

Increased Temperature Enhances the Antimicrobial Effects of Daptomycin, Vancomycin, Tigecycline, Fosfomycin, and Cefamandole on Staphylococcal Biofilms[∇]

Stefan Hajdu,¹ Johannes Holinka,² Sonja Reichmann,³ Alexander M. Hirschl,⁴
Wolfgang Graninger,³ and Elisabeth Presterl^{3*}

Department of Trauma Surgery,¹ Department of Orthopaedic Surgery,² Department of Medicine I, Division of Infectious Diseases,³
and Department of Medical Microbiology,⁴ Medical University of Vienna, Vienna, Austria

Received 25 February 2010/Returned for modification 29 April 2010/Accepted 21 July 2010

Implant-related infections are serious complications of trauma and orthopedic surgery and are most difficult to treat. The bacterial biofilms of 34 clinical *Staphylococcus* sp. isolates (*Staphylococcus aureus*, $n = 14$; coagulase-negative staphylococci, $n = 19$) were incubated with daptomycin (DAP; 5, 25, or 100 mg/liter), vancomycin (VAN; 5, 25, or 100 mg/liter), tigecycline (TGC; 1, 5, or 25 mg/liter), fosfomycin (FOM; 100, 250, or 1,000 mg/liter), and cefamandole (FAM; 50, 100, or 500 mg/liter) for 24 h at three different ambient temperatures: 35°C, 40°C, and 45°C. To quantify the reduction of the biomass, the optical density ratio (ODr) of stained biofilms and the number of growing bacteria were determined. Increasing the temperature to 45°C or to 40°C during incubation with FAM, FOM, TGC, VAN, or DAP led to a significant but differential reduction of the thickness of the staphylococcal biofilms compared to that at 35°C ($P < 0.05$). Growth reduction was enhanced for DAP at 100 mg/liter at 35°C, 40°C, and 45°C (log count reductions, 4, 3.6, and 3.3, respectively; $P < 0.05$). A growth reduction by 2 log counts was detected for FAM at a concentration of 500 mg/liter at 40°C and 45°C ($P = 0.01$). FOM at 1,000 mg/liter reduced the bacterial growth by 1.2 log counts (not significant). The antibacterial activity of antimicrobial agents is significantly but differentially enhanced by increasing the ambient temperature and using high concentrations. Adjuvant hyperthermia may be of value in the treatment of biofilm-associated implant-related infections.

Implant-related infections are severe complications of trauma and orthopedic surgery that frequently require long-term antimicrobial treatment, supportive management, and multiple additional surgical procedures (53). *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS), primarily *Staphylococcus epidermidis*, are the most common organisms associated with implant-related infections after trauma and orthopedic surgery (13).

Bacterial biofilms develop on the surfaces of the implants (12, 13, 30, 41). The biofilm consists of a structured community of bacterial cells enclosed in a self-produced polymeric matrix that adheres to an inert or living surface. The bacteria embedded in the biofilm are quasiprotected by this self-made polymeric matrix; thus, resistance to antimicrobials is increased such that the concentrations needed to kill the biofilm bacteria are 500 to 5,000 times higher than the levels needed to kill planktonic bacteria (8, 12, 13). Although removal of the colonized foreign material may be the most effective means to treat biofilm-associated infections (14), the implant must sometimes be retained because of technical or physiological complications. In this case, any hope for cure or at least the stability of the patient lies in antimicrobial treatment alone.

Antimicrobial agents for the treatment of staphylococcal

infections include beta-lactam antibiotics and, in the case of methicillin resistance, vancomycin (VAN), fosfomycin (FOM), tigecycline (TGC), or daptomycin (DAP) (32, 38). FOM is a small-molecule antibiotic with a wide antibacterial spectrum and excellent tissue penetration, representing an excellent alternative antimicrobial agent for the treatment of deep-seated infections (18, 27). Both TGC and DAP are highly active against Gram-positive cocci resistant to commonly used antibiotics, including methicillin-resistant staphylococci (18, 43). Antimicrobial agents may reduce biofilm and bacterial growth (35, 39). In a previous study, we demonstrated that the antibiotic concentrations achieved under normal physiological conditions did not decrease the growth of established staphylococcal biofilms (21). Bacterial biofilm growth was significantly reduced by the use of antimicrobials at excessive concentrations or when antimicrobials were used in combination with azithromycin (21, 37).

Because the effects of antibiotics on established biofilms are unsatisfactory, other measures to reduce biofilm thickness and to kill biofilm bacteria may be helpful (37). Adjunctive therapy to enhance the activity of an antimicrobial to save an infected but unremovable implant or to improve the engraftment of a new implant after reimplantation may be a benefit for the patient (13, 16). Experimental measures taken to reduce bacterial biofilms included antibiotic combination therapy, particularly with rifampin; therapy with a combination of antibiotics and chemicals like EDTA or *N*-acetylcysteine; or therapy with a combination of antibiotics and physical measures, such as ultrasound or an electric current (7, 15, 39, 51). Low-frequency ultrasound was used to prevent uncontrolled heating and as-

* Corresponding author. Mailing address: Department of Medicine I, Division of Infectious Diseases, Medical University of Vienna, Allgemeines Krankenhaus, Waehringer Guertel 18-20, 1090 Vienna, Austria. Phone: 43 1 40400 4440. Fax: 43 1 40400 4418. E-mail: elisabeth.presterl@meduniwien.ac.at.

[∇] Published ahead of print on 2 August 2010.

TABLE 1. MICs of the isolates investigated

Clinical isolate	Isolation site	No. of isolates	MIC (mg/liter)				
			FAM	FOM	TGC	VAN	DAP
<i>S. aureus</i>	Prosthetic joint infection	13	1 (0.5–64) ^a	4 (0.25–64)	0.125 (0.01–0.25)	0.5 (0.5–1)	0.125 (0.06–0.5)
CoNS	Prosthetic joint infection	6	1 (0.25–2)	16 (4–64)	0.06 (0.01–0.25)	2 (1–2)	0.125 (0.06–0.25)
<i>S. aureus</i>	Preoperative skin	1	0.25	64	0.06	0.5	0.125
CoNS	Preoperative skin	14	0.25 (0.06–16)	32 (0.5–64)	0.03 (0.01–0.5)	0.75 (0.5–2)	0.250 (0.01–0.25)
Control strains							
SEK8	Skin of healthy control		0.25	16	0.125	1	0.25
ATCC 29213	Reference strain		0.5	4	0.06	0.5	0.125
DSM3269	Reference strain		0.5	1	0.5	2	0.25

^a Values in parentheses are the MIC₅₀ ranges for the clinical isolates.

sociated tissue damage (5, 6). For physical therapy, however, ultrasound of 1 MHz (unpulsed) had been described to have a beneficial effect on osteoarthritis (45). Further, application of heat has traditionally been used in physical therapy with various rates of success and minimal harm (9).

Increasing the temperature induced the formation of thicker biofilms (40). Although the amount of heating was never measured in the ultrasound or electric current experiments, heating may have an influence on the effects of antibiotics on biofilms. Thus, we investigated the effects of stepwise increases in the ambient temperature together with antibiotic treatment on biofilm thickness and bacterial growth. Static biofilms of clinical isolates from orthopedic implant infections or preoperative skin isolates, including *S. aureus* and CoNS (*S. epidermidis*, *S. lugdunensis*, *S. hominis*, and *S. capitis*), were incubated at an ambient temperature of 35, 40, or 45°C for 24 h with DAP, VAN, FOM, TGC, or FAM. Three concentrations of each of the antimicrobial agents were investigated.

(Some of the data presented here were presented as poster K2069 at the 48th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy-Infectious Diseases Society of America 46th Annual Meeting, San Francisco, CA, 2009.)

MATERIALS AND METHODS

The University Hospital of Vienna in Vienna, Austria, is a 2,200-bed primary and tertiary-care teaching hospital. Overall, 35 staphylococci were tested: 19 isolates from implant-related infections (*S. aureus*, *n* = 13; *S. epidermidis*, *n* = 5; and *S. capitis*, *n* = 1) and 15 skin isolates (*S. aureus*, *n* = 1; *S. epidermidis*, *n* = 9; *S. lugdunensis*, *n* = 4; *S. hominis*, *n* = 1) taken from the thigh before surgical disinfection and incision for hip arthroplasty after femoral neck fracture. Because of the low numbers of non-*S. epidermidis* isolates, *S. epidermidis*, *S. lugdunensis*, *S. capitis*, and *S. hominis* were grouped together as CoNS. Biofilm-producing strains *S. aureus* ATCC 29213 and *S. epidermidis* DSM3269 served as reference strains. In addition, a biofilm-producing *S. epidermidis* isolate (isolate KO8) from the skin of non-hospital-associated healthy volunteer was tested as the internal control. Antibiotic susceptibility was determined by the disk diffusion method on cation-adjusted Mueller-Hinton agar (bioMerieux, Marcy l’Etoile, France). Resistance to oxacillin to detect methicillin resistance was determined by incubating the plates with disks containing oxacillin at 5 µg and at temperatures of 30°C and 37°C.

Antimicrobial agents. Commercially available DAP, VAN, FOM, TGC, and FAM were purchased from Novartis (Vienna, Austria), Lilly (Vienna, Austria), Astropharma (Vienna, Austria), Wyeth-Lederle (Vienna, Austria), and Sandoz (Kundl, Austria), respectively.

Determination of MICs. Before the biofilms were treated with DAP, VAN, FOM, TGC, or FAM, the MICs of each isolate to be tested were determined. The determination of MICs was done according to the protocol of the Clinical and Laboratory Standards Institute using the microtiter plate method (11).

According to this protocol, cation-adjusted Mueller-Hinton medium and Mueller-Hinton broth (MHB) containing 50 µg/ml calcium were used for *in vitro* susceptibility testing of DAP. For the susceptibility testing of FOM, glucose-6-phosphate (25 mg/liter) was added (Table 1).

Thermostability testing. To test for thermostability, all antimicrobials in powder and in solution, as used for MIC testing, were incubated at 45°C for 1 and 24 h in three independent experiments. For those experiments *S. aureus* ATCC 29213 and a clinical isolate of *S. epidermidis* (OR1) were used. The increase in the MICs—if any—was not significant (Table 2).

Biofilm model. Biofilms were studied using the static microtiter plate model established by Christensen et al. (10). The *S. aureus* and CoNS isolates were prepared in MHB at a concentration of a McFarland 0.5 standard and diluted 1:100 with MHB. Each well of a 96-well polystyrene flat-bottomed microtiter plate was filled with 50 µl of diluted bacteria and 50 µl supplemented MHB (containing calcium), and the plate was incubated for 24 h in ambient air at 35°C. The medium and planktonic cells were removed. The biofilms in the wells were fixed with formalin (37%, diluted 1:10) plus 2% sodium acetate, and each well was stained with 150 to 250 µl 1% crystal violet for 5 min. The stained biofilms were then washed two times with approximately 300 µl distilled water. The wells were then visually checked for the presence or absence of a biofilm, based on the presence of staining at the bottom of the well. The mean optical density (OD) was used for quantification using a routine microtiter plate reader at a 550-nm wavelength. All biofilm experiments were done 4 times for each isolate to minimize variability in the OD measurements. To ascertain the biofilm formation, biofilms were grown on cover slides using a 24-well plate. After 24 h, the biofilms were fixed with 2% glutaraldehyde, and biofilm formation was verified by scanning electron microscopy. Established biofilms grown for 24 h were used for the experiments.

Incubation of the biofilms with DAP, VAN, FOM, TGC, or FAM at ambient temperatures of 35°C, 40°C, and 45°C. To test the effects of the antimicrobials, the 24-h biofilms were incubated with DAP, VAN, FOM, TGC, or FAM at three concentrations and at three different temperatures (35°C, 40°C, or 45°C) for 24 h.

TABLE 2. Thermostability of the antimicrobials with incubation for 24 h at 45°C as powders as well as in solution

Strain	Antibiotic	Median (range) MIC ₅₀ (mg/liter)		
		Standard	After incubation with antimicrobial agent ^a	
			As powder	In solution
<i>S. aureus</i> ATCC 29213	Cefamandole	0.25 (0.25–0.25)	0.25 (0.25–0.25)	0.5 (0.25–0.5)
	Fosfomicin	0.5 (0.25–1)	1 (0.5–1)	0.5 (0.5–1)
	Tigecycline	1 (1–1)	1 (1–1)	1 (1–1)
	Vancomycin	0.5 (0.5–2)	0.5 (0.5–1)	1 (0.5–1)
<i>S. epidermidis</i> OR1	Daptomycin	0.25 (0.25–0.5)	0.5 (0.5–1)	1 (0.5–1)
	Cefamandole	1 (1–2)	1 (1–2)	2 (1–2)
	Fosfomicin	32 (16–32)	32 (32–32)	32 (16–32)
	Tigecycline	1 (1–2)	1 (1–1)	2 (1–2)
	Vancomycin	2 (2–2)	2 (1–2)	2 (1–2)
	Daptomycin	0.5 (0.25–1)	0.5 (0.5–1)	1 (0.5–1)

^a Incubation was for 24 h at 45°C.

Three concentrations of each antimicrobial agent were tested: DAP at 5, 25, and 100 mg/liter; VAN at 5, 25, and 100 mg/liter; TGC at 1, 5, and 25 mg/liter; FOM at 100, 250, and 1,000 mg/liter; and FAM at 50, 100, and 500 mg/liter. The lower concentrations of the antimicrobial agents were chosen with regard to their achievable trough or peak levels in serum. To investigate the impact of a very high concentration, those concentrations were investigated.

The OD was used to measure the biofilm thickness. To correct for the individual biofilm formation of each isolate, the OD ratio (ODr), which was the OD of the untreated biofilm/OD of the treated biofilm, was calculated. The baseline ODr of the untreated biofilm is set equal to 1. The explanation for this was that, using the uncorrected ODs of the biofilms, the intra-assay variability was determined from quadruplicate analyses of the treated biofilms. The median coefficient of variance (CV) for all 34 staphylococcal isolates was 20.5% (range, 0.6 to 110.5%). The median CV for intra-assay variability for *S. aureus* isolates was 15.76% (range, 0.62 to 110.6%), and that for CoNS isolates was 20.78% (range, 3.65 to 6%). The interassay (day-to-day) variability was based on bacterial growth without antibiotic (control condition) on two different days: the median CV was 45.8% (range, 13.44 to 102.1%). The median CV for interassay (day-to-day) variability for the *S. aureus* isolates was 44.7% (range, 39.7 to 50.7%), and that for CoNS isolates was 48.8% (range, 13.4 to 102.1%). Taking the ODr of all isolates, the median CV for intra-assay variability was 28.5% (range, 5 to 117%). The median CV for intra-assay variability for *S. aureus* isolates was 26.7% (range, 5 to 117%), and that for CoNS isolates was 28.2% (range, 9.9 to 108.7%). The interday variability was not calculated because by taking the ODr equal to the OD of the treated biofilm/OD of the untreated biofilm, the baseline (untreated) value was equal to 1, thus excluding any variability. Given the intersubject variability, the ODr was used for the calculations.

Efficacies of antimicrobial agents against biofilms in all experiments. To test for viable bacteria in the biofilms in all experiments, the biofilms were not fixed and dyed but were scraped off and resuspended in MHB, seeded onto Columbia agar, and examined for growth. The numbers of bacteria in suspension were enumerated by serial dilutions, and 0.1 ml of each dilution was inoculated onto blood agar plates. The plates were incubated at 35°C in ambient air and were read after 48 h. Each test was performed in duplicate, and the results were expressed as the mean \log_{10} numbers of CFU per ml (mean \log_{10} bacterial count).

Statistical methods. The significance of the differences was assessed by means of the chi-square test for categorical variables. For subgroups and nonnormally distributed variables, the Mann-Whitney U test was used. To determine the changes due to the different antibiotic combinations and concentrations of every isolate, a general linear model for repeated measurement was calculated. All tests were performed using SPSS for Windows, release 17 (SPSS). A *P* value of <0.05 was considered significant for the reduction of the biofilm ODr. For the reduction of growth, a decrease in the colony count of >2 log counts was considered significant.

RESULTS

Biofilm density. The biofilm density increased at the two higher incubation temperatures. The optical densities of the untreated native biofilms were 0.62 ± 0.34 at 35°C, 1.67 ± 0.79 at 40°C, and 1.65 ± 0.8 at 45°C (mean \pm standard deviation [SD]). For the *S. aureus* isolates, the optical densities of the untreated native biofilms were 0.53 ± 0.25 at 35°C, 1.86 ± 0.71 at 40°C, and 2.19 ± 0.64 at 45°C; and for the CoNS isolates, the optical densities of the untreated native biofilms were 0.68 ± 0.38 at 35°C, 1.54 ± 0.82 at 40°C, and 1.27 ± 0.69 at 45°C. For the isolates from implant infections, the optical densities of the untreated native biofilms were 0.52 ± 0.18 at 35°C, 1.8 ± 0.76 at 40°C, and 1.91 ± 0.79 at 45°C. For the isolates from preoperative skin, the optical densities of the untreated native biofilms were 0.74 ± 0.44 at 35°C, 1.51 ± 0.81 at 40°C, and 1.32 ± 0.69 at 45°C.

Incubation with antimicrobial agents. Increasing the temperature to 45°C or to 40°C during incubation with the antimicrobial agents led to significant but differential reductions in the biofilm thickness in comparison to that at 35°C and that for the untreated biofilm (Fig. 1; Table 3).

Cefamandole, fosfomycin, and daptomycin. FAM, FOM, and DAP reduced the biofilm mass with increasing temperature. After incubation at 45°C, FAM and FOM at all three concentrations tested reduced the biofilm ODr significantly compared to the biofilm ODr after incubation at 35°C (for FAM at the three concentrations, ODr values were 0.86 ± 0.29 , 0.63 ± 0.19 , and 0.65 ± 0.28 , respectively, at 45°C versus 1.29 ± 0.61 , 1.12 ± 0.52 , and 1.27 ± 0.59 , respectively, at 35°C; for FOM at the three concentrations, ODr values were 0.78 ± 0.29 , 0.69 ± 0.26 , and 0.63 ± 0.28 , respectively, at 45°C versus 1.07 ± 0.57 , 1.01 ± 0.52 , and 0.78 ± 0.38 at 35°C; values are means \pm SDs; *P* < 0.05). Compared to the results for the untreated biofilms, FAM (100 mg/liter and 500 mg/liter) and FOM (250 mg/liter and 1,000 mg/liter) achieved significant reductions in biofilm growth at 45°C and 40°C. At 40°C, DAP at all three concentrations reduced the biofilm ODr significantly compared to the ODr at 35°C (ODr values, 0.9 ± 0.25 , 0.56 ± 0.25 , and 0.40 ± 0.33 , respectively, at 40°C versus ODr values of 1.02 ± 0.48 , 0.71 ± 0.46 , and 0.5 ± 0.39 , respectively, at 35°C; *P* < 0.05). At the concentration of 100 mg, the biofilm ODr was already significantly reduced at 35°C. An increase in the temperature did not bring any further reduction in the ODr (Table 1; Fig. 1A). Antimicrobials administered at very high concentrations were more effective at reducing the biofilm ODr compared to the ODr of the untreated biofilms, particularly at 40°C and 45°C. DAP at concentrations of 25 mg/liter and 100 mg/liter achieved significant reductions in the biofilm ODr values at 35°C, 40°C, and 45°C.

Tigecycline and vancomycin. TGC and VAN achieved variable effects with increasing temperature. At 45°C, TGC at concentrations of 1 mg/liter and 5 mg/liter achieved significant reductions in the biofilm ODr compared to the ODr of biofilms incubated with TGC at 35°C. TGC at concentrations of 5 mg/liter and 25 mg/liter achieved significant reductions in the biofilm ODr compared to the ODr of the untreated biofilm at 45°C only. VAN at all three concentrations at 45°C and VAN at concentrations of 25 mg/liter and 100 mg/liter at 40°C reduced the biofilm ODr compared to the biofilm ODr at 35°C. However, at 35°C, biofilms incubated with VAN were exceedingly thicker than the untreated biofilms. At 40°C but not at 45°C, VAN (25 mg/liter and 100 mg/liter) reduced the biofilm thickness compared to that of the untreated biofilms.

Antibacterial efficacy. The colony counts of the untreated biofilms at 35°C, 40°C, and 45°C were $1.5 \times 10^8 \pm 1.3 \times 10^7$, $1.3 \times 10^8 \pm 1.1 \times 10^7$, and $2.8 \times 10^7 \pm 2.7 \times 10^7$, respectively (means \pm SDs). For DAP and FAM, bacterial growth was significantly reduced but was not eradicated with increases in the incubation temperature and antibiotic concentration. After incubation at 35°C, a significant reduction in the amount of bacterial growth was observed only with DAP at a concentration of 100 mg/liter (log reduction, 2) and not with any other antibiotic tested in the study. The reduction of growth in biofilms was enhanced at 45°C for DAP at 25 mg/liter (log reduction, 1.2; *P* was not significant) and at 35°C, 40°C, and 45°C for DAP at 100 mg/liter (log count reductions, 4, 3.6, and 3.3, respectively; *P* < 0.05). Bacterial growth reduction by 2 log counts was detected for FAM at a concentration of 500 mg/liter at 40°C and 45°C (*P* = 0.01). FOM at 1,000 mg/liter reduced the bacterial growth by 1.2 log counts (*P* was not

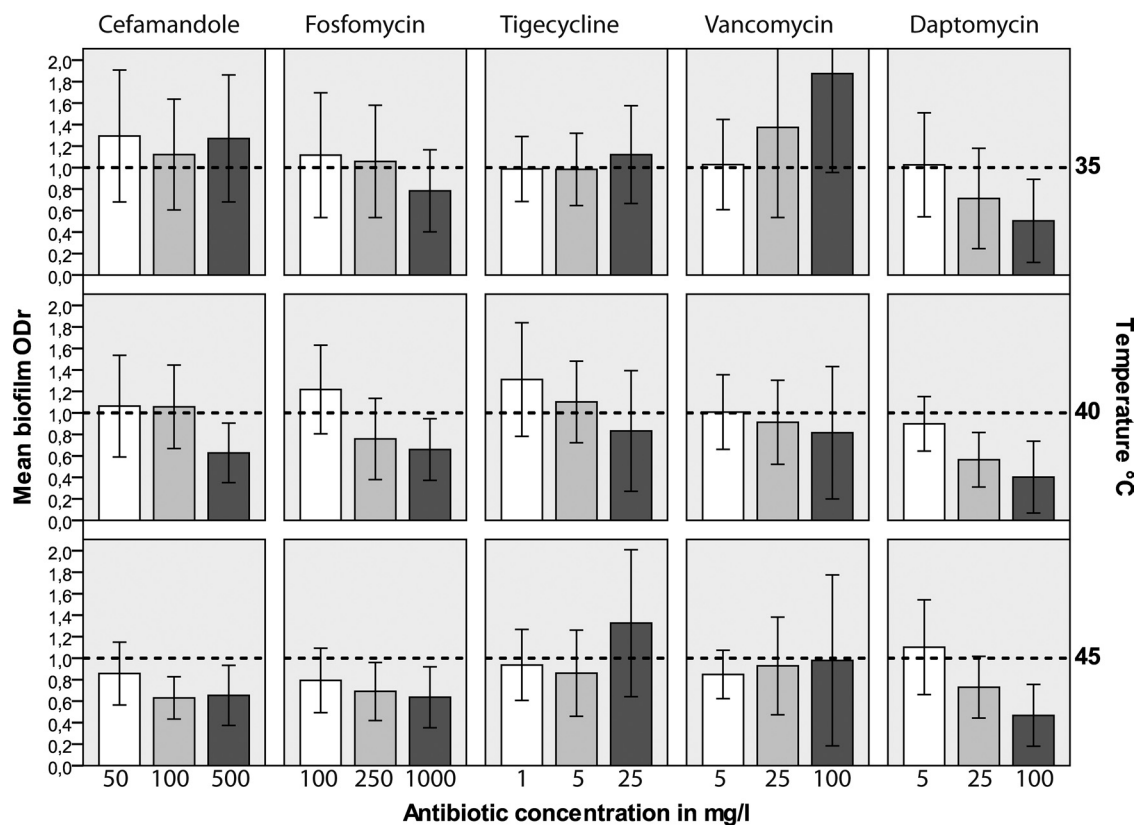


FIG. 1. Mean reduction of the biofilm thickness given as the ODr values obtained with cefamandole, fosfomycin, tigecycline, vancomycin, and daptomycin as a function of the incubation temperature. The error bars indicate ± 1 SD. The baseline ODr of the untreated biofilm is 1 (dashed lines).

significant). No growth reduction was observed for VAN and TGC at any concentration or any incubation temperature (Fig. 2).

DISCUSSION

Temperature is a basic requirement for bacterial growth. Most clinically significant microorganisms are mesophiles that grow optimally at temperatures of between 25°C and 40°C (33). Microbial biofilms present ubiquitous strategies to survive in hostile environments and are influenced by many factors. These factors include the species, number, and physiology of the microorganisms themselves; the presence and concentrations of nutrients or toxins; as well as environmental conditions, such as shear forces, pH, and temperature (20, 40). Ambient temperature has a remarkable influence on microbial biofilms. Increased ambient temperature decreases the biofilm formation of *Streptococcus intermedius* (1) and the rate of recovery of sonicated cultures (31) and induces bacterial stress and increased vulnerability in *Pseudomonas putida* (48). Increasing the temperature to 42°C led to an increase in the biofilm density in an *S. epidermidis* mutant derived from a clinical isolate (40). This effect is confirmed by the findings of the present study, because untreated biofilms incubated at 40°C or 45°C exhibited greater densities than biofilms incubated at 35°C. However, the activity of disinfectants was described to be increased at increased temperatures (44). In the present study, we investigated the influence of increases in the

TABLE 3. Changes in ODr of biofilms incubated with cefamandole, fosfomycin, tigecycline, vancomycin, and daptomycin at ambient temperature of 35°C, 40°C, or 45°C for 24 h

Antibiotic	Concn (mg/liter)	Mean ODr change \pm SD		
		35°C	40°C	45°C
Cefamandole	50	1.29 \pm 0.61	1.07 \pm 0.46	0.86 \pm 0.29 ^{a,b}
	100	1.12 \pm 0.52	1.06 \pm 0.38	0.63 \pm 0.19 ^{a,b,c}
	500	1.27 \pm 0.59	0.62 \pm 0.27 ^{a,b,c}	0.65 \pm 0.28 ^{a,b,c}
Fosfomycin	100	1.07 \pm 0.57	1.22 \pm 0.41	0.78 \pm 0.29 ^{a,b}
	250	1.01 \pm 0.52	0.75 \pm 0.38 ^{a,b,c}	0.69 \pm 0.26 ^{a,b,c}
	1,000	0.78 \pm 0.38 ^a	0.65 \pm 0.28 ^{a,c}	0.63 \pm 0.28 ^{a,b,c}
Tigecycline	1	0.98 \pm 0.3	1.31 \pm 0.52	0.93 \pm 0.33 ^b
	5	0.99 \pm 0.33	1.1 \pm 0.38	0.86 \pm 0.40 ^{a,b}
	25	1.12 \pm 0.45	0.83 \pm 0.56 ^{a,b}	1.32 \pm 0.68
Vancomycin	5	1.04 \pm 0.41	1 \pm 0.35	0.85 \pm 0.22 ^{a,b}
	25	1.37 \pm 0.82	0.91 \pm 0.39 ^b	0.93 \pm 0.45 ^b
	100	1.87 \pm 0.92	0.81 \pm 0.61 ^{a,b,c}	0.98 \pm 0.79 ^b
Daptomycin	5	1.02 \pm 0.48	0.9 \pm 0.25 ^a	1.1 \pm 0.44
	25	0.71 \pm 0.46 ^{a,c}	0.56 \pm 0.25 ^{a,b,c}	0.73 \pm 0.29 ^{a,c}
	100	0.5 \pm 0.39 ^{a,c}	0.40 \pm 0.33 ^{a,b,c}	0.47 \pm 0.29 ^{a,c}

^a Significant reduction of biofilm OD compared to OD of the untreated biofilm. The reference ODr of the untreated biofilm is 1 ($P < 0.05$).

^b Significant reduction of biofilm OD compared to OD of biofilm incubated with the respective antibiotic at 35°C ($P < 0.05$).

^c Significant reduction of biofilm OD compared to OD of biofilm incubated with the lowest concentration of the respective antibiotic ($P < 0.05$).

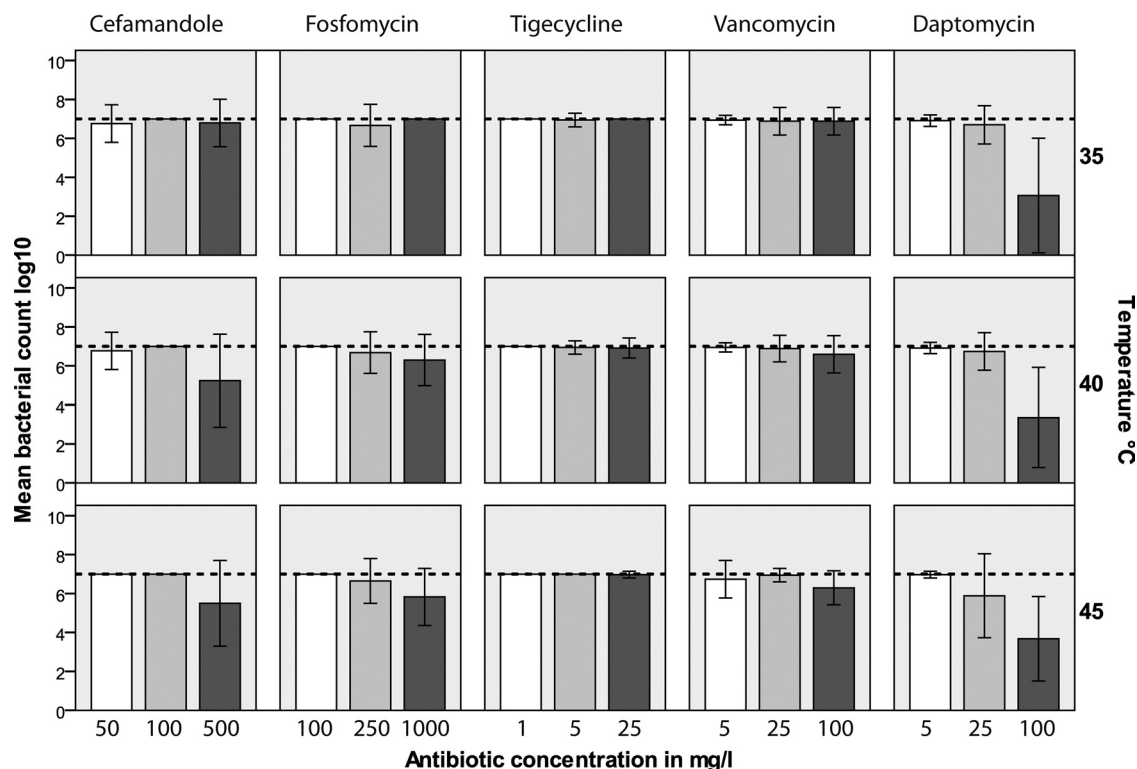


FIG. 2. Mean reduction of bacterial growth after incubation with cefamandole, fosfomycin, tigecycline, vancomycin, and daptomycin as a function of the incubation temperature. The error bars indicate ± 1 SD. The colony count of the untreated biofilm is 10^7 CFU/ml (dashed lines).

incubation temperature to 40°C and 45°C on the effects of antimicrobial agents on established biofilms.

Incubation with the antibiotics at 40°C or 45°C achieved significant reductions in the biofilm thickness and bacterial growth (Table 3; Fig. 1). Similar to the findings of a study of electric current and the activities of antimicrobial agents against *Pseudomonas aeruginosa*, *S. aureus*, and *S. epidermidis* biofilms by del Pozo et al. (15), the effects of increases in temperature were different for the different antibiotics in the present study. For VAN and TGC, TGC incubated at 45°C achieved a significant reduction in the biofilm ODr compared to the ODr of biofilms treated with TGC at 35°C but not compared to the ODr of untreated biofilms at 35°C. At 45°C, VAN reduced the biofilm ODr compared to the biofilm ODr at 35°C, but at 35°C, biofilms that were treated with VAN were exceedingly thicker than the untreated biofilms. Subinhibitory concentrations of tetracycline were reported to enhance the expression of the *icaABCD* gene cluster, which is responsible, although not exclusively, for biofilm production in staphylococci. This may explain the effects of TGC, for TGC shares part of its chemical structure with tetracycline, but for VAN, enhancement of *icaABCD* operon expression has not yet been observed (40).

For FOM, the increase in temperature led to a significant reduction in biofilm thickness but not of bacterial growth. There was a decrease in the biofilm thickness, as shown for DAP and FAM (Fig. 1 and 2). For DAP, the concentration was pivotal. DAP at a concentration of 25 mg/liter or 100 mg/liter reduced the biofilm density at 35°C, 40°C, and 45°C compared to that achieved with DAP at 5 mg/liter and that of the un-

treated biofilm (Table 3). The reduction in the biofilm thickness due to DAP at a concentration of 25 mg/liter or 100 mg/liter is already significant, so an increase in the temperature does not have any further effect on the biofilm thickness. However, with regard to bactericidal activity, an increase in the temperature to 45°C decreases the bacterial growth for DAP at 25 mg/liter (Table 3; Fig. 1 and 2).

A moderate increase in the ambient temperature increased the activities of the antibiotics DAP, FAM, and FOM, yet eradication of the bacteria was not observed for any antibiotic even at the very high concentrations tested. As the lower concentrations used were chosen according to the serum levels reached in clinical practice, these levels might be reached after application of the dosages used. However, when plasma protein binding is taken into account, the TGC or DAP dosages would rise to very high concentrations (Table 4), yet these calculations are based on the results of studies with a great variety of populations whose pharmacokinetics might be different from those of patients with implant infections. Additionally, the very high concentrations used in the present study may not be attainable in synovial fluid after systemic administration, although doses of DAP higher than the recommended dose of 6 mg/kg of body weight, for example, have already been investigated for the treatment of implant infections (46). However, topical application of highly concentrated antibiotics through the use of either intra-articular administration, antibiotic-impregnated polymethylmethacrylate, or bonding of the antimicrobials on implant surfaces may be an option. The VAN included in cement spacers used in two-stage revisions of

TABLE 4. Hypothetical i.v. dosage, corrected for plasma protein binding, of antibiotics needed to reach the *in vitro* concentrations tested in the present study

Antibiotic	Dose administered i.v.	Mean concn (mg/liter)	% protein binding	Hypothetical i.v. dose	Reference(s)
Cefamandole	1,000 g	Serum, 77.7; blister, 22.1 (peak)	74	Serum, 8 g for 50 mg/liter, 16 g for 100 mg/liter, 80 g for 500 mg/liter	Armstrong et al. (2), Fong et al. (17)
Fosfomycin	8 g (100 mg/kg)	Serum, 394.7 (peak); bone tissue, 96.4	Negligible	Serum, 2 g for 100 mg/liter, 6 g for 250 mg/liter, 24 g for 1,000 mg/liter; bone, 8 g for 100 mg/liter	Ode et al. (34), Schintler et al. (47)
Tigecycline	100 mg	Serum, 0.198; bone, 0.077; synovial fluid, 0.116 (after 4 h)	71–97	Serum, >7–10 g for 1 mg/liter, >35–50 g for 5 mg/liter; bone, >70–100 g for 1 mg/liter	Rodvold et al. (43)
Vancomycin	1 g	Plasma, 12.9; cancellous bone, 2.66; cortical bone, 11.53 (variable interval after dosing)	10–50	Serum, 0.5–1 g for 5 mg/liter, 12.5–10 g for 25 mg/liter; cancellous bone, 2–4 g for 5 mg/liter, 10–20 g for 25 mg/liter	Garazzino et al. (19)
Daptomycin	12 mg/kg	Serum, 164.8 (peak) and ca. 20 (trough)	90–93	Serum, 8 mg/kg for 5 mg/liter; 40 mg/kg for 25 mg/kg	Benvenuto et al. (3)

infected hip implants was tolerated at concentrations of >1,500 µg/ml (23). However, the safety and efficacy of at least DAP, TGC, FAM, and FOM for topical use are not well studied. Rifampin, which is probably the single most important drug currently used against biofilm infections of arthroplasties (49), was not investigated in the present study because of the existence of many experimental and clinical data (50). Although rifampin is very active against bacterial and staphylococcal biofilms in particular, resistance to rifampin is increasing in many organisms and not only staphylococci (22, 28).

The question is how heat can be applied topically and used for a long period without risking thermal burns. Hyperthermia treatment is used in cancer therapy, and application of heat has been described to be helpful in preventing postoperative wound infections (25, 29). Shortwave ultrasound and electric current are used for acceleration of infected wound healing and the treatment of septic nonunions (4, 36, 52). Ultrasound and electric current modulate bacterial biofilms and the effects of antibiotics on biofilm bacteria. Both electric current and ultrasound produce heat either by resistivity or by reflection on the surface. Evidently, treatment with ultrasound influences the formation and/or the thickness of the biofilms (7). In the present study, the application of low-frequency (28.48-kHz) ultrasound plus antibiotics was shown to reduce the colony counts of *Escherichia coli* but not those of *Pseudomonas aeruginosa*. In another study with an animal model of staphylococcal implant infection, the same group used low-frequency (<500-kHz) ultrasound because it did not produce heating to the same levels as the higher-frequency (>1-MHz) ultrasound used for medical imaging and physical therapy (6). Heating of implants was carefully avoided—although the level was not measured—because tissue damage was reported even with low-frequency ultrasound, although there was no significant heating of the skin (42). Heating by the use of ultrasound will work only if the frequency used is appropriate for the respective implant surface. Electric current was shown to enhance the activities of antimicrobial agents (15). However, to create heat, an alternating current might be applied to the implant at an appropriate frequency. Other physical measures that could be used to create heating at the implant surface could be microwaves or lasers, both at appropriate frequencies, or, as described recently, magnetic resonance scanning (26). However, as

evidence of clinical safety is not yet available, the increase in the temperature to 45°C, particularly for a prolonged period, may be associated with thermal burn injuries. Thus, topical thermal treatment of the implant surface might be possible only with implants with specifically modified surface properties (embedded antibacterial nanoparticles, ferromagnetic particles, or beads) or, e.g., with implants made of improved alloys, accompanied by careful monitoring of the surface temperature via sensors or magnetic resonance scanning computing algorithms. Future research and experiments will be needed to build a solid foundation for safe clinical applications.

In conclusion, the moderate increase in the incubation temperature to 45°C resulted in a decrease in biofilm thickness with treatment with DAP, VAN, TGC, FOM, and FAM and significant reductions in bacterial growth with DAP at 25 mg/liter and 100 mg/liter and FAM at 500 mg/liter. For all antibiotics and for DAP in particular, high antimicrobial concentrations were the most effective. To find a feasible approach to the treatment of implant-related infections in trauma and orthopedic surgery, experimental studies are needed to investigate the most effective physical measures to be applied; the most effective means of controlling the delivery of heat to the infected implant surface by the use of either ultrasound, microwaves, electric current, or magnetic resonance scanning; and the effects of local heat in combination with antibiotics.

ACKNOWLEDGMENT

None of us has any conflict of interest to declare.

REFERENCES

- Ahmed, N. A., F. C. Petersen, and A. A. Scheie. 2008. Biofilm formation and autoinducer-2 signaling in *Streptococcus intermedius*: role of thermal and pH factors. *Oral Microbiol. Immunol.* **23**:492–497.
- Armstrong, G. C., R. Wise, R. M. Brown, and J. Hancox. 1981. Comparison of ceftazidime and cefamandole pharmacokinetics and blister fluid concentrations. *Antimicrob. Agents Chemother.* **20**:356–358.
- Benvenuto, M., D. P. Benziger, S. Yankelev, and G. Vighiani. 2006. Pharmacokinetics and tolerability of daptomycin at doses up to 12 milligrams per kilogram of body weight once daily in healthy volunteers. *Antimicrob. Agents Chemother.* **50**:3245–3249.
- Brighton, C. T., J. Black, Z. B. Friedenberg, J. L. Esterhai, L. J. Day, and J. F. Connolly. 1981. A multicenter study of the treatment of non-union with constant direct current. *J. Bone Joint Surg. Am.* **63**:2–13.
- Carmen, J. C., J. L. Nelson, B. L. Beckstead, C. M. Runyan, R. A. Robison, G. B. Schaalje, and W. G. Pitt. 2004. Ultrasonic-enhanced gentamicin trans-

- port through colony biofilms of *Pseudomonas aeruginosa* and *Escherichia coli*. *J. Infect. Chemother.* **10**:193–199.
6. **Carmen, J. C., B. L. Roeder, J. L. Nelson, B. L. Beckstead, C. M. Runyan, G. B. Schaalje, R. A. Robison, and W. G. Pitt.** 2004. Ultrasonically enhanced vancomycin activity against *Staphylococcus epidermidis* biofilms in vivo. *J. Biomater. Appl.* **18**:237–245.
 7. **Carmen, J. C., B. L. Roeder, J. L. Nelson, R. L. Ogilvie, R. A. Robison, G. B. Schaalje, and W. G. Pitt.** 2005. Treatment of biofilm infections on implants with low-frequency ultrasound and antibiotics. *Am. J. Infect. Control* **33**:78–82.
 8. **Ceri, H., M. Olson, D. Morck, D. Storey, R. Read, A. Buret, and B. Olson.** 2001. The MBEC assay system: multiple equivalent biofilms for antibiotic and biocide susceptibility testing. *Methods Enzymol.* **337**:377–385.
 9. **Chou, R., and L. H. Huffman.** 2007. Nonpharmacologic therapies for acute and chronic low back pain: a review of the evidence for an American Pain Society/American College of Physicians clinical practice guideline. *Ann. Intern. Med.* **147**:492–504.
 10. **Christensen, G. D., W. A. Simpson, J. J. Younger, L. M. Baddour, F. F. Barrett, D. M. Melton, and E. H. Beachey.** 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J. Clin. Microbiol.* **22**:996–1006.
 11. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, M7-A7, 6th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
 12. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. *Science* **284**:1318–1322.
 13. **Darouiche, R. O.** 2004. Treatment of infections associated with surgical implants. *N. Engl. J. Med.* **350**:1422–1429.
 14. **Darouiche, R. O., M. D. Mansouri, P. V. Gawande, and S. Madhyastha.** 2009. Antimicrobial and antibiofilm efficacy of triclosan and dispersin B combination. *J. Antimicrob. Chemother.* **64**:88–93.
 15. **Del Pozo, J. L., M. S. Rouse, J. N. Mandrekar, M. F. Sampedro, J. M. Steckelberg, and R. Patel.** 2009. Effect of electrical current on the activities of antimicrobial agents against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* biofilms. *Antimicrob. Agents Chemother.* **53**:35–40.
 16. **Falagas, M. E., A. M. Kapaskelis, V. D. Kouranos, O. K. Kakisi, Z. Athanassa, and D. E. Karageorgopoulos.** 2009. Outcome of antimicrobial therapy in documented biofilm-associated infections: a review of the available clinical evidence. *Drugs* **69**:1351–1361.
 17. **Fong, I. W., E. D. Ralph, E. R. Engelking, and W. M. Kirby.** 1976. Clinical pharmacology of cefamandole as compared with cephalothin. *Antimicrob. Agents Chemother.* **9**:65–69.
 18. **Frossard, M., C. Joukhadar, B. M. Erovic, P. Dittrich, P. E. Mrass, M. Van Houte, H. Burgmann, A. Georgopoulos, and M. Muller.** 2000. Distribution and antimicrobial activity of fosfomicin in the interstitial fluid of human soft tissues. *Antimicrob. Agents Chemother.* **44**:2728–2732.
 19. **Garazzino, S., A. Aprato, L. Baietto, A. D'Avolio, A. Maiello, F. G. De Rosa, D. Aloj, M. Siccardi, A. Biasibetti, A. Masse, and P. G. Di.** 2008. Glycopeptide bone penetration in patients with septic pseudoarthrosis of the tibia. *Clin. Pharmacokinet.* **47**:793–805.
 20. **Goller, C. C., and T. Romeo.** 2008. Environmental influences on biofilm development. *Curr. Top. Microbiol. Immunol.* **322**:37–66.
 21. **Hajdu, S., A. Lassnigg, W. Graninger, A. M. Hirschl, and E. Presterl.** 2009. Effects of vancomycin, daptomycin, fosfomicin, tigecycline and ceftriaxone on *Staphylococcus epidermidis* biofilms. *J. Orthop. Res.* **27**:1361–1365.
 22. **Hellmark, B., M. Unemo, A. Nilsson-Angustinson, and B. Soderquist.** 2009. Antibiotic susceptibility among *Staphylococcus epidermidis* isolated from prosthetic joint infections with special focus on rifampicin and variability of the *rpoB* gene. *Clin. Microbiol. Infect.* **15**:238–244.
 23. **Hsieh, P. H., Y. H. Chang, S. H. Chen, S. W. Ueng, and C. H. Shih.** 2006. High concentration and bioactivity of vancomycin and aztreonam eluted from Simplex cement spacers in two-stage revision of infected hip implants: a study of 46 patients at an average follow-up of 107 days. *J. Orthop. Res.* **24**:1615–1621.
 24. **Huang, Y. T., C. H. Liao, L. J. Teng, and P. R. Hsueh.** 2007. Daptomycin susceptibility of unusual gram-positive bacteria: comparison of results obtained by the Etest and the broth microdilution method. *Antimicrob. Agents Chemother.* **51**:1570–1572.
 25. **Hynnen, K.** 2010. MRI-guided focused ultrasound treatments. *Ultrasonics* **50**:221–229.
 26. **James, J. R., Y. Gao, V. C. Soon, S. M. Topper, A. Babsky, and N. Bansal.** 2010. Controlled radio-frequency hyperthermia using an MR scanner and simultaneous monitoring of temperature and therapy response by (1)H, (23)Na and (31)P magnetic resonance spectroscopy in subcutaneously implanted 9L-gliomas. *Int. J. Hyperthermia* **26**:79–90.
 27. **Joukhadar, C., N. Klein, P. Dittrich, M. Zeitlinger, A. Geppert, K. Skhirtladze, M. Frossard, G. Heinz, and M. Muller.** 2003. Target site penetration of fosfomicin in critically ill patients. *J. Antimicrob. Chemother.* **51**:1247–1252.
 28. **Martin, A., S. Panaiotov, F. Portaels, S. Hoffner, J. C. Palomino, and K. Angeby.** 2008. The nitrate reductase assay for the rapid detection of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. *J. Antimicrob. Chemother.* **62**:56–64.
 29. **Melling, A. C., B. Ali, E. M. Scott, and D. J. Leaper.** 2001. Effects of preoperative warming on the incidence of wound infection after clean surgery: a randomised controlled trial. *Lancet* **358**:876–880.
 30. **Mermel, L. A., B. M. Farr, R. J. Sherertz, I. I. Raad, N. O'Grady, J. S. Harris, and D. E. Craven.** 2001. Guidelines for the management of intravascular catheter-related infections. *J. Intraven. Nurs.* **24**:180–205.
 31. **Monsen, T., E. Lovgren, M. Widerstrom, and L. Wallinder.** 2009. In vitro effect of ultrasound on bacteria and suggested protocol for sonication and diagnosis of prosthetic infections. *J. Clin. Microbiol.* **47**:2496–2501.
 32. **Monzon, M., C. Oteiza, J. Leiva, M. Lamata, and B. Amorena.** 2002. Biofilm testing of *Staphylococcus epidermidis* clinical isolates: low performance of vancomycin in relation to other antibiotics. *Diagn. Microbiol. Infect. Dis.* **44**:319–324.
 33. **Murray, P. R., E. J. Baron, J. H. Tenover, M. A. Tenover, and R. H. Tenover (ed.).** 2003. Manual of Clinical Microbiology, 8th ed. ASM Press, Washington, DC.
 34. **Ode, B., S. Haidl, B. Hoffstedt, M. Walder, and J. Ursing.** 1988. Fosfomicin versus ampicillin in the treatment of acute pyelonephritis. *Chemioterapia* **7**:96–100.
 35. **Olson, M. E., H. Ceri, D. W. Morck, A. G. Buret, and R. R. Read.** 2002. Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can. J. Vet. Res.* **66**:86–92.
 36. **Peters, E. J., L. A. Lavery, D. G. Armstrong, and J. G. Fleischli.** 2001. Electric stimulation as an adjunct to heal diabetic foot ulcers: a randomized clinical trial. *Arch. Phys. Med. Rehabil.* **82**:721–725.
 37. **Presterl, E., S. Hajdu, A. M. Lassnigg, A. M. Hirschl, J. Holinka, and W. Graninger.** 2009. Effects of azithromycin in combination with vancomycin, daptomycin, fosfomicin, tigecycline, and ceftriaxone on *Staphylococcus epidermidis* biofilms. *Antimicrob. Agents Chemother.* **53**:3205–3210.
 38. **Presterl, E., A. Lassnigg, B. Parschall, F. Yassin, H. Adametz, and W. Graninger.** 2005. Clinical behavior of implant infections due to *Staphylococcus epidermidis*. *Int. J. Artif. Organs* **28**:1110–1118.
 39. **Raad, I., H. Hanna, Y. Jiang, T. Dvorak, R. Reitzel, G. Chaiban, R. Sherertz, and R. Hachem.** 2007. Comparative activities of daptomycin, linezolid, and tigecycline against catheter-related methicillin-resistant *Staphylococcus bacteremic* isolates embedded in biofilm. *Antimicrob. Agents Chemother.* **51**:1656–1660.
 40. **Rachid, S., K. Ohlsen, W. Witte, J. Hacker, and W. Ziebuhr.** 2000. Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesion expression in biofilm-forming *Staphylococcus epidermidis*. *Antimicrob. Agents Chemother.* **44**:3357–3363.
 41. **Ramage, G., M. M. Tunney, S. Patrick, S. P. Gorman, and J. R. Nixon.** 2003. Formation of *Propionibacterium acnes* biofilms on orthopaedic biomaterials and their susceptibility to antimicrobials. *Biomaterials* **24**:3221–3227.
 42. **Rediske, A. M., B. L. Roeder, M. K. Brown, J. L. Nelson, R. L. Robison, D. O. Draper, G. B. Schaalje, R. A. Robison, and W. G. Pitt.** 1999. Ultrasonic enhancement of antibiotic action on *Escherichia coli* biofilms: an in vivo model. *Antimicrob. Agents Chemother.* **43**:1211–1214.
 43. **Rodvold, K. A., M. H. Gotfried, M. Cwik, J. M. Korth-Bradley, G. Dukart, and E. J. Ellis-Grosse.** 2006. Serum, tissue and body fluid concentrations of tigecycline after a single 100 mg dose. *J. Antimicrob. Chemother.* **58**:1221–1229.
 44. **Russell, A. D.** 2003. Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. *Lancet Infect. Dis.* **3**:794–803.
 45. **Rutjes, A. W., E. Nuesch, R. Sterchi, and P. Juni.** 2010. Therapeutic ultrasound for osteoarthritis of the knee or hip. *Cochrane Database Syst. Rev.* **CD003132**.
 46. **Schaad, H. J., M. Bento, D. P. Lew, and P. Vaudaux.** 2006. Evaluation of high-dose daptomycin for therapy of experimental *Staphylococcus aureus* foreign body infection. *BMC Infect. Dis.* **6**:74.
 47. **Schintler, M. V., F. Traummuller, J. Metzler, G. Kreuzwirt, S. Spendel, O. Mauric, M. Popovic, E. Scharnagl, and C. Joukhadar.** 2009. High fosfomicin concentrations in bone and peripheral soft tissue in diabetic patients presenting with bacterial foot infection. *J. Antimicrob. Chemother.* **64**:574–578.
 48. **Srivastava, S., A. Yadav, K. Seem, S. Mishra, V. Chaudhary, and C. S. Nautiyal.** 2008. Effect of high temperature on *Pseudomonas putida* NBRI0987 biofilm formation and expression of stress sigma factor RpoS. *Curr. Microbiol.* **56**:453–457.
 49. **Trampuz, A., and W. Zimmerli.** 2005. New strategies for the treatment of infections associated with prosthetic joints. *Curr. Opin. Invest. Drugs* **6**:185–190.
 50. **Uckay, I., D. Pittet, P. Vaudaux, H. Sax, D. Lew, and F. Waldvogel.** 2009. Foreign body infections due to *Staphylococcus epidermidis*. *Ann. Med.* **41**:109–119.
 51. **Venkatesh, M., L. Rong, I. Raad, and J. Versalovic.** 2009. Novel synergistic antibiofilm combinations for salvage of infected catheters. *J. Med. Microbiol.* **58**:936–944.
 52. **Zichner, L.** 1981. Repair of nonunions by electrically pulsed current stimulation. *Clin. Orthop. Relat. Res. Nov-Dec.* **115**–121.
 53. **Zimmerli, W., A. Trampuz, and P. E. Ochsner.** 2004. Prosthetic-joint infections. *N. Engl. J. Med.* **351**:1645–1654.