Effects of antibiotics on biofilm and unattached cells of a clinical Staphylococcus aureus isolate from bone and joint infection

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INTRODUCTION
Orthopaedic joint replacement is an increasingly common surgical procedure worldwide, sometimes complicated by the advent of prosthetic joint infections (PJIs), which entail major morbidity (Darouiche, 2004). PJIs are mainly caused by Staphylococcus aureus and Staphylococcus epidermidis (Del Pozo & Patel, 2009). The prevalence of meticillin-susceptible S. aureus (MSSA) and meticillin-resistant S. aureus (MRSA) can vary highly from one hospital to another, but overall in European countries, including France, S. aureus accounts for 13–44.1 % of PJIs, with the majority being MSSA (Titicat et al., 2013; Aggarwal et al., 2014). All species are known to form biofilm on the implants, which impairs the action of antibiotics and immune cells. These mechanisms are major sources of persistent infections.

The high resistance of biofilm bacteria to antibiotics was initially attributed to reduced drug penetration into the biofilm core caused by a physical barrier formed of extracellular polymeric substances (Suci et al., 1994). However, reduced bacterial growth rate, active starvation response and changes in bacterial gene expression also contribute

Received 21 April 2015
Accepted 3 July 2015

Abbreviations: CC, clonal complex; CLSI, Clinical and Laboratory Standard Institute; MBC, minimum bactericidal concentration; MBEC, minimum biofilm eradication concentration; MRSA, meticillin-resistant Staphylococcus aureus; MSSA, meticillin-susceptible S. aureus; PJI, prosthetic joint infection; MIC, minimal inhibitory concentration.

Treatment of orthopaedic infections remains challenging owing to the inability of antibiotics to eradicate biofilms and prevent their regrowth. The present study characterized the effects of 12 antibiotics on in vitro biofilm formed by a representative strain of meticillin-susceptible Staphylococcus aureus (MSSA) isolated from a bone infection. Determination of the minimum biofilm eradication concentrations indicated that in vitro eradication of 24 h-old biofilms required concentrations up to 51 200 times higher than MICs. The influence of the same panel of antibiotics was also investigated on biofilm formation at concentrations including the breakpoints, by numbering viable cells in the suspensions (individual cells) and the biofilm biomass. Except for fusidic acid, the presence of antibiotics during the initial steps of biofilm formation resulted in significant decreases in the number of sessile viable bacteria at the highest concentrations tested. Ceftarolin, daptomycin, fosfomycin, gentamicin, ofloxacin, rifampicin and vancomycin were the most effective drugs. Confocal microscopy analysis indicated that daptomycin was more efficient at bacteria lysis than gentamicin and vancomycin. However, viable individual cells were still detectable in the assays performed with ceftarolin, fosfomycin, ofloxacin, rifampicin and vancomycin at concentrations for which no sessile cells were detected. Although none of the molecules tested was effective at classical therapeutic concentrations against 24 h-old MSSA biofilms, all except fusidic acid were able to impair biofilm formation at concentrations near the breakpoints. However, presence of viable individual unattached cells could imply a significant risk of microbial dissemination and increased risk of infections.
to biofilm resistance (Evans et al., 1990; Mah et al., 2003; Zhang & Mah, 2008; Nguyen et al., 2011). This inherent resistance makes biofilm-associated infections very difficult to overcome (Stewart & Costerton, 2001; Donlan & Costerton, 2002).

Sub-MICs of antibiotics such as β-lactam antibiotics have been shown to induce biofilm formation by MSSA (Ng et al., 2014), so that bacterial cells may become buried deep within biofilms during the course of routine antibiotic therapy (Odenholt, 2001; Singh et al., 2010).

The choice of antibiotic therapy is currently based on data obtained by methods using planktomic bacteria, which are not representative of biofilm bacteria. The present study aimed to determine the drug concentrations required to eradicate in vitro S. aureus biofilm and/or to inhibit biofilm formation using a representative clinical strain of MSSA isolated from a bone infection.

**METHODS**

**Bacterial strains and growth conditions.** Thirty strains of MSSA isolated from patients with monomicrobial PJIs and hospitalized in Lyon, France, were initially characterized. Among these, strain LYO-S2 was isolated in 2001 from a patient suffering from a PJI after total knee arthroplasty. The PJI was classified as early (time from arthroplasty to symptoms <3 months) or chronic (time from initiation of symptoms to diagnosis >4 weeks). A two-stage revision of infected knee arthroplasty was performed. Initial treatment consisted of cefotaxime (6 g day\(^{-1}\)) and vancomycin (2 g day\(^{-1}\)) for 4 days, followed by oxacillin (12 g day\(^{-1}\)) and fosfomycin (12 g day\(^{-1}\)) for 55 days. Ofloxacin (400 mg day\(^{-1}\)) and pristinamycin (4 g day\(^{-1}\)) were then orally administered for 88 days. The outcome was favourable during 3-year clinical follow-up. All isolates were stored at −80°C in trypticase soy (TS, Oxoid) broth containing 15% glycerol (v/v). They were subcultured onto Columbia agar (bioMérieux) at 37°C for 18–24 h.

The molecular characterization of the isolates was performed using a DNA microarray (S. aureus genotyping – Identibac; Alere Technologies) according to the manufacturer’s instructions. The Ridom Staph type standard protocol and the Ridom SpaServer (http://www.ridom.com) were used for spa typing.

*S. aureus* ATCC 29213 was used as a quality control strain for MIC. Bacteria were grown on Mueller–Hinton (MH) and TS agar (Conda), and in MH (Conda) and modified MH (mMH) (bioMérieux) broth.

The panel of antibiotics used comprised cefoxitin (Forest Laboratories), daptomycin (Novartis), fosfomycin (Ercros), gentamicin (Unipex), fusidic acid, erythromycin, oxacillin, rifampicin (Sigma-Aldrich), linezolid (Pharmacia & Upjohn), ofloxacin (MP Biomedicals), pristinamycin (Aventis Pharma) and vancomycin (Alpharma).

**MIC and minimum bactericidal concentration (MBC) determination.** MICs were determined as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2013). MBCs were determined by subculturing broth dilutions of the MIC assay on TS agar.

**Determination of minimum biofilm eradication concentration (MBEC).** MBECs corresponded to the lowest concentrations giving rise to a negative subculture from 24 h-old biofilm. MBECs were determined using MBETM high-throughput assay plates (Innovotech) according to the manufacturer’s instructions.

**Effect of antibiotics on early biofilm formation.** Biofilms were formed on polystyrene pegs of the MBEC device using a bacterial inoculum of \(8 \times 10^5\) c.f.u. ml\(^{-1}\) in mMH broth containing twofold dilutions of antibiotics. After 4 h of incubation at 37°C with shaking (4 rocks min\(^{-1}\)), viable bacteria from the suspensions (individual unattached cells or cells shed from the surface of the biofilm) were enumerated by plating serial dilutions on TS agar. In parallel, biofilms formed on the pegs were rinsed with saline and transferred to new microtitre plates, and the number of sessile viable cells was counted after sonication (10 min at room temperature) by plating serial dilutions on TS agar.

**Biofilm confocal microscopy analysis.** The effects of antibiotics on biofilm formation were observed on biofilms formed on glass strips (Fisher Scientific) in a 24-well polystyrene microtitre plate

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg ml(^{-1}))*</th>
<th>MBC (µg ml(^{-1}))</th>
<th>MBEC (µg ml(^{-1}))†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftaroloxin</td>
<td>0.25</td>
<td>0.25</td>
<td>6400</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>0.25</td>
<td>1</td>
<td>&gt;25 600</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1</td>
<td>&gt;16</td>
<td>6400</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>4</td>
<td>ND</td>
<td>&gt;25 600</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>0.25</td>
<td>&gt;64</td>
<td>1600</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1</td>
<td>8</td>
<td>6400</td>
</tr>
<tr>
<td>Linezolid</td>
<td>1</td>
<td>&gt;128</td>
<td>6400</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>0.25</td>
<td>2</td>
<td>12 800</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>0.5</td>
<td>&gt;64</td>
<td>25 600</td>
</tr>
<tr>
<td>Pristinamycin</td>
<td>0.5</td>
<td>&gt;8</td>
<td>400</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.016</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>2</td>
<td>2</td>
<td>3200</td>
</tr>
</tbody>
</table>

*The MIC values of the antibiotics against *S. aureus* ATCC 29213 were within the CLSI accuracy range.
†The MBEC determinations were obtained using 24 h-old biofilm.
Antibiotics on biofilm and unattached cells of *S. aureus*
Fig. 1. Enumeration of viable bacteria within biofilms formed by S. aureus strain LYO-S2 (grey) and in the biofilm supernatants (black) in the presence of 12 antibiotics at different concentrations after 4 h incubation. Antibiotic concentrations (μg ml⁻¹) are indicated along the x-axes and cell viability along the y-axes (c.f.u. ml⁻¹; threshold 10 c.f.u. ml⁻¹). Each determination was performed three times. The significant differences between c.f.u. ml⁻¹ values were calculated using one-way ANOVA with Dunnett’s multiple comparison test. Significant differences: *P<0.05, **P<0.01 and ***P<0.001. The horizontal bars below the concentration scale indicate the breakpoints as defined by the CLSI (black) or the European Committee on Antimicrobial Susceptibility Testing (grey). Results are shown as means ± SEM.

When biofilms were formed in the presence of antibiotics, viable bacteria significantly decreased after 4 h of incubation in both the suspensions and biofilms for all antibiotics tested, except for fusidic acid with sessile cells (Fig. 1). The numbers of unattached and sessile viable bacteria were below the threshold of detection for daptomycin and gentamicin at the highest concentrations tested. By contrast, ceftarolin, fosfomycin, ofloxacin, rifampicin and vancomycin gave rise to undetectable levels of sessile cells at the highest drug concentrations tested, while unattached cells remained cultivable.

Confocal microscopy analysis showed dense and highly structured biofilms in the control, with mostly live cells (Fig. 2a). These structures were impaired when biofilms were formed in the presence of daptomycin and vancomycin, but the presence of gentamicin did not seem to greatly modify the biofilm architecture.

When added to 24 h-old biofilms, vancomycin, daptomycin and gentamicin did not modify the biofilm architecture, but were effective in killing bacteria within the biofilms, particularly daptomycin (Fig. 2b).

RESULTS

Among the 30 strains characterized by determination of their clonal complex (CC), seven belonged to CC45, and the other isolates were distributed over CC5 (six isolates), CC30 (six isolates), CC15 (four isolates) and minor CCs. Twelve isolates harboured an agr-1 allele, whereas agr-2, agr-3 and agr-4 alleles were detected in ten, seven and one isolates, respectively. Regarding the presence of antibiotic resistance genes, the bla operon and fosB were detected in the majority of the isolates (22 and 18, respectively) and four of them harboured ermC. Numerous genes encoding microbial surface components recognizing adhesive matrix molecules were also detected, including fnbA, sdrC, sdrA and the ica operon. All strains were able to form biofilm (data not shown) and further experiments were carried out with an isolate chosen from among the most common type, i.e. strain LYO-S2 (CC45 and agr-1 allele).

The MICs of the 12 tested antibiotics ranged from 0.02 to 2 μg ml⁻¹ and the MBCs from 0.25 to >128 μg ml⁻¹ (Table 1). In vitro eradication of 24 h-old biofilms required concentrations (MBECs) up to 51 200 times higher than the MICs (Table 1).

DISCUSSION

The MSSA clinical strain tested in this study exhibited high levels of biofilm formation in vitro even after short-duration (4 h) incubation (Fig. 1). The biofilm formation ability of this strain was evaluated in the presence of 12 antibiotics from different families. None induced biofilm formation, and in this regard the strain behaved like those described previously, with an inverse relation between basal biofilm production and antibiotic-driven biofilm inducibility (Kaplan et al., 2012; Ng et al., 2014). Interestingly, no biofilm was detectable after 4 h incubation in the presence of fosfomycin, ofloxacin and vancomycin, rifampicin and ceftarolin at concentrations in the range of the breakpoint concentrations. However, viable unattached cells were still detectable in the suspensions covering the biofilm, even at the highest antibiotic concentrations tested. Since these drugs are mostly active against dividing bacteria, our results suggest that their presence impaired the bacterial process required for initial biofilm formation within the active population, without hampering the non-dividing population. Population heterogeneity has been observed in S. aureus culture as a result of phenotype switching occurring during planktonic exponential-phase growth in the absence of selective pressure, which led to the emergence of subpopulations of slow-growing antibiotic-resistant cells (Edwards, 2012;
Fig. 2. Confocal observations of antibiotic-treated biofilms of *S. aureus* LYO-S2. Observations were performed after formation of biofilm in the presence of vancomycin (0.125 µg ml⁻¹), gentamicin (0.125 µg ml⁻¹) or daptomycin (0.06 µg ml⁻¹) (a) and after treatment of 24 h-old biofilms with vancomycin (800 µg ml⁻¹), gentamicin (1600 µg ml⁻¹) and daptomycin (200 µg ml⁻¹) (b). z, Biofilm thickness.
Bui et al., 2015). Numerous studies have determined the impact of antibiotics on biofilm formation and sessile cell viability (Kaplan et al., 2012; Ng et al., 2014), but the concomitant outcome of non-adhering bacteria, a small portion of the population that might participate in the development of chronic and recurrent infections, has been poorly investigated.

In our study, no cultivable cells were observed after 4 h incubation of the strain LYO-S2 with daptomycin or gentamicin, in both the biofilm and the suspensions, even at antibiotic concentrations as low as the breakpoint concentrations (Fig. 1), but the drugs had no (gentamicin) or little (daptomycin) effect on the viability of 24 h-old biofilms (Table 1, Fig. 2). Daptomycin has already been shown to act faster and more effectively than vancomycin or other antibiotics such as gentamicin against MSSA biofilms (Meije et al., 2014), probably owing to differences in biofilm matrix penetration capacities (Siala et al., 2014). However this study, along with previous in vitro and in vivo experiments, demonstrated that the effective concentrations required for biofilm eradication are unattainable by conventional antibiotic administration (Cha et al., 2011).

In order to avoid re-infection, antibiotic treatment is necessary after removal of infected prosthetic material. Therapeutic molecules should therefore be effective against both sessile and free-living cells to avoid emergence of septic metastasis. None of the molecules tested in this in vitro study was effective at conventional concentrations against all different forms of MSSA cells. Hence, high-dose combined therapies, such as the combination of fosfomycin and daptomycin as described by Miró et al. (2012) could be given priority; only clinical studies will assess their in vivo benefit.

ACKNOWLEDGEMENTS

A CIFRE fellowship from bioMérieux and the Association Nationale de la Recherche et de la Technologie (ANRT) for C. M. is gratefully acknowledged.

REFERENCES


