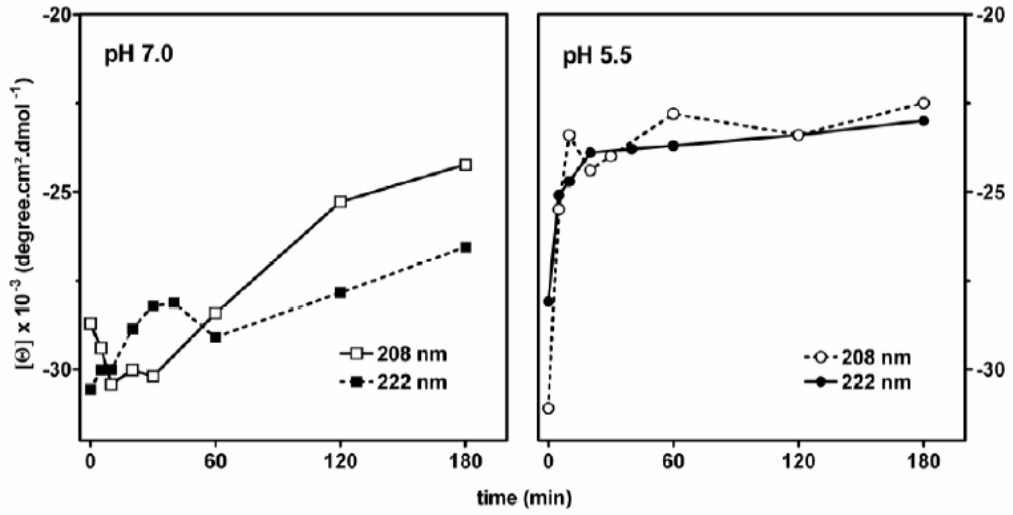


Fig. 5. Change in PBP 2a molar ellipticity observed over time at pH 7.0 (left panel) or pH 5.5 (right panel) upon exposure to oxacillin and recorded at 208 (open symbols) or 222 nm (closed symbols). Reactions were carried out with 30 μM oxacillin at 25°C. The spectrum of oxacillin has been subtracted from all data points.

DISCUSSION

The importance of MRSA as a human pathogen has been of considerable significance since the mid 90s and has become only more significant in the last years. A recent report states that, the incidence rate of invasive MRSA in 2005 in the US was 31.8 per 100,000, with considerably higher values for elderly, resulting in a mortality rate of 6.3 per 100,000 (5). It is, therefore, a major public health problem. Considering that this bacterial pathogen takes its toll at a global scale and exacts a considerable societal cost (33), it is clearly one of the most important pathogens presently. As reviewed recently (18), MRSA has exhibited a most remarkable ability in expanding its genome and in enhancing its ability to counter antibacterial agents. This factor has single-handedly been at the root of the emergence of this pathogen as a leading clinical scourge.

It is imperative that we elucidate the biochemical means to resistance of MRSA to antibiotics in order to rationally devise strategies to counter this organism. The methicillin-resistant phenotype of *S. aureus* is arguably one of the most important subjects for study, because the resistance profile is so broad that it includes all β-lactam antibiotics that are clinically available today. Up to now, β-lactams have remained the most important and generally accepted class of antibiotics, being cited as first line drugs in many types of infections (34). Hence, it bodes poorly for humanity that this entire class of antibiotics has been eliminated as treatment option for infections by MRSA.

The present study was undertaken based on the initial observation that MRSA becomes as susceptible as MSSA to cloxacillin (a β-lactamase-resistant penicillin) or meropenem (a carbapenem) after phagocytosis by human THP-1 macrophages (21). This unanticipated observation could be explained by the fact that *S. aureus* survives after phagocytosis by macrophages and indeed thrives in phagolysosomes. In the phagolysosomal environment bacteria are exposed to acidic pH, and neutralization of this pH abolishes this effect (21). This observation with intraphagocytic MRSA could,

therefore, be related to the long known effect of acidic pH on the restoration of the susceptibility of MRSA towards β-lactams, when grown in broth (20). The influence of acidic pH on MRSA susceptibility may have a broad significance since (i) it has now been reported for several other β-lactams in macrophages and in other models of intracellular infection (Lemaire *et al*., in preparation); and (ii) survival of *S. aureus* in phagocytic cells has been suggested to be a main cause of the relapsing and recurrent character of many staphylococcal infections (35;36). Ironically, establishing that MRSA is the offending organism of the infection that the physician attempts to treat leads to the discontinuation of β-lactams in favor of vancomycin (a glycopeptide) or linezolid (an oxazolidinone) (34). Yet, we know, today, that both antibiotics are poorly effective against intracellular forms of *S. aureus*, not only with laboratory or reference strains (37;38), but also and perhaps more importantly, with multi-resistant isolates as seen in persistent infections (Lemaire *et al*., submitted for publication). Understanding how β-lactams could exert activity against MRSA under these conditions could, therefore, help in implementing therapies aimed at eradicating these intracellular forms. As we shall see, there would also appear to be a degree of commonality with the mechanistic attributes of the processes in acidic pH towards MRSA and that of the new cephalosporins active against MRSA at neutral pH.

Restoration of activity of β-lactams towards MRSA at acidic pH was successively ascribed to two potential reasons. First, to a reduced or even outright absence of the expression of PBP 2a (15), which, as described earlier, is poorly inhibited by β-lactams (39) and provides the transpeptidase activity when other PBPs are inhibited by β-lactams. Second, was a proposal for the lack of the transpeptidase activity of PBP 2a at the lower pH (21). The first possibility was dismissed by the measurement of the expression of *mecA* and by the immunodetection of the encoded protein, both of which were unaffected by the acidic pH (21). While the second hypothesis cannot be ruled out, the data presented in this report offer an alternative, yet more plausible explanation. We proposed that under the acidic pH the active site of PBP 2a is more accessible to β-lactams antibiotics. The active site of this PBP is, indeed, sheltered under normal conditions, which is the reason for the lack of effective inhibition of the enzyme by conventional β-lactams. Improved accessibility at lower pH was documented in this study by the effect of the pH on the protein conformation, by the attenuation of the dissociation constant for the antibiotic and by the enhanced rate of enzyme for acylation in the presence of the antibiotic. If PBP 2a shows facilitated acylation by β-lactams at acidic pH, we need, however, to explain why the protein could not be detected by autoradiographic analysis of PBPs prepared from bacteria grown at acidic pH in the presence of [^{3}H]-benzylpenicillin (15). An ongoing work suggests that PBP 2a could be unstable under the conditions used for sample preparation and, therefore, escapes detection. This is why we examined the binding of Bocillin FL not only to growing bacteria (where it could bind to all PBPs), but also to purified PBP 2a. The increase in Bocillin FL binding when moving from neutral to acidic pH in the latter condition matches nicely with the decrease in MICs of penicillin V under a similar pH shift.

The magnitude of the lowering of the dissociation constant and the enhancement of the rate of acylation upon exposure of PBP 2a to nitrocefin at acidic *vs.* neutral pH might seem modest at first glance. However, analysis of the β-lactam resistant Streptococcal PBP2x in comparison with the natural, sensitive PBP2 and of a reverse, sensitive mutant (Sp328 PBP2x) has shown that switching from resistance to susceptibility and vice-versa can be achieved by variations in the acylation rate constants of merely 2- to 6-fold (40). The magnitude of the changes in the kinetic parameters measured here need also be examined in the context of the decreases in MIC values for nitrocefin when switching pH from 7 to 5 (3 $\log_2$ decrease). Moreover, nitrocefin itself proved already fairly active against MRSA at neutral pH (MIC of 2 mg/L). Unfortunately, molecules such as penicillin G (this study) or meropenem or cloxacillin (33), which all are poorly active against MRSA at neutral pH but show a large decrease of MIC when tested at pH 5.5 are non-chromogenic and, therefore, more difficult to use in this type of experiments. Moreover, these molecules are somewhat labile at acidic pH, which would make the experiments much more difficult to interpret. We also need to compare the values of the second-order rate constant (k_2/K_d) at acidic pH (150 ± 14 $s^{-1}M^{-1}$) with those recorded by Fuda and co-workers (145 and 205 $s^{-1}M^{-1}$) for three experimental anti-MRSA cephalosporins with MICs of 1-8 mg/L (23).

The comparison between the influence of acidic pH on the binding of β-lactams to PBP 2a and the mode of action of the anti-MRSA cephalosporins find also parallels in that they both manifest

conformational changes in the protein. Our data show that incubation of PBP 2a with oxacillin at acidic pH induces conformational changes consistent with a loss of α-helical content (increase in ellipticity in the minima at 208 nm and at 222 nm), which has also been observed with the three experimental anti-MRSA cephalosporins studied by Fuda and co-workers (23). The same has been noted for ceftobiprole (25), the first anti-MRSA cephalosporin to reach the final stages of clinical development (41). Of note is that (i) the changes seen here occur almost immediately upon the addition of oxacillin and remained stable thereafter subsequent to protein acylation; (ii) experiments were performed under conditions that were shown earlier to exclude artifacts that could have affected the circular dichroism spectra such as precipitation of the acyl-enzyme species, or aggregation or oligomerization of the protein. Although the occurrence of conformational change during exposure of β-lactam to PBP 2a is now well documented, we hesitate to quantify this effect for reasons explained by Fuda *et al*. (32). In essence, there exists compelling reason that conformational change does occur with PBP 2a on exposure to antibiotics, and what we see here is that a similar effect is seen as a function of reduced pH when compared to neutrality, consistent with what is seen with the anti-MRSA cephalosporins.

This indicates a commonality of effects of a global environmental variable such as pH and the binding of a specific ligand on PBP 2a conformation for facilitating access to its active site. It would be interesting to examine whether (i) similar conformational changes could be initiated by other environmental variables or other ligands (which might be the case for the peptidoglycan itself [29]) and (ii) whether the effect of acidic pH and an anti-MRSA cephalosporin could have additive, or perhaps even synergic on killing of MRSA. This might have a significance for effective killing of these bacteria, especially if considering not only their intracellular forms, but also habitats where the pH may reach a sufficiently low value, such as skin surfaces (pH 4.2 to 5.9), mouth (pH 5 to 7), vagina (pH 4.2 to 6.6) and urinary tract (pH 4.6 to 7). It is interesting in this context to note that the transglycosylase activity of PBP2, which is needed for biosynthesis of peptidoglycan in MRSA prior to the action of PBP 2a in cross-linking two existing strands of it, exhibits maximal catalysis at pH 4.5 to 5.5 (42). Hence, a full knowledge of chemistry of both catalysis and inhibition under acidic pH is quite relevant to elucidation of the properties of this important bacterial pathogen. The lowering of pH from 7.0 to 5.0 entails 100-fold enhancement of the acidic condition. No doubt such a change will manifest other effects in a growing bacterium and we cannot limit the consequence of what we see with the entire bacterium to a single event, namely the structural consequences that we have outlined for PBP 2a. For instance, we showed here for Bocillin FL, and in a previous report for [^{3}H]radiolabelled penicillin G (21), that acidic pH increases the binding of the antibiotic to the bacterium to a much larger extent than what is seen with purified PBP 2a. Thus, acidic pH may also favor the binding of β-lactams to other PBPs, which will account for the decrease in MIC seen with MSSA. Based on the copy numbers, exposure to β-lactams at pH 5.5 in MRSA should involve primarily PBP 2a which is the most abundant (18). PBP 2a function is a pivotal for bacterial survival. While it is virtually this enzymatic function that converts *S. aureus* from a very common bacterium to one that has presented a difficult phenotype in an infectious setting, not all molecules, however, need to be inhibited before bacteria would die (18). The observations with PBP 2a reported herein should provide the impetus for additional study in effective control of MRSA by intervention of the function of this important enzyme.

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FOOTNOTES

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III - Modulation of the cellular accumulation and the intracellular activity of daptomycin by the P-glycoprotein efflux transporter

Publication #5: Modulation of cellular accumulation and intracellular activity of daptomycin towards phagocytosed Staphylococcus aureus by the P-glycoprotein (MDR-1) efflux transporters in human THP-1 macrophages and Madin-Darby canine kidney cells.

Sandrine Lemaire, Françoise Van Bambeke, Marie-Paule Mingeot-Leclercq, and Paul M. Tulkens

The first aspect of this project was to explore the intracellular pharmacokinetic and pharmacodynamic of daptomycin, a cyclic lipopeptide agent. In this report, we show that this antibiotic (i) penetrate cells, reaching an apparent intracellular concentration lower than the extracellular one at equilibrium; (ii) exerts concentration-dependent activity against *S. aureus* internalized by human THP-1 macrophages or MDCK [Madin-Darby Canine Kidney] cells.

Additionally, we report that the cellular accumulation of daptomycin, and thereby its intracellular activity, is partially defeated through P-glycoprotein (P-gp, MDR-1) efflux transporters, one of the main members of the ATP-binding cassette (ABC) efflux proteins superfamily. This conclusion stems from four converging pieces of evidences gained from independent approaches, namely (i) the use of well-known inhibitors (verapamil, elacridar) and activator of P-gp; (ii) the direct measurement of cellular content of daptomycin versus $DiOC_2$ (a well-known substrate of P-gp) in the presence of P-gp modulators; (iii) the comparison of the cellular accumulation of daptomycin in cell-type with various expression of P-gp (THP-1 versus MDCK cell line surexpressing MDR-1 proteins); and (iv) the silencing of mdr1 (the gene encoding P-gp in humans) expression by specific siRNA (small interfering RNA).

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Modulation of the Cellular Accumulation and Intracellular Activity of Daptomycin towards Phagocytized *Staphylococcus aureus* by the P-Glycoprotein (MDR1) Efflux Transporter in Human THP-1 Macrophages and Madin-Darby Canine Kidney Cells[▽]

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P-glycoprotein (P-gp; MDR1), a major efflux transporter, recognizes various antibiotics and is present in macrophages. We have examined its effect on the modulation of the intracellular accumulation and activity of daptomycin towards phagocytized *Staphylococcus aureus* (ATCC 25923) in human THP-1 macrophages, in comparison with MDCK epithelial cells (wild type and MDCK-MDR1 overexpressing P-gp; the bulk of the protein was immunodetected at the surface of all three cell types). Daptomycin displayed concentration-dependent intracellular activity (Hill equation pattern) in THP-1 and MDCK cells with (i) 50% effective drug extracellular concentration (EC_{50}; relative potency) and static concentrations at 9 to 10 times the MIC and (ii) maximal efficacy (E_{max}; CFU decrease at infinite extracellular drug concentration) at 1.6 to 2 log compared to that of the postphagocytosis inoculum. Verapamil (100 μM) and elacridar (GF 120918; 0.5 μM), two known inhibitors of P-gp, decreased daptomycin EC_{50} (about threefold) in THP-1 and MDCK cells without affecting E_{max}. Daptomycin EC_{50} was about three- to fourfold higher and accumulation in MDCK-MDR1 commensurately lower than in wild-type cells. In THP-1 macrophages, (i) verapamil and ATP depletion increased, and ouabain (an inducer of *mdr1* [the gene encoding P-gp] expression) decreased the accumulation of daptomycin in parallel with that of $DiOC_2$ (a known substrate of P-gp); (ii) silencing *mdr1* with duplex human *mdr1* siRNAs reduced the cell content in immunoreactive P-gp to 15 to 30% of controls and caused an eight- to 13-fold increase in daptomycin accumulation. We conclude that daptomycin is subject to efflux from THP-1 macrophages and MDCK cells by P-gp, which reduces its intracellular activity against phagocytized *S. aureus*.

Active efflux of drugs from eukaryotic cells is now recognized as a major determinant in the modulation of their pharmacokinetic properties in relation to absorption, distribution, and excretion, and needs, therefore, to be fully taken into account in the assessment of their activity and toxicity (20, 27, 40, 49, 66). In this context, P-glycoprotein (P-gp), one of the main members of the superfamily of the ATP-binding cassette (ABC) efflux proteins, has been shown to recognize antibiotics (59, 61), and could therefore modulate their intracellular activity. Thus, P-gp-mediated efflux was found to partially defeat the cellular accumulation and to reduce the activity of azithromycin towards *Staphylococcus aureus* phagocytized by murine J774 macrophages (46, 47).

In the present study, we have examined and assessed the role of P-gp in the modulation of the intracellular accumulation and activity of daptomycin in human THP-1 macrophages. To better ascertain the specificity of the effects seen to the activity of P-gp, we also used Madin-Darby canine kidney (MDCK) cells, for which stable lines overexpressing P-gp are available (12). Originally described in the mid-1980s as LY 146032 (11), daptomycin has become the lead member of the new class of the so-called acidic lipopeptide antibiotics. These are characterized by a membrane-related mode(s) of action (53), which results in a marked bactericidal activity against multiresistant gram-positive organisms, including methicillin-resistant *S. aureus* (14, 23). Intracellular survival of *S. aureus* is often considered as an important determinant in the relapsing and recurrent character of staphylococcal infections (see references 13 and 31 for a discussion). Yet, the intracellular uptake and activity of daptomycin have been little explored so far (63). We show here that daptomycin exerts concentration-dependent activity against intracellular *S. aureus* in the two types of cells studied but that its accumulation, and thereby its activity, is partially defeated by efflux through P-gp.

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MATERIALS AND METHODS

Materials. Daptomycin was obtained as laboratory samples for microbiological evaluation from Novartis Pharma AG, Basel, Switzerland. Verapamil, ouabain, gemfibrozil, and 3-ethyl-2-[5-(3-ethyl-2(3H)-benzoxazolylidene)-1,3-pentadienyl]-iodide ($DiOC_2$) were from Sigma-Aldrich (St. Louis, MO). Elacridar (GF120918) was the generous gift of GlaxoWellcome Research and Development (Laboratoire Glaxo Wellcome, Les Ulis, France). Unless stated otherwise, cell culture media and sera were from Invitrogen, antibodies from Sigma-Aldrich, and other products from Merck KGaA (Darmstadt, Germany).

Bacterial strain and susceptibility testing. *S. aureus* ATCC 25923 (methicillin sensitive) was used throughout our experiments. Daptomycin MIC, as determined by microdilution in Mueller-Hinton broth supplemented with 50 mg/liter $CaCl_2$ (15), was 0.125 mg/liter (determinations made in triplicate; value checked for consistency throughout our experiments).

Cell cultures and assessment of cell viability. Experiments were performed with (i) THP-1 cells (ATCC TIB-202), a human myelomonocytic cell line displaying macrophage-like activity (57) and obtained from the American Tissue Collection (LGC Promochem Ltd., Teddington, United Kingdom), and (ii) MDCK wild-type cells (29) and MDCK cells transfected with human *mdr1*

(MDCK-MDR1) (12), both obtained from P. Borst (The Netherlands Cancer Institute, Division of Molecular Biology and Centre of Biomedical Genetics, Amsterdam, The Netherlands). Maintenance of cell viability in the presence of daptomycin and P-gp inhibitors was assessed by trypan blue exclusion test (validated against the accepted method of measuring lactate dehydrogenase release used in our previous studies) (4) with CSA-13, a membrane-damaging antibiotic (25). Verapamil was dissolved in water, and gemfibrozil and elacridar were dissolved in dimethyl sulfoxide (DMSO; we checked that the concentrations of DMSO brought in the final culture medium [0.5%] did not interfere with the cell accumulation of daptomycin).

Cell infection and assessment of intracellular activities of daptomycin. Infection and assessment of intracellular activity in THP-1 cells were performed exactly as previously described (26). Infection of MDCK and MDCK-MDR1 cells was performed following the protocol previously described for J774 macrophages (45), except that (i) phagocytosis was allowed to take place for 2 h with an inoculum of 10^7 bacteria/ml, and (ii) nonphagocytized bacteria were removed by extensive washing only (to avoid using gentamicin, which caused a marked loss of cell viability if used immediately after phagocytosis). For both cell types, the postphagocytosis inoculum comprised between 1.8 and 2.7×10^6 CFU per mg of cell protein, a value close to what was obtained in our previous studies (3, 26). Intracellular activity was assessed as previously described for J774 cells.

Assay of cell-associated daptomycin and $DiOC_2$ and calculation of apparent cellular-to-extracellular concentration ratios. Daptomycin and $DiOC_2$ were assayed by fluorimetry, according to previously described procedures (51, 54), with the following modifications and performance characteristics. For daptomycin, excitation and emission wavelengths were set at 380 and 425 nm, and linearity was obtained between 1 and 250 mg/liter ($R^2 > 0.99$). For $DiOC_2$, excitation and emission wavelengths were set at 485 nm and 620 nm, and linearity was obtained between 0.05 and 10 mg/liter ($R^2 > 0.99$). We checked in pilot experiments that verapamil, gemfibrozil, and ouabain did not interfere with this assay. In contrast, elacridar intrinsic fluorescence made it impossible to assay daptomycin in its presence. Cell protein was assayed in parallel using the Folin-Ciocalteu/biuret method (30). The cell-associated contents in daptomycin and $DiOC_2$ were expressed by reference to the total cell protein content and converted into apparent concentrations using a conversion factor of 5 μl per mg of cell protein as commonly used for cultured cells. The level of accumulation of each compound was then expressed as the ratio of this apparent cell concentration to the corresponding extracellular concentration.

Measurements of free cell Ca^{2+} concentrations. Calcium-sensor dye Fluo-3 (19, 36), under its acetoxymethyl ester form (Fluo-3-AM; Invitrogen), was dissolved in DMSO with the nonionic detergent Pluronic F-127. THP-1 macrophages were harvested, suspended in Ca^{2+}-free Hanks balanced salt solution supplemented with 5 g/liter bovine serum albumin, and exposed for 30 min at 25°C to 5 μM Fluo-3-AM in the same medium supplemented with 1 mM probenecid (to reduce cell efflux of Fluo-3 after its intracellular deesterification). Cells were thereafter washed in Ca^{2+}-free Hanks balanced salt solution-bovine serum albumin-probenecid medium, incubated for 30 min at 25°C in the same medium, and thereafter incubated without (controls) or with either 100 μM verapamil or 250 μM gemfibrozil or ionomycin (50 μM; a Ca^{2+} ionophore [6]). Whole-cell fluorescence was then read continuously for up to 60 min at room temperature (λ_{exc}, 485 nm; λ_{em}, 530 nm) using a Packard FluoroCount microplate reader instrument (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA).

Modulation of P-gp expression by siRNA (gene silencing). THP-1 macrophages (10^7 cells) were transfected with 0.75 μg of duplex human *mdr1* small interfering RNA (siRNA) (Santa-Cruz Biotechnology, Heidelberg, Germany). We used a Nucleofector II electroporation apparatus (Amaxa Biosystems, Cologne, Germany), following the general procedure described for THP-1 cells (program V-001), with cells suspended in Royal Park Memorial Institute (RPMI) 1640 culture medium supplemented with 10% fetal bovine serum and either (i) 1.25% DMSO (medium a) (32) or (ii) 25 mM HEPES, pH 7.4 (medium b) (43). We checked that both media allowed for an efficient penetration of the nonpermeant dye trypan blue in cells when added to the electroporation buffer (83.4 ± 2.6 of cells stained versus 1.1 ± 0.1 for controls [no electroporation] with medium a; 87.7 ± 0.2 versus 3.4 ± 1.32 with medium b). Cells returned to culture medium and reincubated for 1 h after electroporation before being again exposed to trypan blue excluded the dye for >80%, indicating a satisfactory cell membrane resealing and maintenance of viability.

Western blotting. For analysis of P-gp expression, cells (~10^7) were washed three times with ice-cold phosphate-buffered saline (pH 7.4) and suspended in 500 μl of lysis buffer (64). The samples were then subjected to three successive cycles of freezing-thawing (−80°C for 5 min and 37°C for 5 min), sonicated (for three cycles of 5 s with 30-s intervals at 4°C), and finally centrifuged for 10 min at 14,000 rpm and at 4°C in an Eppendorf 5415C microcentrifuge (Eppendorf AG, Hamburg, Germany) equipped with the standard rotor F-45-24-11. Protein concentration of the supernatant was determined by a modified Bradford's procedure (39) using the Quick Start Bradford protein assay kit 3 from Bio-Rad Laboratories (Hercules, CA). Western blotting was performed following the procedure described previously (34) with a mouse monoclonal anti-P-gp (MDR) antibody (1/500) as primary antibody (Sigma-Aldrich catalog no. P7965; according to its supplier, this antibody detects specifically human MDR1 P-gp but not MDR3) and polyclonal horseradish peroxidase-labeled anti-mouse (1/1,000) as secondary antibody (equiloading of the gels was controlled with monoclonal mouse anti-β-actin antibody [Sigma-Aldrich catalog no. A2228; 1/2,000]). Bands were revealed with the SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, IL). Films were scanned and subjected to a densitometric analysis using Image J software (version 1.3.1; available from the Research Service branch of the National Institute of Mental Health at http://rsb.info.nih.gov/ij).

Confocal microscopy. P-gp was detected on formalin-fixed and saponin-permeabilized cells using the same mouse monoclonal anti-P-gp as for Western blotting and fluorescein isothiocyanate-labeled goat anti-mouse antibodies (Santa Cruz Biotechnology), with counterstaining for actin with 5 units of rhodamine-labeled phalloidin (Invitrogen, Carlsbad, CA), following the general techniques previously described for J774 macrophages (48, 58) with minor adaptations. Observations were made with MRC1024 confocal scanning equipment (Bio-Rad, Richmond, CA) mounted on an Axiovert confocal microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analyses. Curve-fitting analyses were made using GraphPad Prism version 4.02 for Windows (GraphPad Prism Software, San Diego, CA). Analysis of variance was made with GraphPad Instat version 3.06 (GraphPad Prism Software), and analysis of covariance with XLStat version 7.5.2 (Addinsoft SARL, Paris, France).

RESULTS

Intracellular activity of daptomycin in THP-1 macrophages and modulation by efflux transporter inhibitors. In the absence of published detailed analysis of the intracellular activity of daptomycin in macrophages, we first conducted 24-h dose-response studies using a wide range of extracellular concentrations to obtain information on key pharmacological descriptors (50% effective concentration [EC_{50}]; static concentration [C_{static}]; maximal efficacy [E_{max}]). We also used our previous studies (4, 26), which were necessary for the correct interpretation of the present data. Results are shown in Fig. 1A, with the pertinent regression parameters presented in Table 1. Daptomycin displayed clear-cut concentration-dependent activity, with EC_{50} and C_{static} values close to each other at about 9 to 10 times the MIC and an E_{max} value at about −1.6 log CFU. We then selected a daptomycin extracellular concentration of 1 mg/liter (i.e., close to the EC_{50} and C_{static} values) to test for the influence on daptomycin activity of two well-known P-gp inhibitors, namely, the Ca^{2+} channel antagonist verapamil, which is largely nonspecific, and elacridar, a much more specific inhibitor (16). Results of experiments conducted at fixed concentrations (verapamil, 100 μM; elacridar, 0.5 μM) over 5 and 24 h are shown in Fig. 1B. Both inhibitors markedly enhanced the intraphagocytic activity of daptomycin, with an effect already detectable at 5 h only. This was not due to a direct action of verapamil or elacridar on bacteria, since the presence of these inhibitors alone did not markedly affect the intraphagocytic growth of *S. aureus* (they did not affect the MIC of daptomycin, either [data not shown]). The influence of verapamil and elacridar on the activity of daptomycin was then studied in more detail by running complete dose-response studies over a 24-h period. In the first series of experiments, verapamil or elacridar was added at a fixed concentration (100

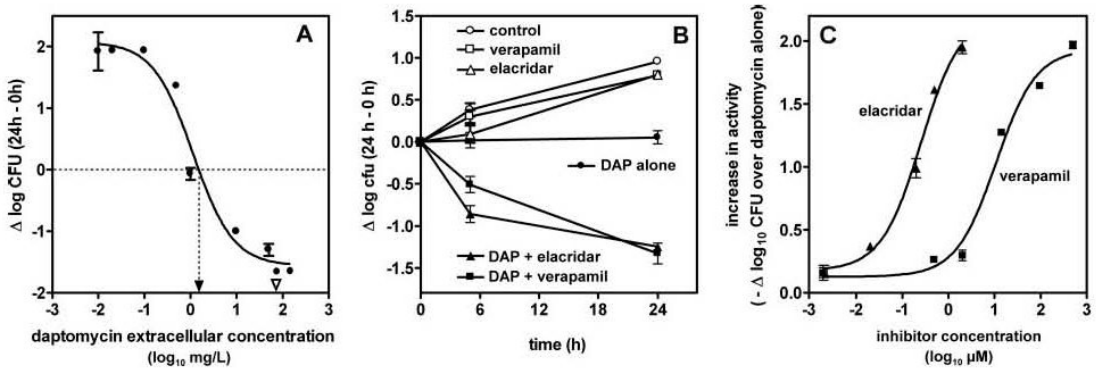

FIG. 1. Intracellular activity of daptomycin towards *S. aureus* ATCC 25923 in THP-1 macrophages. (A) Dose-response curves over a wide range of extracellular concentrations. The ordinate shows the change in the number of CFU (Δ log CFU) per milligram of cell protein at 24 h compared to the postphagocytosis inoculum. A sigmoidal (slope factor, 1) function was used for regression (see Table 1 for goodness-of-fit and regression parameters). The dotted horizontal line indicates a static effect, which was reached for the extracellular concentration shown by the vertical dotted line. For reference, the open triangle on the abscissa indicates the serum C_{max} (total drug) observed in volunteers receiving the clinically recommended dose of 4 mg/kg of body weight daptomycin (77 mg/liter) (67). (B) Influence of time and of the presence of efflux transporter inhibitors on the rate and the extent of the activity of daptomycin at a fixed extracellular concentration. The ordinate is as in panel A. Control, no treatment; DAP, daptomycin (1 mg/liter); verapamil (100 μM); elacridar (GF 120918; 0.5 μM). (C) Influence of the concentration of verapamil or elacridar on the activity of daptomycin (1 mg/liter) measured at 24 h. The ordinate shows the increase in activity defined as the difference between the change in CFU observed in the presence of the inhibitors minus what is observed with daptomycin alone (the graph shows the negative value of this difference to avoid describing increases in activity by decrements in the ordinate). All values are means ± standard deviations (n = 3; when not visible, the standard deviation bars are smaller than the symbols).

or 0.5 μM, respectively) to media containing increasing concentrations of daptomycin (over the same range as that used to generate the data of Fig. 1A). Results, shown in a synoptic fashion in Table 1, demonstrate that both inhibitors significantly increased the relative potency of daptomycin (thus causing a reduction of the EC_{50}) without affecting its E_{max} (no significant change in E_{max} value). In the second series of experiments, daptomycin was used at a fixed concentration of 1 mg/liter (shown to cause an almost static effect), and verapamil or elacridar was added at increasing concentrations (from 2 nM to 10 μM [elacridar] and 500 μM [verapamil]). Results presented in Fig. 1C show that both inhibitors acted in a concentration-dependent fashion and were able to bring the intracellular activity of daptomycin (present in the extracellular medium at a concentration of 1 mg/liter only) to the value observed for a daptomycin concentration of 100 mg/liter in cells incubated in the absence of inhibitors (and corresponding to its E_{max} [Table 1]). To check for the specificity of the changes in daptomycin activity observed with verapamil and elacridar with respect to P-gp, all experiments were repeated with gemfibrozil, an inhibitor of MRPs, the second major family of ABC transporters capable of transporting antibiotics in macrophages (34), but no significant effect was observed.

Intracellular activity of daptomycin in wild-type MDCK cells versus MDCK cells overexpressing P-gp (MDCK-MDR1) and modulation by efflux transporter inhibitors. In the first

TABLE 1. Pertinent regression parameters and statistical analysis of the dose-response curves of daptomycin alone (see Fig. 1A) or daptomycin plus transporter inhibitors (actual data not shown) towards *S. aureus* ATCC 25923 phagocytized by THP-1 macrophages or MDCK cells[a]

Condition	EC_{50} ± SD (mg/liter)[b,c]	C_{static} (mg/liter)[d]	E_{max} ± SD (log_{10} U)[b,e]	R^2
THP-1 cells				
Daptomycin alone	1.14 ± 0.14 A	1.3	−1.60 ± 0.16 A	0.977
Daptomycin + verapamil (100 μM)	0.31 ± 0.13 B	0.4	−1.82 ± 0.16 A	0.978
Daptomycin + elacridar (0.5 μM)	0.27 ± 0.17 B	0.4	−1.67 ± 0.23 A	0.964
MDCK cells				
Wild type, daptomycin alone	0.62 ± 0.09 C	0.6	−1.70 ± 0.07 A	0.993
Wild type, daptomycin + verapamil (100 μM)	0.18 ± 0.06 B	0.2	−2.01 ± 0.06 B	0.996
MDCK-MDR1, daptomycin alone	2.67 ± 0.07 D	2.1	−1.77 ± 0.06 A	0.999
MDCK-MDR1, daptomycin + verapamil (100 μM)	0.70 ± 0.15 E	0.6	−1.78 ± 0.02 A	0.989

[a] Regression parameters obtained by nonlinear regression using the Hill equation with a slope factor of 1.
[b] For statistical analysis (per column; one-way analysis of variance by the Tukey's test for multiple comparisons between each parameter for all conditions), values with different letters are significantly different from each other ($P < 0.05$).
[c] Concentrations causing a reduction of the inoculum halfway between the extrapolated initial (E_0) and maximal (E_{max}) values.
[d] Concentration resulting in no apparent bacterial growth (no change in CFU compared to the postphagocytosis inoculum; determined by graphical intrapolation).
[e] CFU decrease at 24 h from the corresponding postphagocytosis inoculum, as extrapolated for a daptomycin concentration at infinity, all CFU counts were much above the minimal detection level.

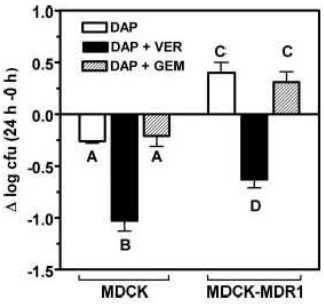

FIG. 2. Activity of daptomycin at a fixed extracellular concentration (1 mg/liter) towards *S. aureus* ATCC 25923 in MDCK (wild-type) and MDCK-MDR1 (overexpressing P-gp) cells in 24 h. The ordinate shows the change in the number of CFU (Δ log CFU) per mg of cell protein. DAP, daptomycin alone; DAP + VER, daptomycin plus verapamil (100 μM); DAP + GEM, daptomycin plus gemfibrozil (250 μM). All values are means ± standard deviations ($n = 3$). Statistical analysis: values of bars with different letters are significantly different from each other (analysis of variance; $P < 0.05$).

series of experiments, the activity of daptomycin in MDCK and MDCK-MDR1 cells infected with *S. aureus* was examined using a fixed extracellular concentration of 1 mg/liter in the absence and in the presence of verapamil (100 μM) or gemfibrozil (250 μM). Results are shown in Fig. 2. Concentrating first on wild-type cells, we see that daptomycin, which at the concentration used was almost bacteriostatic against intracellular *S. aureus*, had its activity markedly enhanced by verapamil, as in THP-1 macrophages. Moving now to MDCK-MDR1 cells, we see that daptomycin, at the same concentration as that used for wild-type cells, was unable to control the intracellular bacterial growth. Yet, the addition of verapamil allowed for the appearance of significant activity. Interestingly enough, the increase in activity obtained with verapamil was similar in MDCK and MDCK-MDR1 cells (about a 1.3-log decrease in CFU), although the basal levels were different. Finally, gemfibrozil was without significant effect on daptomycin activity with either cell line. In the second series of experiments, we ran full daptomycin dose-response curves in infected wild-type MDCK and MDCK-MDR1 cells in the presence and in the absence of verapamil (100 μM). Results shown in a synoptic fashion in Table 1 demonstrate that verapamil increased the relative potency of daptomycin about 3.5-fold (in both cell lines), as in THP-1 macrophages. There was also a small increase in E_{max} in wild-type MDCK cells.

Modulation of the cellular accumulation of daptomycin and $DiOC_2$. Based on the results presented so far, we hypothesized that verapamil increased the intracellular antibacterial effect of daptomycin in THP-1 macrophages and MDCK cells by enhancing its cellular accumulation (through impairment of a P-gp-mediated efflux mechanism). The cellular accumulation of daptomycin was therefore examined under conditions known to either impair (addition of verapamil or ATP depletion) or to enhance (addition of ouabain) (7) the activity of P-gp. These experiments had to use a large extracellular concentration of daptomycin (250 mg/liter) because of the low sensitivity of our assay method (and the unavailability of radiolabeled compound). Results shown in Table 2 demonstrate that (i) MDCK-MDR1 cells accumulated significantly less daptomycin than wild-type MDCK cells, and (ii) verapamil and ATP depletion (tested for THP-1 macrophages only) enhanced the accumulation of daptomycin (and, interestingly enough, to the same extent [about 3.6-fold in all conditions]), whereas ouabain markedly decreased it. As in all other experiments, gemfibrozil had no effect.

Since the large concentrations of daptomycin used could have caused saturation of the putative P-gp efflux transporter, we also examined the influence of verapamil, ouabain, and gemfibrozil on the accumulation of a known and specific substrate of P-gp, namely, $DiOC_2$ (24, 35), which could be used at a much lower concentration (1 mg/liter). In pilot experiments, we observed that the effect of verapamil on $DiOC_2$ accumulation was not immediate but increased over time. We therefore measured in parallel the accumulation of $DiOC_2$ (at the extracellular concentration of 1 mg/liter) and that of daptomycin (at the extracellular concentration of 250 mg/liter) over a period of 1 and 5 h in control cells versus cells coincubated with verapamil, ouabain, or gemfibrozil. Results presented in Fig. 3 show that verapamil caused an increase, and ouabain a decrease, of the accumulation of $DiOC_2$, which was highly correlated with that of daptomycin. In contrast, the effect of gemfibrozil was minimal.

Influence of verapamil concentration on restoration of daptomycin activity and accumulation in MDCK-MDR1 cells. The data of Tables 1 and 2 show that the addition of 100 μM verapamil to MDCK-MDR1 cells did not bring the daptomycin EC_{50} value and accumulation level to the same values as for

TABLE 2. Modulation of the cellular accumulation of daptomycin in THP-1 macrophages and MDCK cells[a]

Condition	Apparent cellular-to-extracellular concn ratio ± SD (mg/liter)[d]		
	THP-1 cells	MDCK cells[c]	
		Wild type	MDCK-MDR1
Daptomycin alone	0.21 ± 0.05 A	0.23 ± 0.13 A;a	0.08 ± 0.11 A;a
Daptomycin + verapamil (100 μM)	0.75 ± 0.05 B	0.83 ± 0.20 B;a	0.30 ± 0.05 B;b
ATP depletion[b]	0.82 ± 0.07 B		
Daptomycin + ouabain (1 μM)	0.12 ± 0.04 C	0.04 ± 0.01 C;a	0.03 ± 0.01 C;a
Daptomycin + gemfibrozil (250 μM)	0.18 ± 0.06 A;C	0.18 ± 0.06 A;a	0.07 ± 0.06 A;a

[a] Cells incubated for 24 h with 250 mg/liter daptomycin.
[b] Cells incubated for 1 h with 5 mM NaN_3 and 60 mM 2-deoxyglucose before being exposed to daptomycin in the presence of the same inhibitors.
[c] Empty cells, exposure of MDCK and MDCK-MDR1 cells to the inhibitors used for ATP depletion in THP-1 cells caused massive cell detachment.
[d] For statistical analysis, one-way analysis of variance by the Tukey's test for multiple comparisons between conditions (analysis per column; capital letters) or between cells (analysis per row for MDCK cells; small letters) was conducted; values with different letters are significantly different from each other ($P < 0.05$).

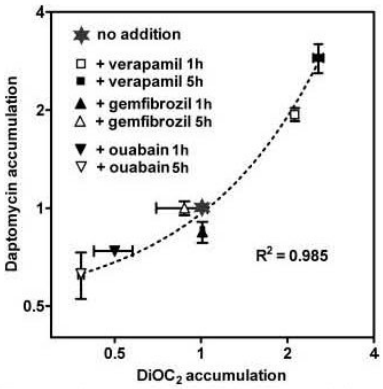

FIG. 3. Comparative modulation of the accumulation of daptomycin and $DiOC_2$ in THP-1 macrophages by verapamil (100 μM), gemfibrozil (250 μM), or ouabain (1 μM). Cells were incubated with daptomycin (250 mg/liter) or $DiOC_2$ (1 mg/liter) alone or in the presence of the modulators for 1 or 5 h. The graph shows the changes in accumulation of daptomycin (ordinate) and of $DiOC_2$ (abscissa) expressed as the fraction of the respective values observed in the absence of the modulator. All values are means ± standard deviations ($n = 3$; when not visible, the standard deviation bars are smaller than the symbols). The dotted line shows the regression curve obtained by using an exponential growth function.

wild-type MDCK cells. This could indicate either an incomplete P-gp inhibition at this verapamil concentration or the presence of another efflux mechanism in MDCK-MDR1 cells. *S. aureus*-infected MDCK-MDR1 cells were, therefore, incubated with 1 mg/liter daptomycin in the presence of increasing concentrations of verapamil (0 to 500 μM). While a verapamil concentration of 100 μM yielded only a modest effect, increasing its concentration to 500 μM yielded a ≈1.5-log CFU decrease (similar to what was seen with 100 μM verapamil with control MDCK cells).

Immunohistodetection of P-gp in THP-1 macrophages and MDCK cells. The previous experiments strongly suggested the presence of an efflux transporter for daptomycin in THP-1 cells that could be potentially identified as P-gp. With only limited data being available as to the presence of this protein in THP-1 macrophages (17), we undertook documenting its presence and localization in our strain of THP-1 macrophages in comparison with MDCK and MDCK-MDR1 cells using immunological techniques. As shown in Fig. 4, the bulk of immunodetected P-gp was in both cases located at the periphery of the cells in close vicinity to actin (used to visualize the cytoskeleton, located as a submembraneous network in association with integrins and other membrane proteins, and determining the cell shape) (41, 44).

Influence of the impairment of P-gp expression through siRNA (gene silencing) on daptomycin accumulation in THP-1 macrophages. Having demonstrated the presence of immunoreactive P-gp in THP-1 (with a localization that could account for an efflux of daptomycin), we examined whether the silencing of its gene (and the corresponding decrease in its cell content) would modulate the accumulation of daptomycin. For this purpose, cells were electroporated with duplex human *mdr1* siRNAs and examined thereafter for the presence of P-gp (by Western blot analysis; performed on lysates from cells incubated for 30 h in control medium after electroporation) and for daptomycin accumulation (measured in cells incubated for 30 h in control medium after electroporation and further incubated for 24 h

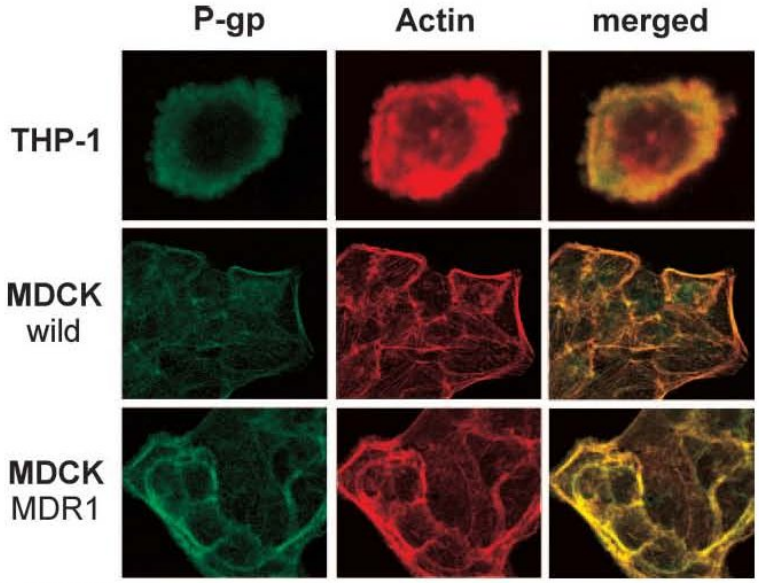

FIG. 4. Immunohistodetection and localization of P-gp in THP-1 macrophages and in MDCK cells (wild type and MDCK-MDR1) by confocal microscopy. Formalin-fixed and saponin-permeabilized cells were exposed to mouse monoclonal anti-P-gp antibodies. Green channel, detection of mouse antibodies with goat fluorescein isothiocyanate-labeled anti-mouse antibodies; red channel, detection of actin with rhodamine-labeled phalloidin; merged images, the two labels are largely but not entirely colocalized.

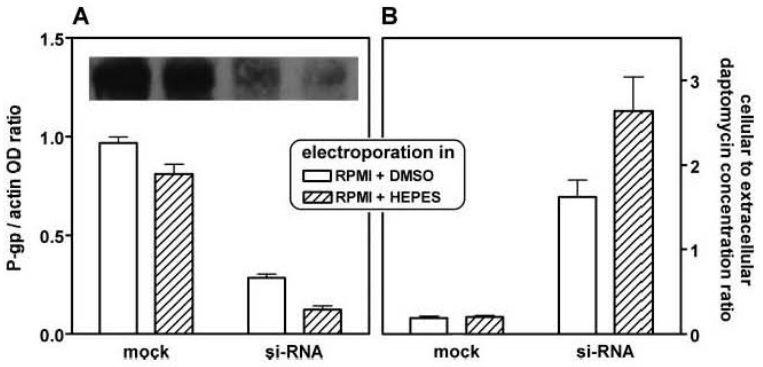

FIG. 5. Modulation of the cell content in P-gp and of daptomycin accumulation in THP-1 macrophages subjected to electroporation without (mock) or with duplex human *mdr1* siRNAs in two different media (RPMI + DMSO, RPMI 1640 medium supplemented with 2.5% DMSO; RPMI + HEPES, RPMI medium supplemented with 25 mM HEPES). (A) Western blot analysis of lysates of cells collected 30 h after electroporation. The picture shows the bands corresponding to the protein as detected by mouse monoclonal anti-P-gp and polyclonal horseradish peroxidase-labeled anti-mouse antibody. The ordinate shows the absorbance ratio of the corresponding band in each condition to that of actin (not shown). OD, optical density. (B) Measurement of the apparent ratio of cellular to extracellular daptomycin concentration in cells incubated in drug-free medium for 30 h after electroporation and thereafter incubated for 24 h with 250 mg/liter daptomycin.

with 250 mg/liter daptomycin). Results are shown in Fig. 5. Electroporation with the duplex human *mdr1* siRNAs (in either of the two media used) markedly reduced the cell content in immunoreactive P-gp, and, in parallel, allowed for a marked increase in daptomycin accumulation.

Correlations between P-gp expression, daptomycin potency, and daptomycin accumulation. Figure 6 shows the correlations established between the amounts of immunoreactive P-gp detected in control THP-1 macrophages, MDCK, and MDCK-MDR1 cells, and THP-1 macrophages subjected to *mdr1* silencing (as determined by Western blot analysis) versus (i) the daptomycin relative potencies (EC_{50}) towards phagocytized *S. aureus* (data of Table 1) and (ii) daptomycin accumulation (data of Table 2 and of Fig. 5) in these cells. In both cases, the correlation was highly significant in spite of the small number of data points. This figure also suggests that the high levels of cellular accumulation of daptomycin noted for THP-1 cells subjected to *mdr1* silencing (see Fig. 5) could only be observed because the P-gp cell content in these cells was much lower than in untreated THP-1 or in wild-type MDCK cells.

Influence of verapamil on cell Ca^{2+} content. Because the antibacterial activity of daptomycin is critically dependent upon a sufficient Ca^{2+} concentration (2, 11), we examined whether the enhancing effect of verapamil on the intracellular activity of daptomycin could not be due to an increase in its intracellular cell Ca^{2+} content. For this purpose, the concentration of free intracellular Ca^{2+} was monitored by means of the calcium sensor Fluo-3 dye in cells exposed to verapamil (100 μM) or gemfibrozil (250 μM). Compared to control cells, verapamil caused a ≈25% reduction of the free Ca^{2+} cellular concentration over 60 min, whereas gemfibrozil was without influence. Ionomycin, an antibiotic also known as a calcium ionophore (6) and used as positive control, caused a 75% increase in apparent free intracellular Ca^{2+} under the same conditions.

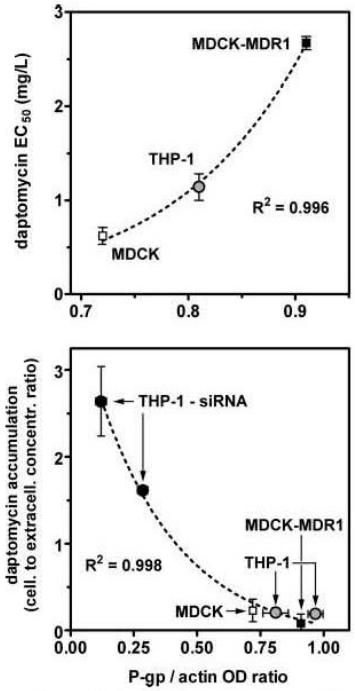

FIG. 6. Correlations between the expression of P-gp (as determined by Western blot analysis and expression as the P-gp/actin optical density [OD] ratio as in Fig. 5) and (i) daptomycin intracellular relative potency and (ii) daptomycin cellular accumulation. (Top) Wild-type MDCK, THP-1, and MDCK-MDR1 cells; EC_{50} values (ordinate) are those listed in Table 1. (Bottom) Comparison between wild-type MDCK, MDCK-MDR1, and THP-1 cells and THP-1 cells subjected to *mdr1* silencing (THP-1-siRNA); accumulation values (ordinate) are those listed in Table 2 and shown in Fig. 5; P-gp expression values (abscissa) are those of the top panel of this figure for MDCK, MDCK-MDR1, and THP-1 cells, and those of Fig. 5 for THP-1-siRNA cells. All values are means ± standard deviations ($n = 3$; when not visible, the standard deviation bars are smaller than the symbols). The dotted lines show the regression curve obtained by using exponential growth (top) or decay (bottom) functions.

DISCUSSION

The present data extend our previous observations underlining the role of the ATP-energized efflux transporters for modulation of the accumulation and activity of antibiotics in macrophages (33, 34, 46, 47). Specifically, we show here that the activity of P-gp in THP-1 human macrophages decreases the relative potency of daptomycin towards phagocytized *S. aureus* by reducing its cellular concentration. This conclusion, and its specificity with respect to P-gp versus other eukaryotic efflux transporters, stems from five converging pieces of evidence gained from independent approaches, namely (i) the use of two well-known inhibitors of P-gp activity, verapamil (42) and elacridar (GF 120918) (17), in comparison with gemfibrozil, a preferential inhibitor of MRP efflux transporters, which are also present in macrophages (34); (ii) the comparison of the behavior of THP-1 macrophages with that of MDCK (wild-type) and of MDCK-MDR1 (overexpressing P-gp) cell lines; (iii) the direct measurement of the cell content in daptomycin and in $DiOC_2$, a well-known substrate of P-gp (55), in the presence of molecules known to impair or to increase P-gp activity (viz. the inhibitors mentioned above and ouabain, an inducer of *mdr1* expression) (7); and (iv) the silencing of *mdr1* (the gene encoding P-gp in humans) expression by specific siRNA. It is intriguing, at first glance, that *mdr1* silencing causes a much larger accumulation of daptomycin than P-gp inhibitors. This may actually indicate that neither verapamil nor elacridar is capable of fully blocking daptomycin efflux. This conclusion is in accordance with the growing evidence for the presence of multiple binding sites in P-gp (9) and with the observation that substrates and inhibitors of the related half-transporter ABCG2 are effluxed by multiple pathways so that inhibitors remain intrinsically poorly effective at pharmacologically achievable concentrations (1).

Originally described in tumor cells in relation to pleiotropic cross-resistance to a wide range of amphiphilic drugs (18), P-gp is also expressed in the apical surface of a variety of epithelia, where it exerts a function of protection against the toxicity of xenobiotics (by excreting them, for instance, in the intestinal lumen or the urine). P-gp has been described in THP-1 macrophages (17), and our observation that the bulk of the protein is located at the cell surface is consistent with a detoxification function. We, however, are facing two difficulties in the context of our study, namely, (i) how daptomycin could be effluxed by P-gp, based on what we know about preferences of this transporter for truly amphiphilic compounds, and (ii) how this efflux could modulate its intracellular activity towards bacteria that sojourn and thrive not in the cytosol but within phagolysosomes.

Association of drugs to P-gp appears to be mediated mainly by lateral diffusion within the inner monolayer of the plasma, thereby maximizing efflux to a wide range of xenobiotics (56). Even though specific structural determinants appear critical for optimal recognition of drugs by P-gp (40, 65), binding constants remain usually weak, since it is the high partition coefficients of the drugs into lipid bilayers that allow them to reach sufficient values in the vicinity of the protein. These considerations should actually make daptomycin a very unlikely substrate for P-gp, since (i) its overall structure is very remote from that of typical P-gp substrates or inhibitors such as doxorubicin and $DiCO_2$ or verapamil and elacridar, and (ii) the calculated daptomycin octanol/water partition coefficient (log *P*) and distribution coefficient (log *D*, a more correct descriptor for predicting lipid solubility of ionizable compounds) are very negative (-4.073 ± 0.25 and -9.56 at pH 7) (Advanced Chemistry Development [ACD/Labs] software, version V8.19 for Solaris, Advanced Chemistry Development, Inc., Toronto, Ontario, Canada, 2007), in sharp contrast to the positive values calculated for most substrates or inhibitors of P-gp (28). The name "peptolide," originally coined to designate daptomycin (11) but used later on also for cyclosporine and related drugs (22), may erroneously suggest a high lipophilicity, which is not true for daptomycin. Actually, the cyclic peptide moiety of daptomycin contains several anionic amino acids that confer a high polarity to this part of the molecule, which is in contrast to the highly hydrophobic amino acids constitutive of the cyclic peptide moiety of cyclosporine. The confusion is further compounded by the fact that cyclosporine and related peptolides are well-known inhibitors of P-gp (21). Whereas daptomycin in solution spontaneously adopts a conformation that does not confer amphiphilic properties, it oligomerizes in the presence of physiological concentrations of calcium to form micelles, which tend to dissociate when interacting with membranes (53). In bacteria, the high content in the negatively charged phospholipid phosphatidylglycerol allows for a deep, Ca^{2+}-facilitated insertion of daptomycin, leading to membrane leakage and cell death. While a similar, toxic process does not occur in eukaryotic cells (as evidenced by the lack of membrane-permeabilizing properties of daptomycin towards the cells used in our study), interaction of Ca^{2+}-daptomycin micelles with other phospholipids is possible and could lead to its recognition as a phospholipid-drug complex by P-gp. Phospholipids, indeed, are among the physiological substrates of P-gp, the transporter acting then as a flippase (70). A similar model of drug-phospholipid complexation for transport of nonlipophilic drugs by efflux proteins has been presented recently to explain the recognition of aminoglycosides (which have calculated log *P* and log *D* [pH 7] values similar to those of daptomycin) (ACD/Labs software, version V8.19) by the AcrB multidrug efflux pump of *Escherichia coli* (69). Interestingly enough, MDR3, another member of the MDR family, is also known to promote phosphatidylcholine translocation through the plasma membrane of fibroblasts (52).

Efflux of daptomycin at the level of the plasma membrane does not directly explain, however, the reduction of its potency against intracellular *S. aureus*, since the bacterium is not located in the cytosol but in the phagolysosomes (26). It could be argued that P-gp localized in the plasma membrane diverts part of the daptomycin from entering cells by diffusion and reduces, therefore, the amount of drug that can thereafter reach the phagolysosomes, as proposed to explain the defeating effect of P-gp towards azithromycin in J774 macrophages (46). But the biophysical properties of daptomycin discussed above are not compatible with the mechanism of diffusion/segregation observed for azithromycin (8), which is an amphiphilic, basic drug. The difficulty is actually compounded by our ignorance, at this stage, of the pathway and mechanism allowing daptomycin to enter cells and reach the phagolysosomes. Further studies will need to examine these aspects of the cellular pharmacokinetics of dap-

tomycin, but their performance is dependent upon the availability of more sensitive bioanalytical tools than are available to us so far. Yet, since the intracellular activity of daptomycin is concentration dependent, the most likely explanation for the effects observed here is that impairing or increasing the activity of P-gp effectively modulates its concentration at the site of infection. We know that daptomycin activity is enhanced by increasing the calcium concentration (10, 11). But such a calcium effect is unlikely to take place here, since verapamil decreased the cell free Ca^{2+} concentration, as anticipated from its known action on calcium channels in macrophages (68).

Moving now to the chemotherapeutic significance of our observations, we first see that daptomycin is active against intracellular *S. aureus*, in spite of its limited cellular accumulation (with apparent cellular-to-extracellular concentration ratios remaining lower than 1). This conclusion, already reached from early studies of daptomycin with *S. aureus*-infected polymorphonuclear neutrophils (63), reinforces our previous conclusions that cellular accumulation per se is not a sufficiently predictive factor of antibiotic intracellular activity (60). The explanation could be that cell-associated daptomycin is accumulated in the same subcellular compartment that phagocytized *S. aureus*. The localization of daptomycin in macrophages has not yet been established, but in vivo studies suggest that daptomycin taken up by proximal tubular cells is partially localized in lysosomes by the endocytosis route (5). Second, we also see that daptomycin is a concentration-dependent antibiotic towards intracellular *S. aureus* as described for extracellular bacteria using appropriate pharmacodynamic models (62). Yet, in spite of this, and in sharp contrast to what is observed with extracellular bacteria, daptomycin never yields a truly intracellular bactericidal effect, since its E_{max} remains lower than the 3 $\log_{10}$ CFU decrease considered the minimum for a drug to be called bactericidal by CLSI criteria. This loss of E_{max} against intracellular *S. aureus* has been a common observation for all antibiotics tested in macrophage models so far disregarding their subcellular localization (see reference 60 for a review) and still needs to receive a satisfactory explanation. Third, our data clearly show that P-gp reduces the potency of daptomycin against intracellular bacteria in relation with its level of expression. Extrapolation to the in vivo situation cannot be ascertained at this stage, since our experiments were conducted under conditions designed to maximize the effects described and used an immortalized cell line, the behavior of which may differ from that of normal macrophages. Yet, this may now help to develop appropriate models to test for such an impact of P-gp expression in vivo. It could also be suggested that concomitant administration of P-gp inhibitors should favorably modulate the activity of daptomycin in difficult-to-treat staphylococcal infections where intracellular survival may play an important role in the maintenance of the infection. This approach, however, seems very unpractical today, in view of the present lack of availability of very safe P-gp inhibitors. Yet, the observation that some fluoroquinolones may act as potent inhibitors of P-gp-mediated efflux in MDCK-MDR1 cells (50) may open new perspectives in this context. A possible caveat, however, is that daptomycin myopathy (one of the drug's most serious side effects) (38) is driven by drug concentrations remaining above some threshold level for an extended period of time (hence directing its clinical use on a once-daily schedule) (37). We cannot exclude that a P-gp inhibitor could amplify this adverse effect. The design and/or selection of new derivatives within the class of acidic lipopeptide antibiotics with a lack of or weak recognition by P-gp could, therefore, also appear as a more useful and a safer approach.

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IV - Insufficient activity of most commonly used anti-staphylococcal agents against *S. aureus* isolates recovered from a patient with persistent bacteriemia and endocarditis: a cause for treatment failure?

Publication #6: Activities of Antistaphylococcal antibiotics towards the extracellular and intracellular forms of *S. aureus* isolates from a patient with persistent bacteriemia and endocarditis.

Sandrine Lemaire, Klaudia Kosowska-Shick, Kathleen Julian, Paul M. Tulkens, Françoise Van Bambeke, and Peter C. Appelbaum

Finally, we used our experimental model of intracellular infection to systematically compare the extracellular and intracellular activities of antibiotics against difficult-to-treat isolates of *S. aureus*.

The present data have been collected on clinical isolates recovered from a patient with prosthetic aortic valve who, as a complication of bacteriemic pacemaker infection, developed an endocardial abscess and persistent bacteriemia.[119] As far as we know, this is the first report describing the intracellular pharmacodynamic of antibiotics against clinical isolates of *S. aureus* recovered from intracellular compartment (heart valves). Using THP-1 macrophages, we provide a potential rationale for the poor clinical and microbiological response in patients infected with these variants, while suggesting potential benefit in using newly developed anti-staphylococcal agents (oritavancin, quinupristin/dalfopristin).

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ABSTRACT

Decreased susceptibility of *S. aureus* to antistaphylococcal agents may be associated with inability to eradicate intracellular forms, which could explain therapeutic failures. We tested this hypothesis using clinical isolates obtained from a patient with persistent staphylococcal bacteremia under therapy. Four isogenic isolates (3 from tissue, 1 from blood) with increased MICs for vancomycin (1 to 4 mg/L) and to daptomycin (1 to 4 mg/L) were collected after an initial 16 days treatment with vancomycin-rifampicin-gentamicin during a subsequent 13-20 days daptomycin-rifampicin-gentamicin treatment. Isolates were tested for MICs and for (i) vancomycin (BODIPY FL-vancomycin) and daptomycin binding, (ii) cell wall turn-over (loss of N-acetyl-D-[1-^{14}C]glucosamine in 30 min after 1 h labelling), (iii) Triton-X-100-induced autolysis. Extracellular (broth) and intracellular (THP-1 macrophages) activities of rifampicin, linezolid, fusidic acid at C_{max}, and of vancomycin, daptomycin, quinupristin/dalfopristin, and oritavancin over a wide range of extracellular concentrations (with pharmacological modelling to determine E_{max}) were measured at 24 h. Increases in vancomycin MICs correlated with increased drug binding, decreased cell wall turn-over, and detergent-induced autolysis. Increases in daptomycin MICs correlated with decreased daptomycin binding. Intracellular activity was weak (E_{max} < 1 $\log_{10}$ cfu decrease) for vancomycin against all isolates and for daptomycin for isolates with MICs > 1 mg/L. Among all antibiotics tested, only quinupristin/dalfopristin and oritavancin provided close to bactericidal intracellular activities (1.6 to 2.5 $\log_{10}$ cfu decreases at C_{max}). Determination of intracellular susceptibility of *S. aureus*, combined with improved methods of diagnostic could be useful when dealing with persistent staphylococcal infections for fine-tuning therapy.

INTRODUCTION

Staphylococcus aureus is an aggressive pathogen that poses a significant public health threat. Treatment of staphylococcal diseases faces two main issues. First, this pathogen has become increasingly resistant to currently available antibiotics, narrowing the choice of useful agents [1,2,22]. In this context, vancomycin-intermediate *S. aureus* (VISA) are being increasingly encountered all over the world and appear to develop from pre-existing methicillin-resistant *S. aureus* in patients undergoing vancomycin therapy [3] These strains are somewhat unique owing to their increased cell wall thickness, the precise mechanism of which is unknown but which is associated with (i) accumulation of free D-alanyl-D-alanine termini in the peptidoglycan (acting as false target sites for vancomycin [13,27]), and (ii) resistance to detergent-induced autolysis [9]. Secondly, there is growing evidence that *S. aureus* has the ability to invade and persist within eukaryotic cells [11,26]. This is reflected clinically by its propensity to cause endocarditis on structurally normal heart valves or metastatic suppurative foci in others sites [34] offering an explanation for frequency and recurrence of such invasive infections.

While the metabolic and phenotypic characteristics of laboratory-generated VISA have been described by several groups [7,9,32], a detailed analysis of isogenic strains has not been frequently attempted [33]. Hence, our study aimed at investigating the global properties of a series of isogenic MRSA recovered from a patient with prosthetic aortic valve who, as a complication of bacteriemic pacemaker infection, developed an endocardial abscess [16]. In addition, giving that the intracellular persistence of *S. aureus* may represent an important nidus for severe endocarditis, we also compared the extra- and intra-cellular activities of several antibiotics commonly recommended or under investigation for the treatment of staphylococcal infections. Concentrating our effort on macrophages using previous established models [6,18], this study provides a potential rationale for the poor clinical and microbiological response in patients infected with these variants, while suggesting potential benefit in using newly developed antistaphylococcal agents.

MATERIALS AND METHODS

Case report

The patient (a 65 year-old, with aortic valve replacement in 1991, coronary artery disease, sick-sinus syndrome, and diabetes mellitus) was hospitalized for fevers that developed two weeks after pacemaker placement, with serosanguinous discharge from pacemaker site, and MRSA bacteremia (vancomycin MIC = 2 µg/mL, daptomycin MIC = 1 mg/L). Details on pertinent clinical events and therapeutic regimens in relation to sample collection are presented in a synoptic fashion in Table 1. Bacteremia was documented over a 33-day span, and resolved only after

debridement of an abscess underneath the aorta sewing ring and aortic valve replacement, strongly suggesting that the maintenance of tissue foci of infection was the main cause of the persistent character of the infection. One year after discharge, the patient was clinically well and showed no sign of relapse.

Table 1: Time course of the infection, successive antistaphylococcal therapies, and vancomycin and daptomycin MICs of *S. aureus* isolated from the blood of the patient at the initiation and during therapy.

Day	Event	Antistaphylococcal treatment	MIC (mg/L) [a]	
			VAN	DAP
0	first hospitalization and blood sampling start initial antibiotic treatment	start vancomycin / gentamicin / rifampicin	2	1
6	removed pacemaker			
13	transfer to Hershey Medical Center isolation of VISA		4	1
15	isolation of DAP-resistant *S. aureus*		2-4 [b]	1-2 [b]
16	vegetation confirmed on prosthetic aortic valve modification of initial treatment	start daptomycin stop vancomycin and rifamicin continue gentamicin		
29	isolation of VISA and DAP-resistant *S. aureus*		4-8 [c]	4 [c]
31	change in antibiotic treatment	start linezolid stop gentamicin and daptomycin		
35	endocardial abscess debrided; aortic valve replaced; sample collection from infected endocardial abscess	transiently add vancomycin and gentamicin	2-8 [d]	1/8 [d]
37-38	change in antibiotic treatment	stop vancomycin and gentamicin continue linezolid add quinupristin-dalfopristin	*	
49	myalgia (related to quinupristin/dalfopristin)	stop quinupristin-dalfopristin continue linezolid add thrimethoprium/sufamethoxazole		
65	hyperkalemia, renal failure	stop thrimethoprium/sufamethoxazole continue linezolid		
77	end of antistaphylococcal treatment	stop linezolid		

[a] determined at the Clinical Laboratory of the Hershey Medical Center (macrobroth dilution method and confirmed by microbroth and E-test methods); the values shown are the extremes observed (see notes a-d).
[b] between day 11th and 27th a total of 14 isolates were obtained: 1 with vancomycin / daptomycin MICs of 2 / 1 mg/L, 12 with vancomycin / daptomycin MICs of 4 / 1 mg/L, and 1 with vancomycin / daptomycin MICs of 4 / 2 mg/L.
[c] between day 29th and 32nd a total of 4 isolates were obtained: 1 with vancomycin / daptomycin MICs of 4 / 1 mg/L, and 3 with vancomycin / daptomycin MICs 8 /4 mg/L.
[d] on day 35, a total of 9 isolates were obtained: 8 with vancomycin / daptomycin MICs of 2 / 1 mg/L, and 1 with both vancomycin / daptomycin MICs of 8 / 8.
*** all blood culture were negative for *S. aureus* (MRSA or VISA) as from day 36.**

Bacterial isolates and reference strains, determination of MICs and of agr polymorphism

The clinical isolates used in the present study (see Table 2) have been described previously [16]. In parallel, we used a fully sensitive S. *aureus* strain (ATCC 25923). A vancomycin-intermediate *S. aureus* strain (NRS 126, obtained from NARSA via Eurofins, Herndon, Va; vancomycin MIC = 4 mg/L) was also used for control purposes. MICs (using both geometric and arithmetic dilutions) were determined as previously described [18] (for oritavancin, all media contained 0.002 % polysorbate [Tween 80 or 2-[2-[3,4-bis(2-hydroxyethoxy)oxolan-2-yl]-2-(2-hydroxyethoxy)ethoxy]ethyl(E)-octadec-9-enoate] as advised by the manufacturer to prevent drug binding to plastic). The accessory gene regulator (*agr*) polymorphism was determined by PCR [31,38].

Binding of vancomycin and daptomycin to whole bacteria

Drug binding was measured using boron dipyrromethene difluoride-labelled vancomycin (Bodipy® FL vancomycin; Invitrogen Corp., Carlsbad, CA), or unmodified daptomycin. Bacteria were exposed to either drug (Bodipy-FL-vancomycin, 1 mg/L; daptomycin, 100 mg/L) for 30 min at 37°C

in Mueller Hinton Broth containing 2 % NaCl and adjusted to pH 7.4, and harvested by centrifugation, washed with ice-cold Phosphate Buffered Saline (PBS) and lysed as described previously [17]. Both drugs were then assayed by fluorimetry (Fluorocount TM [multiplate reader], Packard Instruments, PerkinElmer Life And Analytical Sciences, Inc., Waltham, Mass.), with excitation and emission wavelengths set at 485 and 530 nm for vancomycin (assay linearity: 0.6-4 mg/L; $R^2 > 0.99$), and 380 and 425 nm for daptomycin [19] (linearity: 1-250 mg/L; $R^2 > 0.99$ [due to instrument limitations, we could not use the optimal emission wavelength (460 nm), but this reduced the signal value of about 50 % only]). Protein content was determined in parallel by using the Folin-Ciocalteu / biuret method [20].

Autolysis assay

Triton X-100-stimulated autolysis in 50 mM glycin buffer (pH 8) was assessed after 30 min following a previously described [33].

Cell wall turnover

Cells were labelled for 1 h at 37°C with 5 µg of N-acetyl-D-[1-^{14}C]glucosamine (55 mCi/mmol; Amersham plc, Little Chalfont, Buckinghamshire, UK). After labelling, bacteria were harvested by centrifugation and pellets resuspended in isotope-free medium containing 5 mM of non-radioactive N-acetyl-D-glucosamine [32], and collected either immediately (time 0 h) or after 30 min of incubation at 37°C. After washing in ice-cold PBS, bacteria were resuspended in 10 % ice-cold trichloroacetic acid - 100 mM N-acetyl-D-glucosamine [10] and radioactivity measured by liquid scintillation counting. Cell-wall turnover was the expressed as the percentage of radioactivity released during the 30 min post-labelling incubation period.

Determination of extracellular and intracellular activities.

Extracellular activities were measured in broth as described earlier [30]. Intracellular activities were measured on bacteria phagocytozed by THP-1 macrophages (ATCC TIB-202), a human myelomonocytic cell line displaying macrophage-like activity (American Tissue Collection; supplied through LGC Promochem Ltd, Teddington, UK) as also described in details [6,17]. Typical starting inocula were about 1-2 x 10^6 cfu per ml (broth) or per mg of cell protein (macrophages).

Antibiotics and main reagents

The following antibiotics were obtained as microbiological standard from their corresponding manufacturers: linezolid from Pfizer Inc, New York, NY; quinupristin-dalfopristin (30:70 mass ratio) from Nordic Pharma, Paris, France; oritavacin from Targanta Therapeutics Corp., Cambridge, Mass.; daptomycin from Novartis AG, Basel, Switzerland; teicoplanin from Sanofi-Aventis, Paris, France, rifampicin from Merrell Dow Pharmaceuticals, Strasbourg, France; ciprofloxacin and moxifloxacin from Bayer HealthCare, Leverkusen, Germany. Gentamicin and vancomycin were obtained as the corresponding branded products (GEOMYCIN®; VANCOCIN®) distributed in Belgium for human use by Glaxo-SmithKline s.a., Genval, Belgium. Oxacillin and fusidic acid were purchased from Sigma-Aldrich, St-Louis, MO. Cell culture media and sera were from Invitrogen and other reagents from Sigma-Aldrich or Merck KGaA (Darmstadt, Germany).

Statistical analyses

Curve-fitting analyses were made using GraphPad Prism® version 4.02 for Windows (GraphPad Prism Software, San Diego, CA).

RESULTS

Antibiotic susceptibilities (MICs)

Table 2 shows the clinical isolates used for the present study with their anatomical source and MICs against selected antibiotics (all these isolates were resistant to oxacillin [MIC ≥ 16 mg/L; all strains carried the *mecA* and the *SCCmec* group IV a genes], to fluoroquinolones [ciprofloxacin MIC ≥ 32 mg/L; moxifloxacin MIC ≥ 2 mg/L] and to rifampicin [MIC ≥ 4 mg/L]). Compared to the fully susceptible strain ATCC 25923 and with the exception of strain HMC 546, all the clinical isolates also showed a reduced susceptibility towards vancomycin and teicoplanin. Susceptibilily to daptomycin was also impaired, even for the two VSSA strains (HMC 546 and HMC547) and still more for the two VISA strains. In contrast, the MICs of fusidic acid, quinupristin/dalfopristin and linezolid towards all clinical isolates were low and similar to those observed for of ATCC 25923,

and in the same range as observed in a recent survey of MRSA and VISA isolates [8]. For oritavancin, MICs were 2 to 8-fold larger for the clinical isolates compared to ATCC 25923 but still 2-3 $\log_2$ dilutions lower than those of vancomycin.

TABLE 2. Clinical isolates, source of isolation and susceptibilities to selected antibiotics (all isolates are resistant to oxacillin, ciprofloxacin, moxifloxacin, and rifampicin [see text]) in comparison with a fully-sensitive reference strain.

Isolate / strain*	Source of isolation	MIC (mg/L) [a]						
		vancomycin	Teicoplanin	daptomycin	linezolid	Q/D	Fusidic acid	oritavancin
HMC 546 (#3)	Wound; Aortic valve [b]	1 (1.25)	2	1-2	1	0.5	0.5	0.125
HMC 547 (#6)	Subvalvular tissues [b]	2 (2)	4	3	1	0.5	0.5	0.125
HMC 548 (#10)	Annulus vegetations [b]	4 * (3.5)	8	4	1	0.5	0.5	0.5
HMC 549 (#17)	Blood [c]	4 * (4.0)	8	4	1	0.5	0.5	0.5
ATCC 25923	reference strain	1 (0.9)	1-2	0.125-0.25	1	0.5	0.5	0.06

* numbers between brackets are those used in the original description of the isolates [16]
[a] as determined by geometric ($\log_2$ progression) dilution for all antibiotics by microdilution [8] (for vancomycin, figures between brackets refer to the MICs determined by arithmetic dilutions using 0.25 mg/L increments)
[b] isolated at day 35 (see Table 1)
[c] isolated at day 15 (see Table 1)
* defined as VISA (vancomycin-Intermediate) based on definition of CLSI (MIC ≥2 mg/L [35])

Characterization of vancomycin reduced susceptibility

VISA strains have been reported to contain more cell-wall sub-units able to bind vancomycin [13], to have reduced cell-wall turnover [32], and to be more resistant to Triton-X-100 autolysis [32] in comparison with susceptible strains. These characteristics were, therefore, examined here for 4 selected clinical isolates with different susceptibilities to vancomycin (HMC 546 and 547 [VSSA] and HMC 548 and 549 [VISA]). As shown in Figure 1 (panels A, B, and C), we found that vancomycin binding increased, and cell wall turnover and Triton-X-100-induced autolysis decreased roughly in parallel to the increase in MIC, consistent with the concept that vancomycin decreased susceptibility is related to thickening of the bacterial cell wall.

Earlier studies demonstrated that vancomycin treatment failure is often associated with group II polymorphism of the *agr* locus [24]. All clinical isolates belonged to the *agr* group II based on PCR analysis.

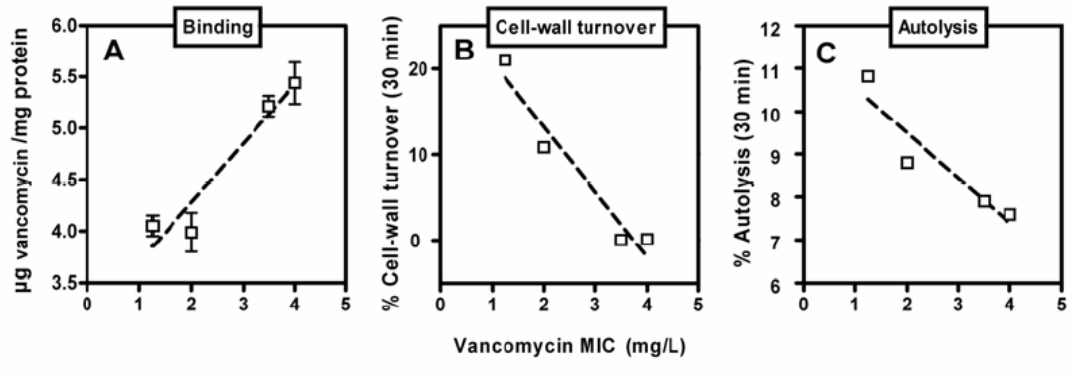

Figure 1. Correlation between vancomycin susceptibility (MIC, as determined by arithmetic dilution) and vancomycin binding (A), cell wall turnover (B), Triton-X-100-induced autolysis (C) of clinical isolates (from left to right in each graph: HMC 546 [MIC=1.25 mg/L; HMC 547 [MIC=2 mg/L]; HMC 548 [MIC=3.5 mg/L], and HMC 549 [MIC=4 mg/L]). All values are means ± SD (n = 3; when not visible, SD bars are smaller than the symbols). The dotted lines show the result of a linear regression analysis of each set of data. Values for the fully-sensitive ATCC 25923 strain (MIC=1 mg/L) were: vancomycin binding, 4.3 ± 0.2 µg/mg prot.; cell-wall turnover, 23.3 ± 0.3 %; autolysis, 12.3 ± 0.2 %.

Characterization of daptomycin resistance

Because all clinical isolates (including the two VSSA HMC 546 and 547) showed MICs above the clinical susceptibility breakpoint (1 mg/L) of EUCAST and of the US-labelling, we measured the binding of the drug to whole bacteria preparations from these isolates (HMC 546 and 549) in comparison with the fully-susceptible reference strain ATCC 25923 and a reference VISA strain (NRS 126). As shown in Figure 2, binding of daptomycin for both clinical isolates was considerably reduced compared to what was observed for the fully-sensitive strain. There was also a clear correlation between reduced binding and increase in MIC when considering all data points.

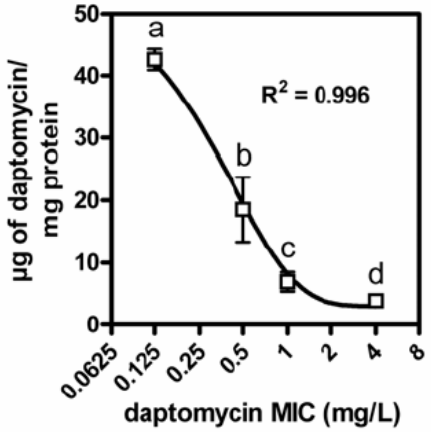

Figure 2. Correlation between binding of daptomycin to whole bacterial preparations (± SD [n=3]) and MIC (as determined by geometric dilution [in duplicate; no difference between individual values]) for (from left to right) the fully-sensitive reference strain ATCC 25923 (MIC = 0.125 mg/L), the VISA reference strain NRS 126 (MIC = 0.5 mg/L) and the two clinical isolates (HMC 546 [MIC = 1 mg/L] and HMC 549 [MIC = 4 mg/L]). The graph shows the regression function obtained by fitting a sigmoid equation to the data. Statistical analysis (ANOVA): data points with different letters are significantly different from each others ($p < 0.01$).

Assessment of the intracellular activities of antibiotics

In earlier studies, we showed that the activity of antibiotics is, generally speaking, markedly impaired intracellularly compared to what can be observed for bacteria maintained in broth or in culture medium [6,29,30]. Since this phenomenon could explain therapeutic failures in spite of a acceptable susceptibility as determined by conventional MIC measurement, we systematically compared the extracellular and intracellular activities of antistaphylococcal antibiotics towards HMC 546 strain in comparison with the fully-susceptible ATCC 25923 using our model of infected THP-1 macrophages.

In the first approach, we compared all drugs at a concentration (total drug) corresponding to their observed or estimated C_{max} (total drug)when administered to humans at conventional doses (rifampicin, 4 mg/L; vancomycin, 50 mg/L); daptomycin, 77 mg/L; linezolid, 20 mg/L; fusidic acid, 50 mg/L; quinupristin-dalfopristin, 10 mg/L; oritavancin, 40 mg/L). Results are presented in Figure 3 and show first that the loss of activity of all antibiotics when tested towards an intracellular form of *S. aureus* was as important for the clinical isolates as for the reference strain (the loss was marginal for fusidic acid, but this antibiotic is essentially bacteriostatic against both forms). For vancomycin, activity was not much different between the reference strain and HMC 546 and HMC 549, indicating that the concentration used (50 mg/L) was giving maximal activity, disregarding the differences in MIC. In contrast, the decreased susceptibility of HMC 546 and 549 towards daptomycin in comparison with the reference strain (MIC = 1-2 vs. 0.12-0.25 mg/L) resulted in this drug loosing almost completely its ability to kill intracellular bacteria even though it was used at a concentration much above its MIC (77 mg/L). Quinupristin/dalfopristin and oritavancin showed an intermediate behaviour, with extracellular activity decreasing in parallel with the increase in MIC, while the intracellular activity was only marginally affected. Linezolid and fusidic acid activities were essentially bacteriostatic in macrophages and their activities was not markedly different between the clinical and the reference strain.

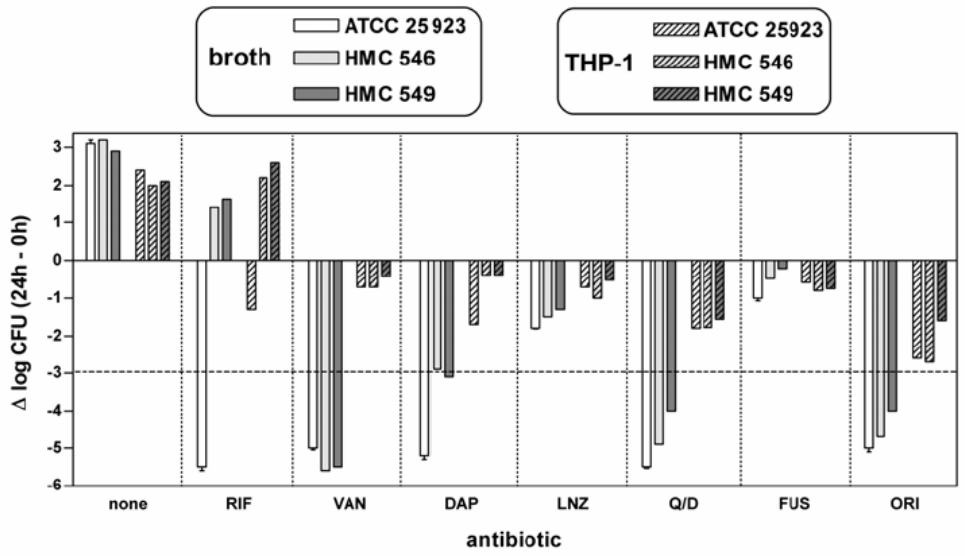

Figure 3. Comparison between extracellular (broth; unhatched bars) and intracellular (THP-1 macrophages; hatched bars) activities of antistaphylococcal antibiotics against the fully-susceptible ATCC 25923 strain (white bars), the HMC 546 isolate (VSSA; light grey bars), and the HMC 549 isolate (dark grey bars). The ordinate shows the change in the number of cfu (Δ log CFU) per ml of culture medium (broth) or per mg of cell protein (THP-1). Each antibiotics was added for 24 h at a concentration corresponding to its known or estimated human serum C_{max} (total drug; rifampicin [RIF, 4 mg/L]; vancomycin [VAN, 50 mg/L]; daptomycin [DAP, 77 mg/L]; linezolid [LNZ, 20 mg/L]; fusidic acid [FUS, 50 mg/L]; quinupristin-dalfopristin [Q/D]; 10 mg/L]; oritavancin [ORI, 40 mg/L]). The dotted horizontal line shows the decrease in cfu (3 log_{10} units) commonly taken as an indication of a bactericidal effect [25].

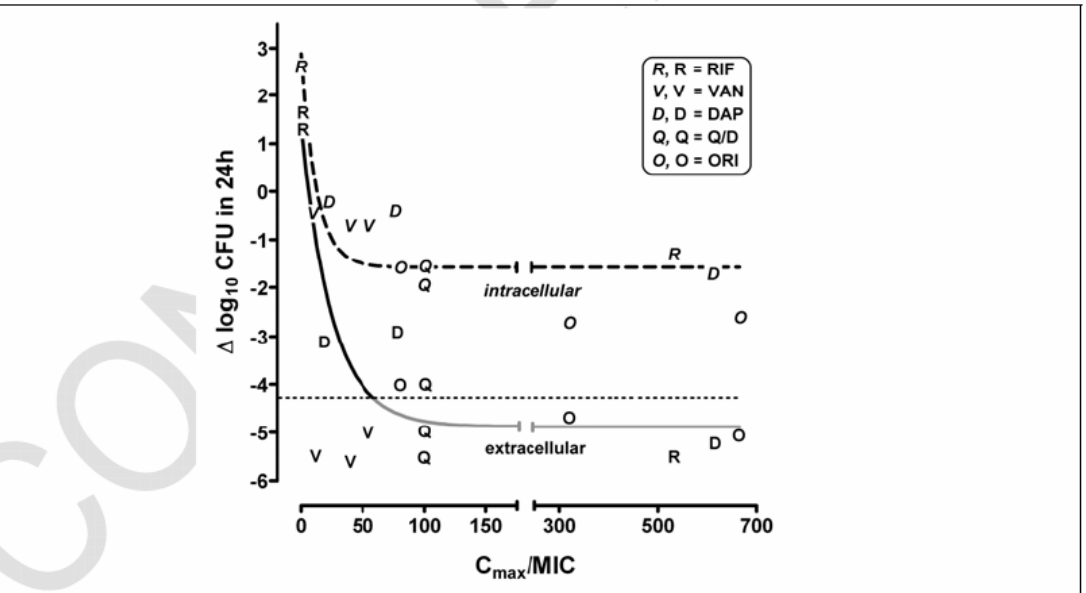

Figure 4. Extracellular and intracellular activities of rifampicin (R), vancomycin (V), daptomycin (D), quinupristin/dalfopristin (Q), and oritavancin (O) towards ATCC 25923, HMC 546, and HMC 549 when plotted against the corresponding C_{max}/MIC ratio (see Figure 3 for activity and C_{max} values and Table 2 for MICs). Data were used to fit "one phase exponential decay" functions (dotted line, intracellular bacteria; solid line, extracellular bacteria) to help identifying the corresponding apparent maximal killing values (-1.6 log_{10} cfu for intracellular and -4.9 log_{10} cfu for extracellular bacteria, compared to the original inoculum) and the C_{max}/MIC ratio at which these are neared. The horizontal dotted line indicates the limit of reliability of bacterial counts by the technique used (see [6] for details). The real maximal killing activity towards extracellular bacteria could, therefore, be more extensive than estimated here.

Finally, the resistance of HMC 546 and 549 towards rifampicin in broth (MIC = 4 mg/L) translated in a complete loss of activity against its intracellular form when used at that concentration. None of the antibiotics tested had a bactericidal activity against the intracellular forms of either the reference or the clinical strains (defined by a 3 log_{10} decrease of the cell-associated cfus [5]) even though oritavancin approached it for ATCC 25923 and HMC 546, but not HMC 549.

Figure 4 shows the data of Figure 3 expressed as a function of the concentration/MIC ratio for each drug showing bactericidal against bacteria in broth (rifampicin, vancomycin, daptomycin, quinupristin/dalfopristin, and oritavacin). It clearly appears that an intracellular activity of about 1.5-2 $\log_{10}$ cfu decrease is maximal and reached once the concentration/MIC ratio exceeds 50 whatever the antibiotic/strain combination considered. In this context, the lower response of daptomcyin and the lack of response of rifampicin with HMC 546 and HMC 549 could be related to the lower concentration/MIC ratios obtained with these combinations.

To gain more insight in the concentration-effect relationships of the antistaphylococcal antibiotics against the clinical isolates HMC 546 (VSSA) and HMC 549 (VISA) in comparison with the fully sensitive reference strain ATCC 25923 [6,30], we ran full dose-response experiments with vancomycin, daptomycin, quinupristin/dalfopristin and oritavancin using a wide range of extracellular concentrations spanning from sub-MIC values to C_{max} or above (vancomycin, 0.005-50 mg/L; daptomycin, 0.01-250 mg/L; quinupristin/dalfopristin, 0.01-25 mg/L; oritavancin, 0.01-40 mg/L). The data were then used to determine the corresponding Hill functions. This approach allows to distinguish between changes affecting the drug efficacy (by calculating the E_{max} parameters [relative efficacies]) with accuracy) from those affecting the drug potency (by calculating the corresponding EC_{50} parameters [relative potencies]) [6]. Data are presented in Figure 5, with the corresponding Hill equation descriptors presented in Table 3 together with the regression parameters, an estimation of the static concentration of each antibiotic (C_s) and the C_{max}/Cs ratios. In all cases, activity was related to concentration, obeying the pharmacological model previously described for the reference strain [6]. This revealed significant differences in relative efficacies (E_{max}) the absolute values of which were significantly (i) larger for quinupristin/dalfopristin and oritavancin compared to vancomycin or daptomycin for all strains; (ii) lower for daptomycin when comparing the two clinical isolates to the ATCC reference strain. Interestingly enough, however, no statistically significant difference in E_{max} was noted between HMC 546 (VSSA) and 549 (VISA) for each of the antibiotic tested, indicating that the reduced susceptibility of the VISA isolate for vancomycin and daptomycin, as detected by the measurement of their MIC, was only relative (and could be compensated by an increase in concentration).

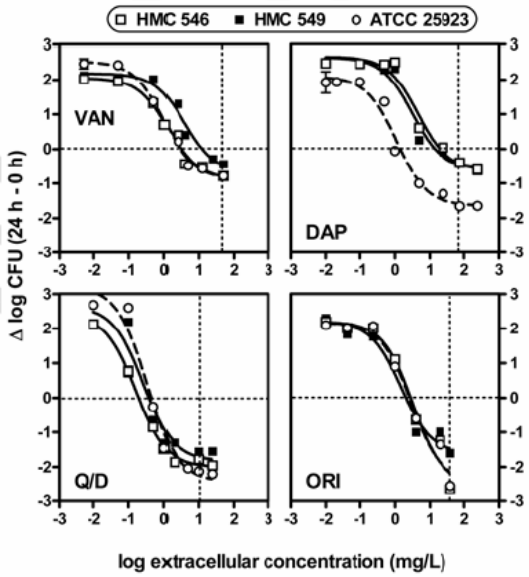

Figure 5. Activities of 4 selected antibiotics (VAN, vancomycin; DAP, daptomycin; Q/D, quinupristin/dalfopristin; ORI, oritavancin) towards the intracellular forms of the reference fully sensitive strain ATCC 25923 (open circles) and of the two clinical isolates HMC 546 (VSSA; open squares) and HMC 549 (VISA; closed squares) after phagocytosis by THP-1 macrophages. Cells were incubated with the antibiotic for 24 h at the concentrations (total drug) indicated on the abscissa (VAN, 0.005-50 mg/L; DAP, 0.01-250 mg/L; Q/D, 0.01-25 mg/L; ORI, 0.01-40 mg/L). The graphs show the change in the number cfu (Δ log CFU) per mg of cell protein. All values are means ± SD (n=3; when not visible, the SD bars are smaller than the size of the symbols). Data were used to fit sigmoidal dose-response curves using a standard model with a slope factor [Hill coefficient] of 1 (see Table 3 for regression parameters). The horizontal dotted line corresponds to a static effect (no net change from the initial post-phagocytosis inoculum; the vertical dotted line indicates the extracellular concentration (total drug) corresponding to the observed or estimated serum C_{max} in patients receiving conventional dosages of the corresponding antibiotic and used for the comparison of activities shown in Figure 3).

TABLE 3. Pertinent regression parameters, statistical analysis, and calculation of static concentrations from the dose-responses curves illustrated in Figure 2.

Antibiotic	Strain	Regression parameter value (95 % confidence intervals) *			Static conc. (mg/L)[3]	C_{max}/Static conc ratio
		E_{max} (mg/L)[1,4]	EC_{50} (mg/L)[2,4]	R^2		
VAN	ATCC 25923	-0.87 (-1.14 to -0.60) a,A	0.99 (0.62 to 1.59) a,A	0.995	2.9	17
	HMC 546	-0.87 (-1.27 to -0.47) a,A	1.33 (0.70 to 2.51) a,A	0.979	3.1	16
	HMC 549	-0.68 (-1.32 to -0.04) a,A	3.79 (1.47 to 9.78) a,A	0.974	12	4.2
DAP	ATCC 25923	-1.64 (-2.04 to -1.26) b,B,E	1.13 (0.59 to 2.16) a,A	0.980	1.4	55
	HMC 546	-0.61 (-1.16 to -0.06) a,A	3.20 (1.66 to 12.5) a,A	0.971	20	3.8
	HMC 549	-0.59 (-1.14 to -0.05) a,A	4.56 (1.11 to 9.16) a,A	0.967	14	5.4
Q/D	ATCC 25923	-2.42 (-3.27 to -1.58) a,C,E	0.34 (0.12 to 0.94) a,C	0.973	0.46	21.7
	HMC 546	-2.01 (-2.25 to -1.78) a,C,E	0.16 (0.11 to 0.25) a,C	0.995	0.19	53
	HMC 549	-1.82 (-2.24 to -1.50) a,C,E	0.26 (0.05 to 1.27) a,C,E	0.934	0.38	26
ORI	ATCC 25923	-2.46 (-3.50 to -1.42) a,C	2.95 (1.02 to 8.49) a,A	0.976	2.6	15
	HMC 546	-2.54 (-3.79 to -1.57) a,C	3.24 (0.93 to 11.2) a,A	0.967	2.7	15
	HMC 549	-1.61 (-2.44 to -0.77) a,C,B	1.51 (0.46 to 4.81) a,A,D	0.968	2.0	20

[1] cfu decrease (in $\log_{10}$ units) at 24 h from the corresponding original inoculum, as extrapolated for antibiotic concentration at infinity.
[2] concentration (mg/liter) causing a reduction of the inoculum halfway between the initial (E_0) and the maximal (E_{max}) values, as obtained from the Hill equation (by using a slope factor of 1).
[3] Concentration (mg/liter) resulting in no apparent bacterial growth (the number of CFU was identical to that of the original inoculum), as determined by graphical interpolation.
[4] Total drug.
* Statistical analysis (one-way ANOVA; Tukey's test for multiple comparisons): values with different letters are significantly different from each others within the pertinent comparison group ($p < 0.05$); lowercase letters, comparison between the two isolates for each condition [antibiotic]; uppercase letters, comparison throughout all conditions.

Differences in relative potencies (EC_{50}) were also noted between vancomycin and daptomycin on the one hand and quinupristin/dalfopristin and oritavancin on the other (variations in the oritavancin responses made, however, this difference not statistically significant for oritavancin). Finally, whereas the C_{max} to static concentration ratio ranking was clearly in favour of daptomycin for the reference strain ATCC 25923, this ranking became quinupristin/dalfopristin > vancomycin ≈ oritavancin >> daptomycin for HMC 546 (VSSA), and quinupristin/dalfopristin > oritavancin >> daptomycin ≈ vancomycin for HMC 549 (VISA).

DISCUSSION

Vancomycin has long been considered as the drug of choice for the treatment of MRSA infections. Yet, isolates of *S. aureus* with reduced vancomycin susceptibility, first reported in Japan [14] appear now widespread [3] and call for both a better knowledge of the biochemical changes associated with this phenotype (improving thereby our diagnostic capabilities) and a critical assessment of the proposed therapies. The present report aims at extending our knowledge of these strains in three main directions.

Firstly, we were able to correlate three critical biochemical properties (binding of vancomycin, cell wall turn-over, and Triton-X-100-induced autolysis) with changes in vancomycin susceptibility in a series of isogenic MRSA isolates obtained from a single patient under vancomycin and daptomycin treatments given in succession [16]. This should allow for a more confident interpretation of these altered properties, rather than variations occurring for unrelated clinical isolates. Thus, we showed that the decrease in susceptibility of the isolates collected during treatment with vancomycin is directly correlated with an increase in the drug binding to the bacteria, a reduction of the cell wall turn-over, and a decreased susceptibility to Triton-X-100 autolysis. This supports the concept that the VISA phenotype developing *in vivo* is due to bacteria building up an "antibiotic-trapping" mechanism through the thickening of their cell wall [27]. Combined with the detection of *agr* group II polymorphism (more frequent in VISA strains [28] and predictor of vancomycin treatment failures [24]), these biochemical characteristics could be used for improving the early and reliable diagnostic of VISA isolates in clinical samples. Further studies, however, will be needed to confirm these correlations on more clinical and laboratory strains and to fully demonstrate the specificity and

selectivity of these biochemical changes with the alterations in susceptibility. Moreover, because these binding experiments or cell wall turnover measurements are difficult to implement in routine practice, simple tests will need to be developed. This may, actually, become critical given the present situation of poor recognition of the reduced susceptibility of *S. aureus* to vancomycin with available automated commercial tests, especially in the case of heterogeneously resistant phenotype [15].

Secondly, the rapid emergence of resistance to daptomycin, although already observed by others [23] is puzzling. The isolates studied here had both cell wall changes (reduction in muramic acid *O*-acetylation) and point mutations (in *mprF*, encoding lysylphosphatidylglycerol synthase) [16]. We now show that increases in daptomycin MIC's correlated with an almost commensurate reduction of drug binding to the bacteria (as assessed by fluorometric determination), which has not been reported so far. Decreased daptomycin susceptibility in VISA isolates is commonly ascribed to the thickening of their cell wall which shields the daptomycin target (phophatidylglycerol) [13]. While this could explain the decreased daptomycin binding seen with HMC 548 or 549, it probably cannot account for our observations with HMC 546, since this isolate was vancomycin-susceptible and showed no increase in vancomycin binding or decreased susceptibility to detergent-induced autolysis. We may, therefore deal here with a true reduction of target abundance or shielding through another mechanism than cell wall thickening. This will need to be explored in more details.

Thirdly, although primarily known as an extracellular pathogen, *S. aureus* has been shown to invade and survive within the eukaryotic intracellular environment [21,34]. Persistence in tissue, most likely intracellularly, probably accounts for several aspects of relapsing staphylococcal diseases, long-term colonization, and persistence of bacteraemia. Our previous studies using reference strains of *S. aureus* with various susceptibility phenotypes [5,6] showed that conventional antistaphylococcal agents are considerably less efficient against intracellular bacteria than against their extracellular counterparts. The present study extends this concept to clinical strains and shows also that the pharmacological model derived from the analysis of the reference strain ATCC 25923 is also valid for these strains. In all cases, the lack of bactericidal effect of the antistaphylococcal agents towards intracellular bacteria could be ascribed to a loss of relative efficacy (E_{max}), explaining why a large intracellular inoculum could persist regardless of the extracellular concentration of the antibiotic [5,6]. Moreover, we also show that a minimal extracellular concentration/MIC ratio of 50 is probably necessary to near a maximal antibacterial effect, and that, even for highly bactericidal antibiotics such as oritavacin, quinupristin/dalfopristin, or rifampicin, this effect is unlikely to be improved by further increasing the drug concentration/MIC ratio. The present study allows also drawing two important conclusions in relation to the treatment failures seen with the patient presented in this study. First, a phenotype of resistance to rifampicin (MIC $\geq$ 4 based on current CLSI breakpoints; no breakpoint is yet defined by EUCAST) makes the drug completely inactive against the intracellular forms of *S. aureus*, even if used at a concentration that matches its MICs towards extracellular bacteria. This was surprising since rifampin accumulates in macrophages (about 20-fold in the model used here [6], and its activity is enhanced by acid pH (about 30-fold decrease in MIC for ATCC 25923 [6]). Second, a reduced susceptibility to vancomycin (MIC > 2 mg/L [15]; see also the current CLSI breakpoint) or to daptomycin (MIC > 1 [15]; see also the CLSI and the EUCAST breakpoints) makes both drugs poorly active against intracellular forms even if used at extracellular concentrations far above their MIC. We did not measure the accumulation of vancomycin and daptomycin in cells infected by HMC 546 or HMC 549 in comparison with those infected with ATCC 25923 and cannot exclude, therefore, that the losses of intracellular activity observed for HMC 546 and 549 are due to pharmacokinetic parameters only. However, differences in pharmacodynamic parameters, due to target modifications, are more likely. Whatever the mechanism, however, it is tempting to speculate that the low intracellular efficacies of rifampicin, vancomycin, and daptomycin towards the clinical isolates studied here is the reason why infection could not be cured, and why the infected tissues, eventually, had to be surgically removed. It would be interesting, in this context, to examine the effects drug combinations towards the intracellular forms if the clinical strains of this patient, since combinations are known to be synergistic against susceptible strains [4]. This is probably most critical for vancomycin and daptomycin, because our pharmacological analysis (Figure 5) shows that vancomycin has only limited efficacy towards all strains and that daptomycin maximal efficacy is reduced for strains showing an elevated MIC in a manner than cannot be compensated by an increase in its concentration.

Our study also shows that fusidic acid, which in many countries is considered as a useful resource for difficult-to-treat MRSA infections caused by organisms with an MIC < 1 or 2 mg/L [12] is actually poorly effective against intracellular forms. Conversely, quinupristin/dalfopristin and oritavancin were quite active against the intracellular forms of all clinical isolates tested, regardless of their resistance phenotype. The superiority of oritavacin compared to vancomycin, including against VISA strains, is consistent with its dual mode of action that involves not only its binding to D-Ala-D-Ala motifs like vancomycin but also transglycosylase inhibition and membrane depolarization and permeabilization [36]. Conversely, the mode of action of quinupristin/dalfopristin, for which synergism between the two components is due to induction an increased ribosome affinity [37], is less suggestive apart from the fact that this combination is bactericidal in broth whereas single components are bacteriostatic only.

While our model has many limitations that have been discussed in previous reports [5,6], such a the use of a constant exposure to the antibiotics, the impossibility to evaluate the influence of the defeating effect of protein binding on drug efficacy, or the many uncertainties concerning relevance to the *in vivo* situation, it may nevertheless prove useful for a comparative assessment of antistaphylococcal drugs and characterization of better agents in the future. Combined with the improved methods of diagnostic suggested above, assay of intracellular activity could also help, as from today, in the choice of appropriate therapies among available agents for difficult-to-treat staphylococcal infections when persistence of intraphagocytic *S. aureus* is suspected.

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DISCUSSION AND PERSPECTIVES

For a long time, *Staphylococcus aureus* has been only considered as an extracellular pathogen. Over the last decades, it became however quite well recognized that this bacterium has also abilities to survive and thrive intracellularly. This intracellular stage is believed to act as a reservoir of surviving *S. aureus* that may account for relapses and recurrences. Routine evaluation of antibiotic activity (such as MIC determinations, time- and dose-kill studies), which is useful to predict the therapeutic outcome when dealing with extracellular *S. aureus*, does not predict efficacy against intracellular bacteria. In this context, our work has focused on the evaluation of series of antibiotics against both extracellular and intracellular forms of *S. aureus,* in order to delineate how their activity could be modified by the intracellular milieu.

1. Interest of the model of intracellular infections

1.1. Choice of the cell type

We were interested in examining whether any differences of *S. aureus* susceptibility to antibiotics may be observed between cells differing from: (i) their origin (human vs. animal cells), (ii) their immune status (professional and non-professional phagocytes); (iii) the subcellular compartment hosting intracellular *S. aureus* (phagolysosomial model [e.g. macrophages, keratinocytes] vs. phagolysosomial – cytosolic model [e.g. endothelial cells]); (iv) the level of expression of efflux transporters (basal level of expression or surexpression).

By and large, our studies have shown little differences, if any, between macrophages (whether activated or not) and keratinocytes with respect to what has been measured in our work (intracellular rate of bacterial growth, intracellular susceptibilities to antibiotics). This may be quite surprising and will need to be further investigated. The simplest explanation may be that we deal with an intraphagosomal infection, and that many of the factors that could modulate cell uptake and activity of antibiotics based on the histological character of the cells and/or their activation may not extend to this compartment. Conversely, our studies and those of A. Olivier (data accepted for the 18th European Congress of Clinical Microbiology and Infectious Diseases [ECCMID, which be held in Barcelona in april 2008]) suggest that endothelial cells may behave very differently. This is probably related to the different subcellular localization of *S. aureus* within these cells, as shown earlier by Menzies and colleagues[153], and more recently confirmed in our laboratory by A. Olivier. Therefore, a key message may be that different cell types can be used for the type of study we have undertaken, but that attention should be paid to the way *S. aureus* interact with them. A priori, we could suggest to utilize, for each type of investigation, the more relevant cell type in relation to the type of infection under study.

1.2. Choice of bacterial isolates

While earlier studies have only considered laboratory-derived strains (mostly sensitive to all antibiotics, which does not reflect at all the current clinical situation), we were interested in examining whether any differences of intracellular killing by antibiotics could be observed between: (i) recent clinical isolates of *S. aureus* differing in their response to antibiotics (β-lactamase producing *S. aureus*, HA-MRSA, CA-MRSA, VISA, VRSA, …); (ii) recent *S. aureus* isolates recovered from presumed *in vivo* intracellular compartments (such as heart valves), (iii) a recent MRSA of animal origin.

Of interest, we observed that the general behavior of *S. aureus* (intracellular rate of bacterial growth, intracellular susceptibility to antibiotics) is actually not markedly influenced by the origin of the strain used, beyond what could be anticipated from its pattern of antibiotic resistance. This justifies, a posteriori, the previous use of well defined, laboratory strains for the pharmacological studies undertaken here. In other words, while it is obviously interesting to use clinical isolates in specific studies, their use does not appear mandatory for delineating and characterizing their general response to antibiotics in the intracellular milieu. The use of resistant strains, on the other hand, allows to critically assessing the capacity of (i) novel antibiotics to exert their activity in this environment (viz. our studies with daptomycin); (ii) conversely, to disclose unanticipated restoration of antibiotic susceptibility of resistant strains to antibiotics when exposed to the conditions prevailing intracellularly (viz. our studies with β-lactams and MRSA). A key message here could be that well defined, laboratory strains can be used for most investigations related to the pharmacology of the intracellular antibiotics, keeping clinical isolates and resistant strains for specific studies aimed at defining the potential of new drugs and/or at delineating new approaches specific to these isolates.

1.3. Pharmacological evaluation of antibiotic activity

The interaction of antibiotics with eukaryotic cells and their influence on the fate of intracellular bacteria is of therapeutic importance. In the screening of potentially effective antibacterial agents against intracellular pathogens, it is crucial to critically examine the cellular properties of antibiotics related to their pharmacokinetic (including antibiotic uptake and efflux in eukaryotic cells, intracellular disposition and metabolism) or pharmacodynamic (such as the capacity of antibiotics to exert activity intracellularly, or the influence exerted by the intracellular milieu on the intracellular fate of *S. aureus*).

1.3.1. Evaluation of the pharmacokinetics of antibiotics

Our study has addressed this aspect only in a limited fashion because we quickly realized that there is only a poor correlation between intracellular activity and accumulation of the antibiotics we have studied. This aspect, critically examined by Van Bambeke and colleagues[257], will need to be further experimentally studied. For instance, our observation that an inhibition of daptomycin efflux by impairment of the activity of the P-glycoprotein does not readily explain its increased activity against intraphagosomal *S. aureus*, because this inhibition is supposed to increase the cytosolic and not the phagolysosomal content of the cell in daptomycin. This apparent paradox can be, however, explained by the recent findings of Fu and Roufogalis. Using a P-gp-enhanced green fluorescent protein (EGFP) stable expression system in human breast cancer cells (MCF-7 cell line)[131], they were able to detect P-gp not only in membranes, but also in acidic organelles. Consequently, this apparent paradox can only be addressed by undertaking detailed studies aimed at tracing the subcellular fluxes and disposition of the drug.

Another point which also needs to be addressed is that all our experiments were made with drug concentrations that have remained constant (apart from their spontaneous degradation) throughout the observation period. This situation is far remote from the one prevailing in patients (unless using continuous infusion). It was a limit imposed by our experimental set-up that will need to be corrected in the future. Yet, it was for us the only way to begin the type of study we report in this Thesis in a systematic and thorough fashion.

1.3.2. Evaluation of the pharmacodynamics of antibiotics

Early studies assessing the intracellular activity of antibiotics generally expose the intracellular bacteria to a fixed concentrations of drug[168,174,223]. In our experiments, the bactericidal pharmacodynamics of antibiotics against intracellular *S. aureus* were analysed by using both time- and dose-response (concentrations of antibiotics ranging from sub-MIC values to clinically meaningful concentrations [C_{max}]) approaches. In addition to their use for preclinical trials, these pharmacodynamic models are also suitable to provide informations: (i) on key pharmacological descriptors (50 % effective concentration [EC_{50}]; static concentration [C_{static}]; maximal efficacy [E_{max}]); (ii) and on the optimal dosing and scheduling of antibiotics in the intracellular milieu.

In this respect, our approach has been quite innovative. Indeed, it is, as far as we have been able to ascertain, the first time the intracellular activity of antibiotics is examined with the tools and models commonly used in pharmacological approaches (use of large

concentration spans, expanding from suboptimal concentrations to concentrations exceeding several fold the MIC; modeling of the data according to what is used in conventional pharmacological dose-response analysis). This has allowed us to make a strong link between microbiology and pharmacology, and to explain how some drugs, such as β-lactams, could be reported as following models that were, apparently, incompatible with basic pharmacological rules (time-dependency). We see indeed that all antibiotics are concentration-dependent, but within a limited concentration span only (essentially from about 0.1 to 50-fold their MIC). Yet, we see that the so-called time-dependent antibiotics (such as β-lactams) are actually used in clinical practice at concentrations that most often largely exceed their MIC. Thus, these drugs are not intrinsically time-dependent only, but are used under conditions at which their concentration-dependency can no longer be clearly evidenced. This point is not of pure academic interest. Strains with elevated MICs towards these antibiotics will indeed begin to show concentration-dependency, a point which has, so far, not be taken into account in the clinical use of these drugs. Conversely, antibiotics that are clearly concentration-dependent for their activity in the current serum concentration range would become time-dependent if their MIC could be substantially lowered. In this context, we suggest that more attention should be spent on the determination of the E_{50} parameter, in relation to the MIC and the actual antibiotic concentrations achievable in patients, since it may be critical to categorize an antibiotic as a concentration or a time-dependent drug, thereby leading to different therapeutic recommendations.

In a broader context, our studies may also help to understand the limits in the action of antibiotics, as quantified by the E_{max} parameter. This parameter is rarely taken into account and/or quantified in routine microbiological testing. Yet, it appears to us very critical, since it tells us not only how effective an antibiotic can be, but, more importantly, which proportion of the original inoculum will never be eradicated whatever the concentration of the antibiotic. The reason why this parameter has long been neglected is probably that E_{max} in broth is often very large and corresponds, practically speaking, to bacterial counts below detection levels in routine clinical microbiology. This, however, is not the case for the intracellular bacteria. While we do not know, today, where and how those bacteria ever succeed to remain untouched by the antibiotics, they certainly represent a potential threat not only in terms of persistence but also as a source of resistant organisms (trough selection of best-fitted organisms due to insufficient killing). In this context, it is clear that an antibiotic with a large E_{max} effect will be the drug of choice if eradication is what is being looked for when initiating an antibiotic treatment.

1.4. Perspectives

1.4.1. Evaluation of antibiotic combinations

Therapeutic strategies towards multi-resistant *S. aureus* have pointed out the interest of using antibiotic combinations for critically ill patients (see for review: [64,69,70,215]). Antibiotic combinations are sometimes used with the specific intent of (i) extending the antimicrobial spectrum, (ii) obtaining a synergistic antimicrobial effect, and (iii) preventing or minimizing the likelihood of emergence of multi-resistant strains. It would therefore be worthwhile to evaluate whether combination of antibacterial agents may improve the relative potency and/or (EC_{50} effect) the maximal efficacy (E_{max} effect) of antibiotics.

1.4.2. Evaluation of the cooperation between antibacterial agents and host immune responses

The possibility that interactions between antibacterial agents and the immune system may contribute to the therapeutic efficacy is widely recognized. However, these interactions have not been so far considered against intracellular *S. aureus*. Phagocytes, multifaceted cells which are key components of cellular immunity, are involved (i) in the immediate defences against pathogens, and (ii) in the regulation and triggering of specific immune responses. They are, thus, of prime interest to widely explore the effect of cytokines and burst oxidative processes on the pharmacological properties of antibiotics against intracellular pathogens. Interestingly, it has been recently demonstrated that γ-interferon or TNF-α, two main cytokines, reduced considerably the intracellular growth of *S. aureus* upon incubation within endothelial cells. In this context, we can presume that a significant improvement of the anti-bacterial therapeutics could be obtained by an optimal cooperation within cytokines, bacteria and antibiotics.[29,183]

1.4.3. Static vs. dynamic models

The usual *in vitro* estimations of antibiotic activity (MICs, dose- and time-kill studies) are only determined using constant concentration of antibiotics (static models), which do not reflect the pharmacokinetic profile of antibiotics that fluctuate over time. Various dynamic models (considering both intrinsic activities of antibiotics, and their pharmacokinetics) have been successfully used against extracellular bacteria (see for review:[38]). In this context, it should be interesting to applicate this dynamic model to our cultured cell lines.

1.4.4. Evaluation of the *in vivo* activity *of* antibiotics

Extrapolation to the *in vivo* situation cannot be established at this stage, since our experiments were only conducted using cultured cell lines. Therefore, our hypothesis will need to be specifically addressed in future *in vivo* studies to obtain more accurate information for clinical perspectives. Interestingly, a remarkable animal model of mixed extracellular-intracellular peritoneal infection has been recently developed by Sandberg and colleagues (European Congress of Clinical Microbiology and Infectious Disease, 2006, Nice, France, Poster 1157), in which mice are infected by peritoneal infection of *S. aureus*, treated with antibiotics, and finally sacrificed.

2. Can we use β-lactams for the treatment of intracellular *S. aureus* infections?

Owing to their poor accumulation within cells, several studies have claimed that β-lactam antibiotics are ineffective intracellularly[8,212] without analyzing in greater depth the relationship between drug concentration (dosing), time of exposure, and chemotherapeutic response of these agents. There is nevertheless consensus on the fact that β-lactams should express an intracellular activity because these agents have been used successfully for the treatment of various intracellular organisms (e.g., *L. monocytogenes)*. In the cases of a β-lactam sensitive *Staphylococcus aureus,* we have firstly shown that a significant reduction of the intracellular inoculum should be obtained when (i) the extracellular concentration of antibiotics markedly exceeded the MIC of the pathogen (which is clinically relevant because serum levels are often quite high), and (ii) the duration of incubation is brought to 24 h (β-lactams are time-dependent antibiotics). Taken together, these results suggest (i) that optimal efficacy should be obtained by prolonged duration of treatment at the maximal dose (to compensate for the lack of accumulation); (ii) there is a place for continuous infusions.

Secondly, we have noticed that β-lactams recover activity against Methicillin-Resistant *S. aureus* when the bacteria were exposed to these antibiotics after phagocytosis by eukaryotic cells. This is of crucial importance, because (i) MRSA is arguably one of the most striking subject of clinical concern, (ii) this behavior in the acidic pH is also relevant to similar conditions prevailing in the skin, mouth, vagina, or urinary tract. Because this restoration of susceptibility has been documented for all of our MRSA isolates, we may reasonably assume that a common character (most related to the presence and expression of PBP2a) is responsible for the effect seen. In addition to facilitating a reduction in β-lactam resistance, recent findings show also that acidic pH improved significantly β-lactam-induced autolysis of

MRSA isolates. Therefore, this novel finding opens interesting perspectives for the understanding and unravelling of this complex phenomenon.

In a broader context, it is somewhat ironic that the diagnostic of MRSA as the offending organism leads to the discontinuation of β-lactams in favor of vancomycin or linezolid, when we know (i) that both antibiotics are poorly active against intracellular forms of *S. aureus (*not only with laboratory strains, but also and perhaps more importantly, with multi-resistant isolates as seen in persistent infections); (ii) that β-lactams could be effective against the intracellular forms of these organisms. Our observations may trigger future *in vivo* studies, in order to (i) assess such phenomenon *in vivo* (with special attention to tissues such as skin and bronchial epithelia where survival of intracellular *S. aureus* may play a critical role); (ii) delineate the potential interest of including a β-lactam in the treatment of MRSA infections prevailing in the acidic milieu (obviously, β-lactams will be ineffective against the extracellular forms of MRSA and their use should, therefore, be always combined with that of other agents acting against these forms) ; and to (iii) delineate the influence of acidic pH on the activity of novel, promising anti-MRSA compounds belonging to the class of β-lactams (such as ceftobiprole). Carefully designed *in vitro* and *in vivo* studies examining this new type of agent or combination therapy to control MRSA infection could be useful in this context.

3. Can we use β-lactam in combination with vancomycin for the treatment of intracellular MRSA infections?

Our study has clearly delineated the potential interest of including a β-lactam to vancomycin in the treatment of recurrent and persistent *S. aureus* infections. However, there have been many contradictory observations with regard to the combination effect of vancomycin and β-lactam antibiotic against *S. aureus*, including both (i) additive and/or synergistic effects, and (ii) antagonistic effects.

On the first hand, some investigators have noted that a combination of glycopeptides and β-lactam antibiotics can demonstrate *in vitro* additive or synergistic effect against MRSA, since they act at different stages of the cell-wall synthesis.[19,198,232] By a variety of *in vitro* tests, Domaracki and colleagues have demonstrated that vancomycin and oxacillin were synergistic against clinical isolates of MRSA when concentrations of antibiotics are equivalent to a multiple of one-fourth to one-half of their corresponding MIC.[63] On the second hand, other investigators have reported antagonistic effects when combining a β-lactam with vancomycin.[15,102,111] As demonstrated by Aritaka and colleagues, β-lactams exhibit a synergistic effect when their concentration is brought to a concentration equivalent to their

MIC, while antagonistic effects were observed at lower, sub-MIC levels. The additive concentrations are therefore difficult to achieve in patients, because it required high dosages of β-lactams (> 128 mg/L).[15]

Despite the apparent limitation described by Aritaka and colleagues, this antagonistic effect has been firstly reported for MRSA isolates showing a reduced susceptibility to vancomycin, which will be obviously associated with a poor response of this agent. Secondly, these data are often obtained using *in vitro* studies, and they still need to be confirmed *in vivo.* This is, obviously, of crucial importance, since Sorrell and colleagues failed to demonstrate any clinical effectiveness of combination therapy with vancomycin and amikacin, even though synergistic effects were demonstrated *in vitro* for this combination. Future studies are therefore required to critically examine (i) the *in vitro* effectiveness of vancomycin and β-lactams under well-defined conditions (MRSA growing at acidic pH, intracellular MRSA), and (ii) the potential benefit of including *in vivo* a beta-lactam to vancomycin in the setting of recurrent and persistent *S. aureus* infections, where intracellular foci of *S. aureus* may play a major determinant. Hence, this application warrants further investigation to establish a rational combined therapy with vancomycin and β-lactams.

4. Can we use daptomycin for the treatment of intracellular *Staphylococcus aureus* infections?

Despite of the poor cellular accumulation of daptomycin within cells, we have observed that this agent achieves a significant reduction of the intracellular inoculum of a daptomycin-susceptible strain of *S. aureus* (isolate ATCC 25923). This observation, already reached from previous studies dealing with *S. aureus*- infected polymorphonuclear neutrophils[16], reinforces our earlier conclusions that cellular accumulation is not *per se* a predictive factor of antibiotic activity in the cellular milieu. Pending a more detailed analysis of daptomycin distribution within macrophages (only *in vivo* studies have been earlier performed, and report a main distribution of this agent within the lysosomes of the proximal tubular cells), these observations open up new perspectives to evaluate the potential *in vivo* relevance of this finding.

Secondly, we have shown that transporters belonging to the MDR-1 subfamilly may partially defeat the cellular accumulation and potency of daptomycin against intracellular forms of *S. aureus,* in relation with their level of expression. Inhibitors of these transporters (verapamil or elacridar) have also be shown to potentiate both the pharmacokinetic (increase of drug accumulation) and pharmacodynamic (decrease of EC_{50}) properties of daptomycin,

without affecting the intracellular growth of the bacteria. Moving now to the chemotherapeutic significance of our observations, these findings open up new perspectives for the extrapolation of these results to the *in vivo* situation. A possible caveat, however, is that the concomitant use of daptomycin and P-gp inhibitors may increase the likelihood of severe daptomycin-induced myopathy. Because we still ignore whether these side-effects may occur upon administration, the design and/or selection of new lipopeptides showing a lack or a weak recognition by P-gp transporters appear to us as a more useful and safer approach.

5. Do we need to reconsider the treatment of intracellular *S. aureus* infections?

The inability of phagocytes to rapidly and completely destroy intracellular *S. aureus* after phagocytosis plays an important role in the dissemination of infections, as well as in the favorable transport of the pathogens across vascular and non-vascular compartments. As described earlier[18,21,168,225], conventional anti-staphylococcal agents are considerably less effective intracellularly than against their extracellular counterparts. Our studies performed on recent clinical isolates recovered from presumed intracellular environment (such as heart valves) extend these observations to various antibiotics, and show that the pharmacological model described earlier by Marcia-Bacay and colleagues[18] is also valid for our clinical isolates. This loss of E_{max} is probably crucial, because it probably accounts for several aspects of relapsing staphylococcal infections.

These *in vitro* studies have also yielded a number of interesting and important findings. Firstly, we show that (i) a phenotype of resistance to rifampicin (a commonly used antimicrobial agent against difficult-to-treat bacterial infections) makes the drug completely ineffective intracellularly, despite its huge cellular accumulation; (ii) a phenotype of resistance to vancomycin and daptomycin makes both drugs poorly active against intracellular forms, even if used at concentrations far above the MICs. Considering now the efficacy of agents that are not affected by resistance mechanisms (gentamicin, fusidic acid, linezolid), we have secondly noticed a generally low intracellular potency of these agents when used against intracellular bacteria. Taken together, this low intracellular potency of presently available antimicrobial agents probably accounts for treatment failure, and suggests why the infected tissues, eventually, had to be removed surgically.

Finally, our studies have also documented the superiority of novel agents (quinupristin/dalfopristin and oritavancin) against these intracellular bacteria, regardless of their resistance phenotype. In this context, selecting molecules with low MIC values and

extended activity against the intracellular forms of *S. aureus* (high E_{max}) appears to be straightforward approaches. Analysis of the pharmacodynamic properties of antibiotics in appropriate models of infected cells (i) may rationalize their use for future *in vivo* studies, (ii) and may help in directing future research to select *in vitro* more potent derivatives.

6. Do we need to reconsider the use of vancomycin against intracellular *S. aureus*?

Vancomycin treatment failure and slow clinical response have long been reported in the setting of recurrent and persistent *S. aureus* infections, such as endocarditis (10 to 20 % of treatment failure)[23]. Although vancomycin is still considered as a first line antibiotic against MRSA in the hospitals, poor clinical outcomes have been documented with both susceptible and resistant strains (VISA, hetero VISA [hVISA]).

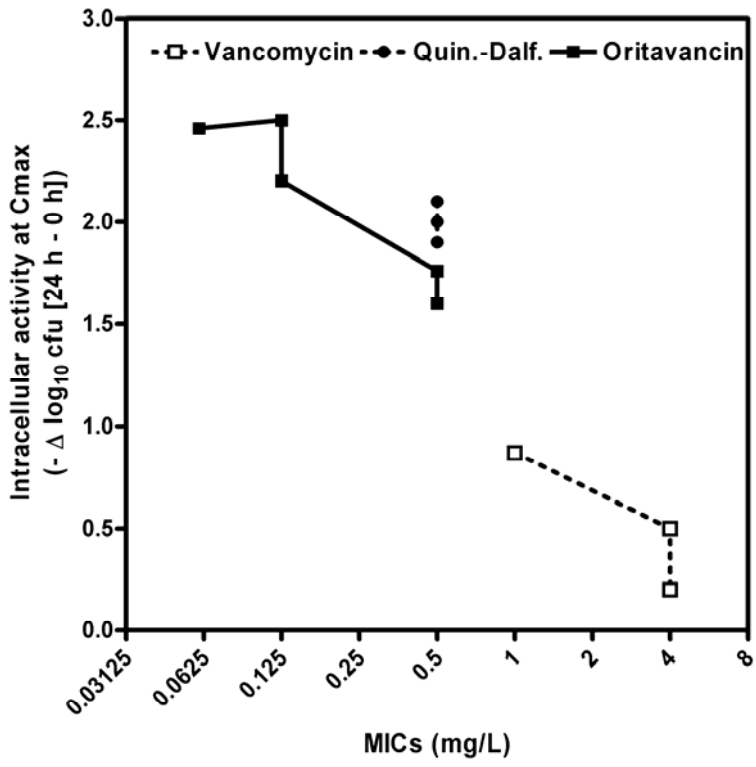

Fig. D.1. Comparative evaluation of (i) the susceptibility of *S. aureus* to antibiotics (vancomycin, quinupristin-dalfopristin, and oritavancin) and (ii) the activity of antibiotics against intracellular forms of *S. aureus* (24 h incubation) at a concentration equivalent to the human Cmax described in the literature (vancomycin, 50 mg/L; quinupristin-dalfopristin, 10 mg/L, and oritavancin, 40 mg/L).

All of these observations are highly relevant to our findings, since we have shown that (i) vancomycin is only poorly active intracellularly (affecting both Emax and static dose), compared to extracellularly), (ii) isolates with reduced susceptibility to vancomycin are

associated with a significantly impaired activity of this agent against intracellular forms (Fig.D.1). In this context, it must be emphasized that the lack of vancomycin bactericidal effect may be an important determinant in the emergence of isolates with decreased susceptibility to vancomycin. Therefore, the possibility that vancomycin may have limited activity should be borne in mind when selecting an anti-staphylococcal agent in the setting of recurrent and persistent illnesses.

7. Can we define the ideal antibiotic?

The choice of antibiotics against intracellular pathogens is of immediate clinical importance. In the ideal situation, an antibiotic should eradicate bacteria from tissues sites, including those localized in intracellular compartments. By defining the pharmacological basis governing the antibacterial activity of antibiotics in the intracellular milieu, we can assume that the ideal antibiotic should (i) have a broad antimicrobial coverage (including bacteria showing a variety of resistance mechanisms) and not only kill extracellular bacteria, but also intracellular ones, (ii) readily enter cells (lack or weak recognition by eukaryotic efflux transporters), (iii) have the same subcellular distribution than *S. aureus* to tightly bind its intracellular target, (iv) express extensive activity in the intracellular milieu (low MIC values at acidic pH, fast and extensive killing), (v) have a selective toxicity (no adverse effects on human), (vi) promote optimal immune functions, and (vi) require no additional surgery.

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ANNEX

CIRCULAR DICHROISM

1. Introduction (See for review : [123,282])

Circular dichroism (CD), a spectroscopic method similar to the optical rotatory dispersion (ORD), allows the determination of the structural integrity of proteins and biomolecules using the far-UV spectra. This spectroscopic procedure measures, as a function of wavelength, the differential absorption of left-handed and right-handed circularly polarized light of an optically active chromophore. This kind of technique, that can discern the subtle differences between enantiomers, is therefore highly sensitive to the three-dimensional features of molecules (hence, its conformation).

2. Theory of Circular Dichroim

Plane polarized light consists of two circularly polarized components of equal intensity. In each component, the electric vector rotates about the direction of propagation, undergoing a full rotation in a distance equal to the wavelength of the light. The absorption of unpolarized light by a sample is described by the absorbance (A), defined as:

$$A = \log (Io/I)$$

Where Io is the intensity of the incident light, and I is the intensity after that the light has travelled a distance l. According to the law of Lambert-Beer, the absorbance is given by the following equation:

$$A = \varepsilon\, c\, l$$

Where ε is defined as the molar extinction coefficient, and c is the molar concentration. In the same way, the molar extinction coefficients can be defined for the right-hand (rcpl) and left-hand (lcpl) circularly polarized light. The molar circular dichroism ($\Delta\varepsilon$) is represented by the following equation:

$$\Delta\varepsilon = \varepsilon_{left} - \varepsilon_{right} = (A_{left} - A_{right})/cl$$

Therefore, the molar circular dichroism is defined as the difference between the extinction coefficients for the two types of circularly polarized light. Most instruments are, however, calibrated in terms of molar ellipticity (Θ, units: degree cm²/dmol), an alternative measure of

CD, that represents the angle whose tangent is the ratio of the minor and major axes of the ellipse.

$$\Theta = 32.98\ \Delta A$$

In order to eliminate the effects of path length and concentration, the molar ellipticity is defined as:

$$[\Theta] = (100.\Theta) / lc = 3298\ \Delta\varepsilon$$

3. Instrumentation

Light from a monochromator is linearly polarized, then passes through a modulating device which converts the plane-polarized light to circularly polarized light and alternating between lcpl and rcpl at the modulation frequency. Because the incident light is modulated between left and right circular polarization, the light intensity detected by the photomultiplier will also be modulated at the same frequency if the sample exhibits chiral properties.

Buffers, salts and other additives must be selected with care for far-UV measurements (10 mM phosphate or Tris can be tolerated). In addition, it is essential to run a baseline CD spectrum for all the added coumpounds (e.g., antibiotics), and substract them from the spectrum of the sample. The baseline should be run under the same conditions (solvent, incubation time, …) as the sample.

4. Applications

Circular dichroim measurements are used for the determination of macromolecules conformation, such as polynucleotides, DNA, polypeptides and proteins. The analysis of CD spectra to obtain information about protein secondary structures has been reviewed extensively. Firsty, the strongest and most characteristic spectrum is that of α-helix, which has two negative bands of comparable magnitude near 222 and 208 nm, and a stronger positive band near 190 nm (Fig.A.1). Secondly, peptides adopting the β-sheet conformation have a less intense and simpler spectrum compared to α-helix, with negative bands near 217 nm and 180 nm, and a positive band near 195 nm (Fig A.1). Conversely, β-turns occur over an even wider range of conformations, which can give rise to a variety of CD spectra (most of β turns should give a CD spectrum which resembles that of β-sheet). Finally, the CD spectra

of unordered polypeptides are characterized by a strong negative band near 200 nm (Fig.A.1)

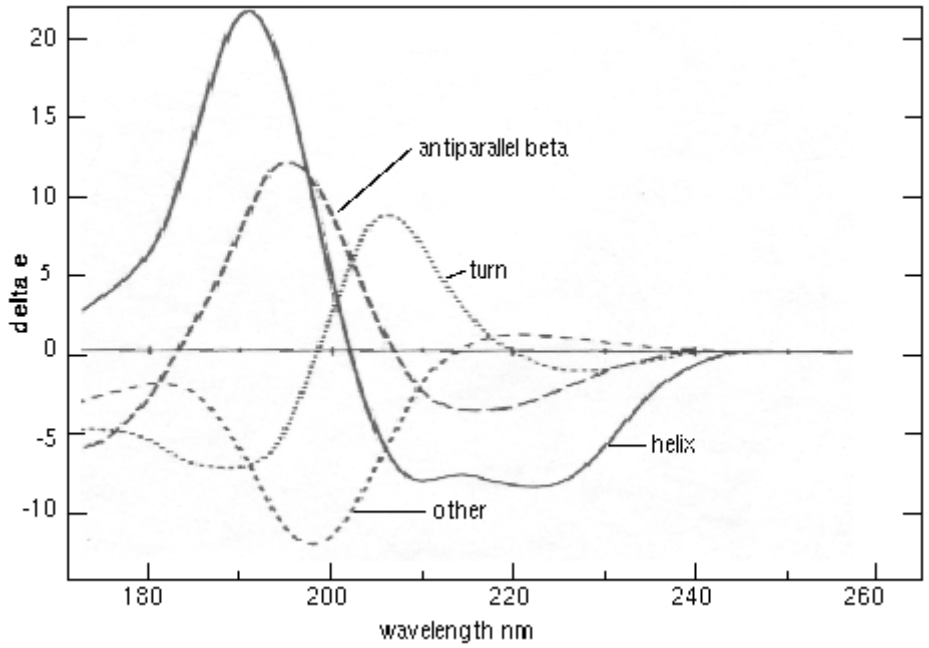

Fig.A.1. Circular dichroism spectra of specific secondary structures (http://www.cryst.bbk.ac.uk/PPS2/course/section8/ss-960531_21.html).

5. Advantages and drawbacks

5.1. Advantages

Circular dichroism is a relatively fast and cost-effective (~ 200 µL of a protein solution of 1 to 100 µM) procedure to gain insight of protein conformation. CD analysis of secondary structure is valuable because (i) it is, in essence, non-destructive (allowing the recuperation of the sample for further experiments), and (ii) less labor-intensive compared to X-ray diffraction or multidimensionnal NMR. This technique allows also the evaluation of structural changes (pH-, temperature-, concentration- dependence) in the timescale of seconds. In this context, the effects of mutations or denaturants can be studied, and the kinetics of protein folding and unfolding can be investigated much easily by CD procedure.

5.2. Drawbacks

CD procedures determine only an average secondary structure. In consequence, there is no information about which part of the molecule adopts a certain conformation. Additionally, CD spectra can only be performed with single, purified proteins.

SUMMARY

Staphylococcus aureus est un germe redoutable fréquemment associé à des pathologies graves, récurrentes et persistantes, probablement en raison de sa survie dans un environnement intracellulaire (phagocytes professionnels et non professionnels). Néanmoins, cet élément est trop rarement pris en compte dans les choix thérapeutiques.

Notre travail de thèse s'est inscrit dans la problématique expérimentale de l'infection intracellulaire par *S. aureus* et de l'activité des antibiotiques dans des modèles de cellules infectées par des souches sensibles ou multi-résistantes. En premier lieu, nous avons développé des modèles stables d'infections intracellulaires en utilisant à la fois (i) des types cellulaires susceptibles de représenter des situations pathologiques où la persistance de *S. aureus* est démontrée (macrophages, cellules endothéliales, kératinocytes, cellules épithéliales bronchiques), et (ii) des isolats de *S. aureus* potentiellement multi-résistants aux antibiotiques. Dès lors, ces modèles nous ont permis d'étudier l'activité d'antibiotiques (molécules conventionnelles vs. molécules nouvelles) par une approche pharmacologique. Dans un deuxième temps, ces modèles nous ont permis d'évaluer l'influence de l'environnement intracellulaire sur l'activité de ces différents antibiotiques. En outre, nous avons mis en évidence que, premièrement, le pH acide intracellulaire (phagolysosomes) permet de restaurer l'activité des antibiotiques de la classe des β-lactames vis-à-vis de souches résistantes à ces agents antimicrobiens (souches MRSA pour Methicillin-Resistant *S. aureus*). Le mécanisme sous-jacent à cette restauration d'activité a été étudié au niveau moléculaire et a permis de mettre en évidence que la Penicillin-Binding Protein2a (PBP2a), une PBP de faible affinité pour les β-lactames, subit à pH acide des modifications conformationnelles permettant son acylation par les β-lactames. Deuxièmement, nos travaux ont mis en évidence que la daptomycine, un nouveau lipopeptide anti-MRSA, est le substrat de la pompe à efflux P-glycoprotéine (P-gp, un des représentants principaux des transporteurs ABC), ce qui reduit son accumulation cellulaire, et en conséquence, son activité vis-à-vis des formes intracellulaires de *S. aureus*. Dès lors, ces résultats mettent en évidence le rôle majeur de ces transporteurs eukaryotes dans la modulation des propriétés pharmacocinétiques des antibiotiques, et dès lors, de leurs propriétés pharmacodynamiques.

Par ces travaux, nous avons mis en évidence que ces modèles expérimentaux d'infections intracellulaires sont des outils précieux pour déterminer l'activité d'antibiotiques vis-à-vis de *S. aureus (*étant donné que celle-ci ne peut être déduite de facteurs microbiologiques ou de l'accumulation cellulaire des antibiotiques), et nous suggérons vivement l'application de ces modèles cellulaires dans l'évaluation pré-clinique de nouveaux antibiotiques.

Staphylococcus aureus is an aggressive organism often associated with severe, recurrent and relapsing illnesses, probably due to its intracellular survival within cells (professional vs. non-professional phagocytes). Nevertheless, this intracellular niche is not fully taken into consideration when selecting appropriate anti-staphylocccal treatment.

In this thesis, we have focused our interest on the survival of *S. aureus* within cells and on the intracellular activity of antibiotics against these intracellular forms (sensitive vs. multi-drug resistant *S. aureus*). In the first part of this thesis, we have developed stable models of cellular infections, using (i) diverse cell types susceptible to mimic pathological situations for which persistence of *S. aureus* has been reported (macrophages, endothelial cells, keratinocytes, bronchial epithelial cells), and (ii) multi-drug resistant isolates of *S. aureus*. These cellular models have been extensively used for the determination of the intracellular activity of antimicrobials (old molecules vs. novel molecules) following a pharmacological approach.

In the second part of this thesis, we have used our cellular models to determine whether the cellular milieu may modulate the intracellular pharmacokinetic and pharmacodynamic properties of antibiotics. Firstly, we have highlighted that the acidic pH prevailing in the phagolysosomes may restore the susceptibility of Methicillin-Resistant *S. aureus* (MRSA) to β-lactams. The mechanism underlying this process has been widely studied at the molecular level, and we have demonstrated that the Penicillin-Binding Protein 2a (PBP2a), a low-affinity PBP for β-lactams, undergoes at acidic pH conformational changes allowing its acylation by β-lactams. Secondly, we have demonstrated that daptomycin, a novel anti-MRSA lipopeptide, is a substrate of P-glycoprotein (P-gp) transporters (one of the main representant of ABC transporters), which may affect its accumulation within cells, and therefore, its intracellular activity. These data underline the major role that efflux plays in the modulation of pharmacokinetics of drugs, and hence, of their pharmacodynamics.

By these observations, we may conclude to the usefulness of cellular models to determine the intracellular activity of antibiotics (since this latter can not be simply deduce from microbiological parameters (MICs, killing curves in broth) or cellular accumulation) and we suggest the implementation of such models in the early development of novel antimicrobials.