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Lysostaphin as a Potential Therapeutic Agent for Staphylococcal Biofilm Eradication

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Abstract

The aim was to study the activity of lysostaphin in monotherapy or in combination with oxacillin, towards biofilms built by clinical and reference *S. aureus* and *S. epidermidis* strains in the wells of microplate, in the chambers of a LabTekII chamber slide or on the polyethylene catheter. MICs of oxacillin and lysostaphin for planktonic bacteria were determined according to the standards of NCCLS. BIC (Biofilm Inhibitory Concentration) was estimated by the MTT assay. The integrity of biofilm treated with antimicrobials was also examined: by staining with FITC and laser scanning fluorescence confocal microscopy and visually by TTC reduction assay. Despite the fact that susceptibility of planktonic cultures of 25 staphylococcal strains to lysostaphin action was various, we have demonstrated the effectiveness of lysostaphin in the treatment of biofilm, built not only on the flat surface of the microplates but also on catheter's surface. The synergistic effect of subBIC lysostaphin+oxacillin was observed for MSSA and MRSA biofilms but not for 1474/01 hVISA strain. Also BIC_{OXA} for *S. epidermidis* RP12 and A4c strains, but not for 6756/99 MRSE biofilms was reduced when lysostaphin was simultaneously used.

Key words: staphylococcal biofilms, antibiotics, lysostaphin

Introduction

Bacterial biofilms are described as polymer-dipped communities of cells which accumulate, in a precisely controlled manner, on the abiotic or biotic surfaces (Lewis, 2001; Fux *et al*., 2003; Boles *et al*., 2004). Some naturally existing biofilms have a protective role for the host tissue homeostasis (biofilms on urogenital epithelium, intestine epithelium, dental plaques), by preventing their colonization by exogenous pathogens (Prakash *et al*., 2003; Boles *et al*., 2004; Fux *et al*., 2003). However, it should be stressed that bacterial and fungal biofilms are also responsible for a number of diseases, such as native valve endocarditis, cystic fibrosis-associated pneumonia, middle ear infections, bone infections, bacterial prostatitis, periodontitis (Hall-Stoodley *et al*., 2004; Götz, 2002; Fux *et al*., 2003). Biofilms are also involved in the pathogenesis of various infections related to implanted medical devices (urinary and vascular catheters, prosthetic heart valves, prosthetic hip/knee, contact lenses *etc.*). Most of these infections have a chronic nature and, because of the intrinsic resistance of the biofilm cells to antibiotics and host defense sytems, such diseases are very difficult to treat effectively. Many hypotheses were considered to explain the high biofilm resistance to antimicrobial agents: restricted penetration, decrease in bacterial metabolism and growth rate, increase in antibiotic-degrading enzymes accumulation and enhancement of exchanging rate of genes encoding for resistance (Lewis, 2001; Hall-Stoodley *et al*., 2004; Fux *et al*., 2003). Currently, the most accepted view is that all the hypotheses are true, but none of them explains the resistance of various biofilms to killing by

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different groups of antimicrobial agents. Nevertheless, additional and now dominating is molecular biology based explanation of antibiotic tolerance rather than resistance, induced within biofilm structure *i.e.* expression of stress response genes, phase variation, persister-state and biofilm-specific phenotype development (Hall-Stoodley *et al*., 2004).

Many research groups investigated, alternative to antibiotics, potential strategies in preventing biofilm formation or its eradication. One possible approach is enzymatic removal of bacterial biofilms, but due to the heterogeneity of the extracellular matrix, in most cases a mixture of enzymes may be necessary for a sufficient degradation of biofilm structure. Nevertheless, this strategy is useful in the eradication of so called environmental biofilms, *e.g.* industrial pipelines (water, oil) or food processing equipment. Enzymatic biofilm disruption is a very attractive idea for the prevention or elimination of pathogenic biofilms causing various medical problems. Enzymatic activity can be directed to biofilm matrix, allowing better penetration of subsequent antimicrobials used or to be directed to pathogen's cell wall components and causing their lysis (Johansen *et al*., 1997; Kaplan *et al*., 2004). Endopeptidases secreted by different bacterial species are examples of enzymes which can be used for this purpose. Two of them are now being thoroughly investigated, the LasA protease produced by *Pseudomonas aeruginosa* and lysostaphin secreted by *Staphylococcus simulans*, both specifically active towards staphylococcal strains (Barequet *et al.*, 2004; Wu *et al*., 2003). Lysostaphin, a 27-kDa endopeptidase which degrades the pentaglycine bridges in peptidoglycan bone of the cell wall, was shown as a potent antistaphylococcal agent, however, it acts much more effectively against *S. aureus* than *S. epidermidis* strains due to differences in cell wall composition of these species. *S. aureus* and coagulase-negative staphylococci (CNS), mainly *S. epidermidis*, are known as the leading species in chronic polymer-associated infections of biofilm nature and resistant to antibiotic treatment. Therefore, we ask the question whether lysostaphin may be considered as an effective biofilm eradicating agent, since its strong activity against planktonic staphylococci is well documented (Climo *et al*., 1998, Kiri *et al*., 2002, von Eiff *et al.*, 2003) and what advantages or limitations the use of lysostaphin alone or with antibiotics, creates.

Experimental

Materials and Methods

Bacteria. The group of 25 staphylococcal strains chosen for investigation consisted of: clinical *S. aureus* (n = 10) and *S. epidermidis* (n = 9) isolates, *S. aureus* ATCC25923 and *S. aureus* ATCC29213 (the reference MSSA strains), *S. aureus* 1474/01 (clinical hVISA, NIPH), *S. epidermidis* ATCC12228 (the reference MSSE strain), *S. epidermidis* RP12 (slime producing clinical isolate, from the collection of A. Ljungh, Dept. Medical Microbiology, University of Lund, Sweden), *S. epidermidis* 6756/99 (clinical MRSE, NIPH). Most clinical strains were isolated from medical device-associated infections (Table I). The organisms were stored in TSB with 15% glycerol at –70°C, and in each experiment the cultures were established from the original stock.

Antimicrobial agents and susceptibility testing against planktonic bacteria. The antibiotic oxacillin (disks 1 μg and tablets 0.1 mg) was purchased from Mast Diagnostics (United Kingdom). Recombinant lysostaphin (from *S. simulans*, No. L 0761) was obtained from Sigma, (St. Louis, USA). The susceptibility of staphylococcal strains to antimicrobial agents was determined by the standard NCCLS disk diffusion and microdilution methods (National Commitee for Clinical Laboratory Standards, M7-A5, 2000). The concentration range of oxacillin used in the study was 0.125–128.0 μg mL^{-1} in CAMHB + 2% NaCl, whilst lysostaphin concentration range tested was 0.0625–64.0 μg mL^{-1} in CAMH + 0.1% BSA (to prevent its nonspecific adherence to plastic surface). To specify the MICs, turbidometric (OD_{600}) bacterial studies were carried out using the multifunction counter Victor2 (Wallac, Finland). MIC was estimated as the lowest concentration of antimicrobial agent which gave OD equal to the medium negative control (below 0.05).

Biofilm formation. *S. aureus* or *S. epidermidis* from the stock cultures were grown for 24 h at 37°C on the agar plate. Next, a single colony of each strain was grown in 5 mL of TSB (Difco) supplemented or not with 0.25% D-(+)-glucose (TSBGlc). The overnight cultures were diluted 1:40 in TSBGlc. A final volume of 200 μL was added to each well of a 96-well tissue culture plate (Nunclon™ Surface, Nunc) or of 500 μL to each chamber of Lab Tek chamber slide II (Nalge-Nunc International, Napervile, Ill., USA). In order to allow bacteria to form biofilms the plates/chambers were incubated for 24 h at 37°C.

Stains for biofilm visualization. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma), TTC (2,3,5-triphenyltetrazolium chloride, Sigma), FITC (Fluorescein isothiocyanate Isomer I, Sigma) were used.

Biofilm MTT-staining. MTT assay was performed according to the method described by Kairo *et al*., (1999) with minor modifications. Briefly, after 24 h lasting biofilm formation the wells of microplate were emptied and filled with 150 μL of PBS per well, then 50 μL of MTT solution (0,3% in PBS) was added and plates were incubated for 2 h at 37°C. At the end of incubation period, MTT was replaced with 150 μL of DMSO and 25 μL of glycine buffer (0.1 M, pH 10.2). For complete dissolving of formed purple formazan crystals the plates were incubated for 15 min. at room temperature, with gentle agitation. The optical density of the wells containing biofilms was determined using a spectrophotometer (550 nm, Victor2 multifunction counter, Wallac, Finland).

Biofilm FITC-staining. Biofilms formed on the slides surface of Lab Tek chamber slide II device were stained with FITC solution (0,1% in PBS) for 20 min. at room temperature. After staining chambers were emptied and very gently washed, once with

Table I
List of staphylococcal strains used in these studies

Strain	Origin / characteristics
S. aureus	
ATCC25923	reference MSSA, ATCC*
ATCC29213	reference MSSA, ATCC
1474/01	clinical hVISA, NIPH**
A3	drain
A7	hip prosthesis associated abscess
B1	orthopedic wound
C1	tracheostomic tube
D5	tracheostomic tube
D8	venous catheter's tip
D13	drain
E1	tracheostomic tube
E4	tracheostomic tube
E7	tracheostomic tube

Strain	Origin / characteristics
S. epidermidis	
ATCC12228	reference MSSE, ATCC
RP12	slime positive, University of Lund, Sweden
6756/99	clinical MRSE, NIPH
A4a	hip prosthesis
A4b	hip prosthesis
A4c	hip prosthesis
C10	blood
C11	blood
C12	blood
C13	blood
C15	blood
C27	blood

* – American Type Culture Collection, ** – National Institute of Public Health, Poland

PBS supplemented with 4% BSA and twice with PBS. After the washing, the chamber slides partitions were removed and the slides were covered with cover glasses. The effects of biofilm FITC-staining were observed using the laser scanning confocal microscope (LSCM, Zeiss).

Biofilm bacterial cultures for antimicrobial test. Biofilms were prepared in 96-well microplates or in chamber slides device as described above. After 24 h of growth, the medium from the wells/chambers was removed by aspiration. The biofilms were treated either with various concentrations of oxacillin or lysostaphin, or with a combination of oxacillin and subMIC concentration of lysostaphin. Antimicrobial agents were diluted in CAMHB supplemented with 2% NaCl and 0,1% BSA. The incubation time was 24 h at 37°C, then the medium containing the antimicrobial agent was gently aspirated and the viability of the biofilm remaining on the surfaces of the wells or chamber slides was stained with MTT or FITC as described above.

Confocal microscopy study. Examination of FITC-stained biofilms treated with antimicrobial agents was performed using a LSM5 (Pascal) Laser Scanning Confocal Microscope (LSCM), equipped with Axiovert 2 (Zeiss) microscope with objective Plan-Apochromat 100x (1.4 oil). Images were recorded at a 488 nm (argon laser) excitation and emission at 530 nm (long pass filter set). Digital image analysis of LSCM optical thin sections was performed with Pascal Zeiss software.

"Catheter study". Intravascular catheter's (Venflon, PTFE, Becton Dickinson, USA) segments (1 cm length) were prepared using sterile instruments, placed into the eppendorf tubes (in triplicate) containing 1 mL of bacterial suspension (overnight culture of *S. aureus* A3- MRSA, diluted 1:40 in TSBGlc), and incubated for 72 h at 37°C. Then catheter's segments were rinsed with PBS and transferred to the new tubes containing: i) medium, ii) oxacillin, iii) lysostaphin, or iiii) oxacillin and subMIC lysostaphin for 3 or 24 h incubation at 37°C. Next, biomaterial segments, gently rinsed with PBS were moved to the fresh TSBGlc medium with TTC (one drop of 1% TTC in PBS) and incubated for 24 h at 37°C. The presence of red stained bacterial biofilm (reduction by live bacteria of colourless TTC to the red insoluble formazan crystals) was estimated as described previously (Sadowska *et al*., 1998).

Data analysis and presentation. Each assay was performed in duplicate or triplicate on at least two occasions. Data were expressed as the mean from 2–3 evaluations obtained from each experiment.

Results

Most *S. aureus* and *S. epidermidis* strains used in this study were clinical isolates, mainly from patients suffering from medical-devices-associated infections, as presented in Table I. These strains, after their initial identification, were obtained from hospital microbiological laboratories. In our hands, strains were reidentified using selected typing methods (hemolysis, clumping factor and tube coagulase tests) and 32ID Staph (BioMerieux) system.

Using the standard antimicrobial susceptibility tests, according to the 2000' guidelines of NCCLS, the MICs of oxacillin and lysostaphin were determined. The obtained MICs values of each strain cell suspension tested on two occasions were similar (differences not exceeding one dilution), therefore single values are presented (Table II). For the suspension cultures of *S. aureus* and *S. epidermidis* strains, oxacillin MICs ranged from 0.250 to >128 μg mL^{-1}. However, most *S. aureus* clinical strains (7/10) were highly resistant, with MIC_{OXA} >128 μg mL^{-1}, whereas most *S. epidermidis* strains (10/12) had MICs range from 1.0 to

Table II
Susceptibility to oxacillin and lysostaphin of *S. aureus* and *S. epidermidis* strains. MIC for planktonic culture determined by disk-diffusion and broth microdilution methods

Strain	Oxacillin		Lysostaphin
	Inhibition (∅mm)	MIC_{OX} μg mL^{-1}	MIC_{LYS} μg mL^{-1}
S. aureus			
ATCC 25923 (MSSA)	16	0.250	0.125
ATCC 29213 (MSSA)	14	0.250	0.250
1474/01 (hVISA)	0	>128.0	0.0625
A3	0	>128.0	0.250
A7	0	>128.0	0.500
B1	14	2.0	0.250
C1	16	1.0	0.250
D5	0	>128.0	0.250
D8	15	1.0	0.250
D13	13	2.0	0.250
E1	0	>128.0	0.250
E4	0	>128.0	32.0
E7	6	>128.0	0.500

Strain	Oxacillin		Lysostaphin
	Inhibition (∅mm)	MIC_{OX} μg mL^{-1}	MIC_{LYS} μg mL^{-1}
S. epidermidis			
ATCC 12228	22	0.125	4.0
RP12	16	1.0	8.0
6756/99 (MRSE)	6	16.0	32.0
A4a	17	2.0	1.0
A4b	17	2.0	2.0
A4c	17	1.0	1.0
C10	0	16.0	8.0
C11	10	2.0	2.0
C12	14	2.0	0.500
C13	14	2.0	16.0
C15	0	128.0	2.0
C27	0	128.0	8.0

16 μg mL^{-1}. These 25 staphylococcal strains underwent tests of susceptibility to lysostaphin. Within *S. aureus* group, lysostaphin MICs ranged from 0.0625 to 0.500 μg mL^{-1}, with median MIC = 2.7 μg mL^{-1} for more than 92% of strains. One exception was *S. aureus* E4 strain whose MIC_{LYS} was 32 μg mL^{-1}. The median MIC for 12 *S. epidermidis* strains was much higher – 7.0 μg mL^{-1}, and for 91% of strains the range started from 0.5 and ended at 16 μg mL^{-1}. Generally, for most staphylococcal strains there was no correlation between MIC_{OXA} and MIC_{LYS} values. However, *S. aureus* E4 which presented the highest MIC_{LYS} = 32.0 μg mL^{-1} was also highly resistant to oxacillin (MIC_{OXA} >128 μg mL^{-1}. A similar result was demonstrated for 6756/99 MRSE strain (MIC_{LYS} = 32.0 μg mL^{-1}, MIC_{OXA} >16.0 μg mL^{-1}). On the other hand, 1474/01 hVISA strain, whose MIC_{OXA} exceeded 128 μg mL^{-1} had the lowest MIC_{LYS} among all 25 tested strains, which was 0.0625 μg mL^{-1}.

The preparation of *S. aureus* and *S. epidermidis* biofilms, on hydrophobic polystyrene (96-well microplate) or negatively charged glass (Lab Tek chamber slide II) surfaces, produced high bacterial yield. After MTT staining, optical density (OD_{550}) readings ranged from 1.8 to 3.1 (Fig. 1), therefore the isolates were categorized as strong biofilm producers. The degree of biofilm formation was commonly higher when bacteria were initially and finally grown in TSBGlc than in TSB without glucose, however, for some strains (mainly among *S. aureus* group) the opposite effect was observed (data not shown). Cells growing as biofilms (in chamber slide devices) stained with FITC, were characterized without the disturbance of their structure using laser fluorescence scanning microscope. LSCM images showed multilayered clumps of bacteria, surrounded by less dense material, attached to the surface (Fig. 3-A, B). *S aureus* strains produced biofilms which were, on average, 2.5 times thinner than those formed by *S. epidermidis*. Biofilm of *S. aureus* ATCC29213, 24 h after set-up, reached 2.66 ± 0.56 μm of thickness, whereas that of *S. epidermidis* RP12 was 6.35 ± 0.86 μm. When cultures incubation time was prolonged, further growth of biofilms was observed and at the end (72 h) biofilm of *S. aureus* ATCC29213 and *S. epidermidis* RP12 reached the thickness of 4.09 ± 0.78 μm and 8.02 ± 0.61 μm, respectively.

In order to test the hypothesis that lysostaphin and beta-lactam antibiotics act as synergistic agents toward staphylococci, the effect of the oxacillin or lysostaphin (alone or in combination) on cell viability in biofilms was examined. BICs (Biofilm Inhibitory Concentration) of antimicrobials were determined by MTT reduction assay measuring the active metabolism of bacteria that survived the antimicrobials action. Biofilms of all 13 *S. aureus*, as well as 12 *S. epidermidis* strains were highly resistant to oxacillin (BICs > 128–256 μg mL^{-1}). The absorbances of the oxacillin treated *S. aureus* biofilms were the same after 24 h as at the time of antibiotic application (OD_{550} 2.9–3.1). Similarly, the absorbances of *S. epidermidis* biofilms showed a minimal range of changes. One exception was biofilm of *S. epidermidis* RP12 strain treated with oxacillin at the

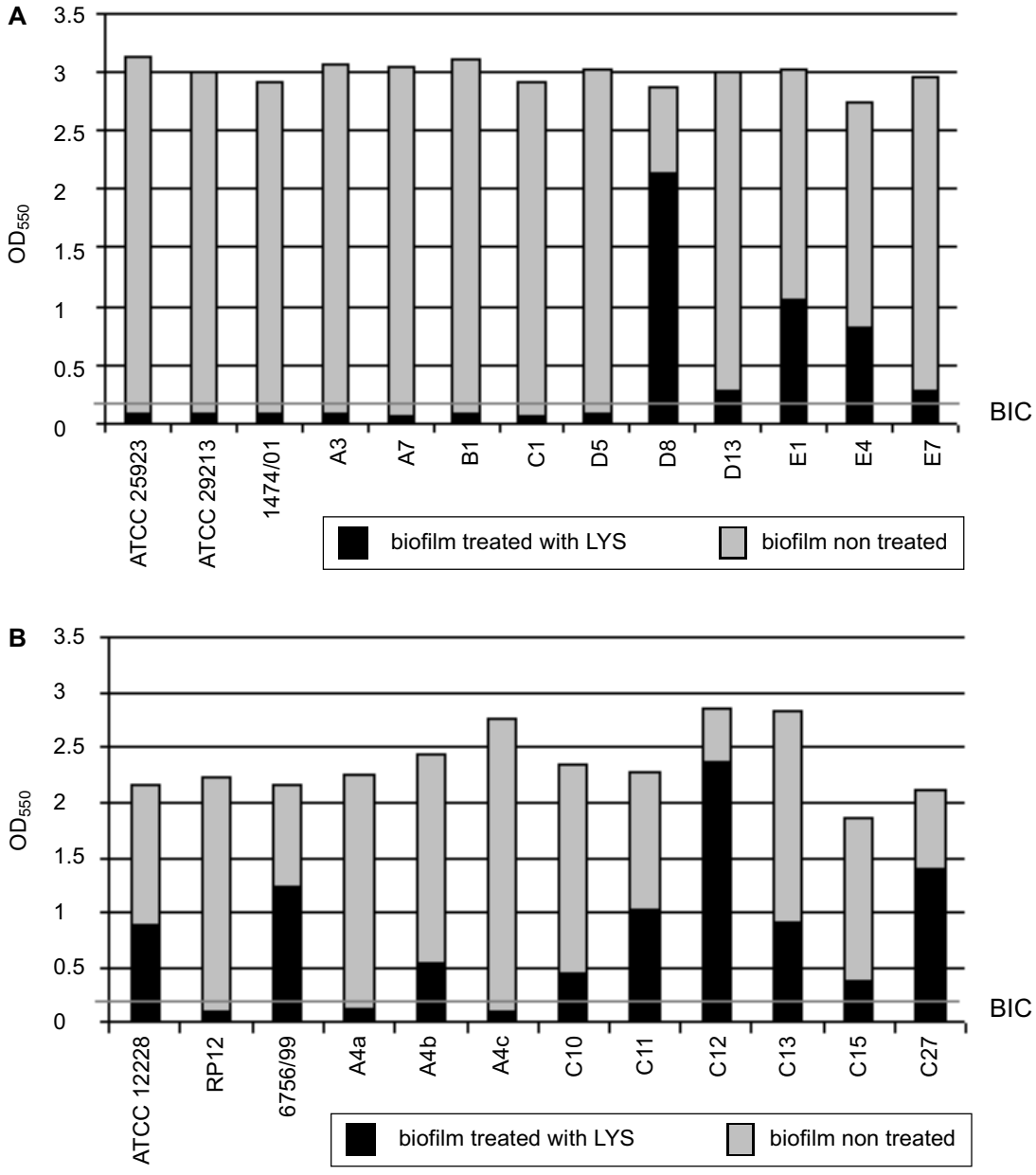

Fig. 1. Biofilm formation of *S. aureus* (A) and *S. epidermidis* (B) strains and their lysostaphin susceptibility, measured by the MTT reduction assay in the wells of 96-well microplate

concentration of 128 μg mL^{-1}, where the absorbance dropped after 24 h from 2.2 to 0.49. For the evaluation of biofilm susceptibility to lysostaphin, two concentration ranges of the enzyme were used, chosen on the basis of different MICs values established for *S. aureus* and *S. epidermidis* strains growing in suspension. For generally more susceptible planktonic cultures of most *S. aureus* ($MICs_{LYS}$ 0.063–0.5 μg mL^{-1}), lysostaphin used against biofilms was at the concentrations of 2–64 μg mL^{-1}. For more resistant planktonic *S. epidermidis* ($MICs_{LYS}$ 0.5–16 μg mL^{-1}), lysostaphin concentration range used for biofilm eradication was 8–256 μg mL^{-1}. *S. aureus* biofilms demonstrated various susceptibility to the lysostaphin; BIC_{LYS} range for 8/13 strains was from 4 to 32 μg mL^{-1}, set when the absorbance dropped near the baseline established for negative control well (OD_{550} = 0.05–0.09). On the other hand, for 5/13 *S. aureus* strains BIC_{LYS} exceeded the maximal concentration used – >64 μg mL^{-1}. *S. epidermidis* biofilms were significantly more resistant to lysostaphin, BIC_{LYS} determined for 10/12 strains was >256 μg mL^{-1}, for one strain (RP12) it was 128 μg mL^{-1} (OD_{550} dropped from 2.237 to 0.092) and for the other one (A4c) – 16 μg mL^{-1} (OD_{550} dropped from 2.762 to 0.096) (Fig. 1).

Further experiments concerned the possible synergistic effect of the antibiotic and subinhibitory concentration of enzyme, towards biofilm cultures. For this purpose, 3 *S. aureus* and 3 *S. epidermidis* strains,

Table III
The influence of oxacillin or/and lysostaphin on biofilm viability, evaluated by MTT reduction assay in 96-well microplate

Strain	BIC_{OX} (μg mL^{-1})	BIC_{LYS} (μg mL^{-1})	$BIC_{OX(+LYS)}$ (μg mL^{-1})
S. aureus			
ATCC 29213 (MSSA)	>128	8	4 (+4)
A3 (MRSA)	>128	8	32 (+4)
1474/01 (hVISA)	>128	4	>128 (+2)

Strain	BIC_{OX} (μg mL^{-1})	BIC_{LYS} (μg mL^{-1})	$BIC_{OX(+LYS)}$ (μg mL^{-1})
S. epidermidis			
RP12	>128	64	32 (+64)
A4c	>128	16	4 (+8)
6756/99 (MRSE)	>128	>128	>128 (+64)

whose biofilms were characterized as differently susceptible to lysostaphin, and, of course, highly resistant to oxacillin, were included in these studies. The applied "biofilm MTT viability" test, revealed non-significant differences between replicate wells, both within and between test dates, therefore single values are presented in Table III. When lysostaphin was used in subBIC (established earlier), together with different oxacillin concentrations, MTT reduction assay revealed that effective inhibition in biofilm growth could be achieved with a much lower antibiotic concentration. This was demonstrated for biofilms of *S. aureus* ATCC29213 (MSSA) and *S. aureus* A3 (MRSA), BIC_{OXA} dropped from >128 to 4.0 and 32.0 μg mL^{-1}, respectively. The synergistic action of oxacillin and lysostaphin was also demonstrated for *S. epidermidis* RP12 and clinical *S. epidermidis* A4c biofilms. Unfortunately, such a good effect was not observed for 1474/01 hVISA and 6756/99 MRSE strains included in this part of the study (Table III, Fig. 2).

The activity of lysostaphin towards biofilm cultures was confirmed by laser scanning confocal microscopy. Microscopic examination of *S. aureus* and *S epidermidis* biofilms, prepared in chamber slides, which were treated with lysostaphin (16 μg mL^{-1}) for 24 h, demonstrated a disruption of *S. aureus* biofilm and loosening of *S. epidermidis* biofilm structure (Fig. 3-A1, B1). Oxacillin alone had no effect on biofilm integrity, even when used at the highest concentration, whereas oxacillin used together with subBIC of lysostaphin caused partial biofilm disruption (data not shown).

The incubation of polyethylene catheter's segments, for 72 h at 37°C, with overnight culture of clinical MRSA strain (*S. aureus* A3) resulted in biofilm formation. Their presence on extra- and intraluminal surfaces of control segments was demonstrated as red stained bacterial deposit, which was a result of the reduction of colourless TTC to the red insoluble formazan crystals by live bacteria (Fig. 4a). When the colonized catheter's segment was immersed in the medium with oxacillin (4 μg mL^{-1}, according to NCCLS borderline concentration describing MRSA) and incubated at 37°C for 24 h, no visible effect of antibiotic action was seen (Fig. 4b). Lysostaphin alone used at a concentration of 8 μg mL^{-1} (BIC_{LYS} established earlier for *S. aureus* A3 strain) caused complete biofilm eradication as soon as after 3 h incubation (Fig. 4c). In order to demonstrate synergistic effect of antibiotic and lysostaphin, catheter's samples colonized by *S. aureus* A3 were incubated at 37°C for 24 h with oxacillin (4 μg mL^{-1}) together with lysostaphin in subBIC concentration (4 μg mL^{-1}). Also in this case total biofilm eradication was achieved (lack of red stained bacterial deposit).

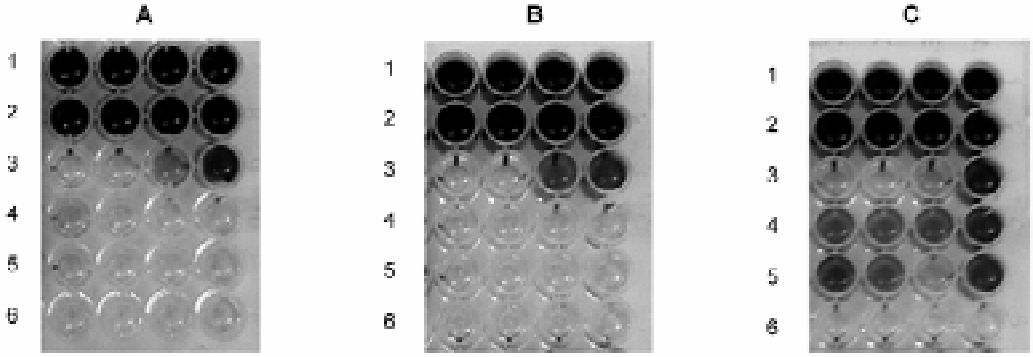

Fig. 2. Effect of lysostaphin (LYS), oxacillin (OXA) or both (LYS+OXA) on (A) *S. aureus* ATCC29213 – MSSA, (B) *S. aureus* A3 – MRSA, (C) *S. aureus* 1474/01 – hVISA biofilms, measured by the MTT reduction assay in the wells of 96-well microplate.

1 (A-C) – biofilm non-treated (positive control); 2 (A-C) – biofilm treated with OXA 128–2 μg mL^{-1}; 3 – biofilm treated with LYS: (A, B) – 32–4 μg mL^{-1}, (C) – 16–2 μg mL^{-1}; 4, 5 – biofilm treated with OXA 128–2 μg mL^{-1} + LYS: (A, B) – 4 μg mL^{-1}, (C) – 2 μg mL^{-1}; 6 – medium + MTT (negative control)

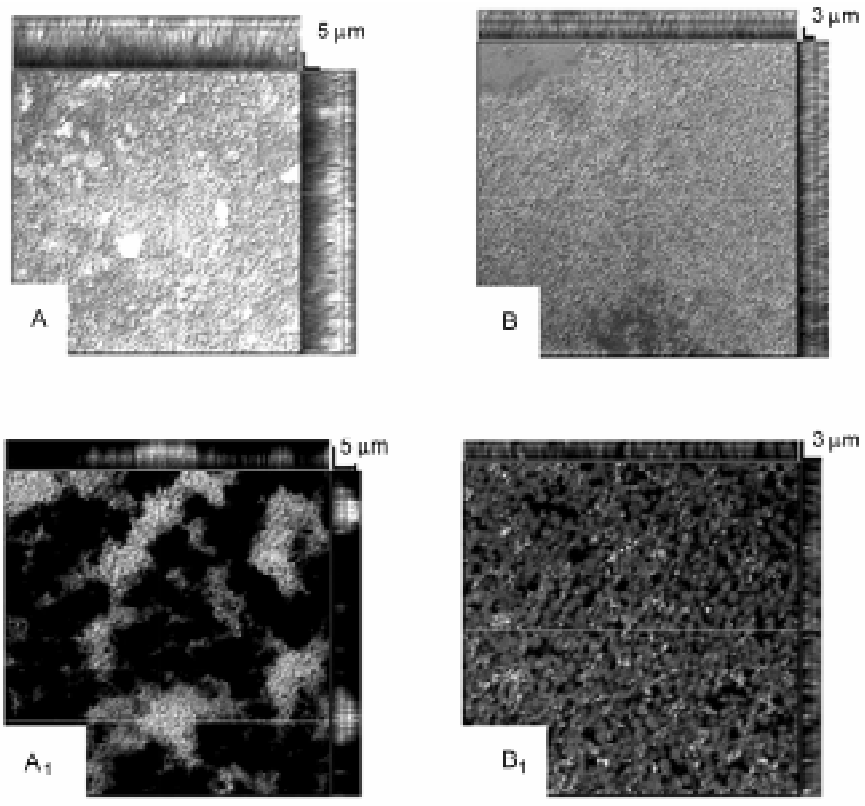

Fig. 3. Laser scanning confocal microscopy (LSCM) images of (A) – *S. aureus* A3 and (B) *S. epidermidis* A4c biofilms, formed in chamber slides and stained with FITC. A_1, B_1 – biofilms of *S. aureus* A3 and *S. epidermidis* A4c, respectively, treated for 24 h with LYS 16 µg mL^{-1}. The square panel are a plain view and the side panels are vertical cross sections, respectively

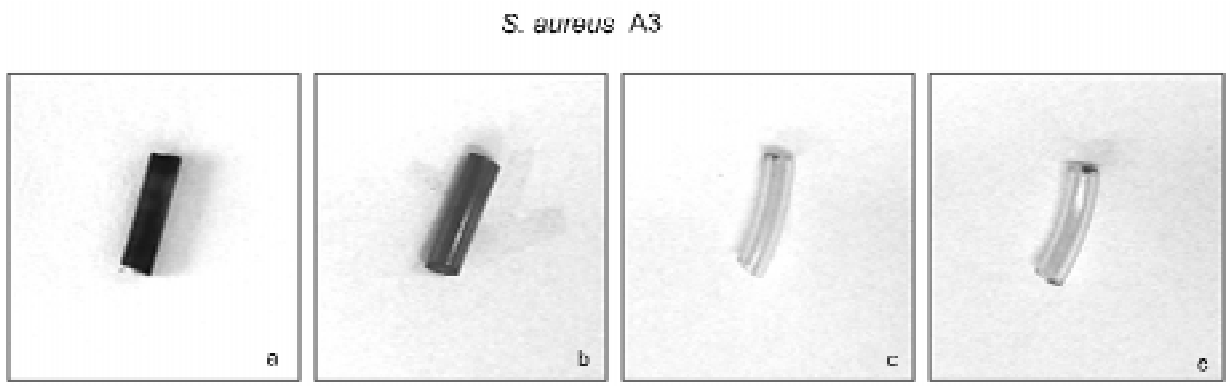

Fig. 4. Eradication activity of oxacillin or lysostaphin alone and in combination, against *S. aureus* A3 biofilm formed on catheter's extra- and intraluminal surfaces, measured by the TTC reduction assay.
a – biofilm non-treated (positive control); b – biofilm treated for 24 h with OXA (4 µg mL^{-1}); c – biofilm treated for 3 h with LYS (8 µg mL^{-1}); d – biofilm treated for 24 h with OXA (4 µg mL^{-1}) + LYS (4 µg mL^{-1})

Discussion

It is now well accepted that bacteria form groups and respond as groups and that individual bacteria in biofilm community rapidly diversify, which increases the capability of the group and provides a form of "biological insurance" (Boles *et al*., 2004). Changeability of the bacteria within biofilm structure causes their extremely high resistance to antimicrobial agents and host immune system (Prakash *et al*., 2003; Fux *et al*., 2004; Hall-Stoodley *et al*., 2004). Biofilm infections are the major medical problems, with *S. aureus*

and coagulase-negative staphylococci, mainly *S. epidermidis*, as the leading species responsible for chronic polymer-associated infections (Götz, 2002; Vuong and Otto, 2002; Costa *et al.*, 2004; Lindsay and Holden, 2004). This was the main reason why in the present paper we wanted to test whether lysostaphin, alone or in combination with oxacillin, may be considered as an effective staphylococcal biofilm eradicating agent, which was indicated for planktonic cells in the reports of many authors (Climo *et al.*, 1998, 2001; Kiri *et al.*, 2002; von Eiff *et al.*, 2003).

Twenty five staphylococcal strains, 13 of *S. aureus* and 12 of *S. epidermidis* were included in this study (Table I and Table II). Within *S. aureus* group, lysostaphin MICs ranged from 0.0625 to 0.500 μg mL^{-1} and for *S. epidermidis* strains the range started from 0.5 and ended at 16 μg mL^{-1}. Such lysostaphin MICs ranges, different for *S. aureus* and *S. epidermidis* strains were also demonstrated by Climo *et al.*, (1998, 2001), Kiri *et al.*, (2002), Wu *et al.*, (2003). It is known that lysostaphin is capable of cleaving the cross-linking pentaglycine bridges in peptidoglycan of cell wall and that differences in the *S. aureus* and *S. epidermidis* susceptibility are mediated by increased incorporation to the muropeptide of serine and alanine, instead of glycine (Climo *et al.*, 1998, 2001; Kiri *et al.*, 2002; von Eiff *et al.*, 2003).

For all 25 staphylococcal strains their biofilm formation ability was evaluated using the MTT assay and laser scanning confocal microscopic (LSCM) observations. Optical density (OD_{550}) readings after MTT staining ranged from 2.1 to 3.1 (Fig. 1), therefore the isolates were categorized as strong biofilm producers, however, as revealed by LSCM, *S. epidermidis* formed more biomass than *S. aureus* (72 h after set-up 8.02 ± 0.61 μm and 4.09 ± 0.78 μm for *S aureus* ATCC29213 and *S. epidermidis* RP12, respectively). In many reports it is stressed that the main factors involved in *S. epidermidis* and *S. aureus* biofilm formation are not the same and they are not known to the same extent (Mack *et al.*, 2004). The same methods (MTT, LSCM) were used for the evaluation of oxacillin or/and lysostaphin action on biofilm viabillity and integrity. A single standard method for the biofilm susceptibility testing is still lacking, so it is very difficult to compare the already published results obtained for biofilms assayed under different conditions. Since we wanted to know how many bacteria survive the incubation of biofilms with antimicrobial agent, our choice was to use MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) measuring active metabolism of cells. The reproducibility of our observations proved that this method was accurate and proper. It was demonstrated that biofilms of both species were highly resistant to oxacillin (BICs > 128–256 μg mL^{-1}), which means that irrespective of different level of susceptibility (low or high MIC_{OXA} of planktonic cells), none of the biofilms was killed by oxacillin used at such a concentration (Fig. 1).

It should be pointed out that the first authors who demonstrated lysostaphin activity against staphylococcal biofilms were Wu *et al.*, (2003). They also proposed the speculation on the possible explanation for the mechanisms of biofilm eradication by lysostaphin, such as rapid lysis of adherent cells, which may be sufficient to destabilize biofilm matrix and allow their detachment. Also, in our study lysostaphin was shown to be effective in biofilm eradication, however with different concentrations used. According to what was described earlier in many reports, the synergistic effect of lysostaphin with oxacillin combination works for planktonic cells (Kiri *et al.*, 2002), and the same effect could be expected against biofilm, which was demonstrated in our study. We have shown that oxacillin at a concentrations of 4.0 and 32.0 μg mL^{-1}, when applied for 24 h with subBIC of lysostaphin, was effective in the killing of biofilm formed by *S. aureus* ATCC29213 and A3 strains, respectively. A similar result (significant decrease in BIC_{OXA} values) was demonstrated for biofilms of two (RP12, A4c) *S. epidermidis* strains (Table III, Fig. 2). However, using lysostaphin in subBIC had also unexpected limitations, since applying it together with oxacillin did not result in the reduction of oxacillin concentration which could be effective in the killing of 1474/01 hVISA and 6756/99 MRSE biofilms. We suggest that unpredictable synergistic effect of lysostaphin low doses, combined with antibotics, could be the main limitation for such a therapeutic strategy.

Similarly to Wu *et al.*, (2003) we have examined biofilm microscopically. In our study laser scanning confocal microscopy of FITC-stained biofilm was used, which allowed us to observe not only differences in *S. aureus* and *S. epidermidis* biofilm thickness and architecture but also antibiotic and lysostaphin influence, more precisely than SEM used by these authors. Our results are in some discrepancy with theirs since lysostaphin used at a concentration of 16 μg mL^{-1} did not clear the biofilm from the surface, however it managed to disrupt it. Wu *et al.*, (2003) demonstrated such an effect using a higher lysostaphin concentration. Nevertheless, we do hope that biofilm structure partially disrupted by the action of lytic enzyme probably could be enough for a more effective antibiotic activity and/or immune mechanisms of the host. The observed synergistic effect of lysostaphin and oxacillin, shown by MTT-reduction assay, was confirmed by the study on "catheter model". It was another method successfully introduced to our study, based on the reduction by live bacteria of tetrazolium salt – TTC to unsoluble red formazan crystals. The presence

of red-stained bacterial biofilm on extra- and intraluminal catheter's surfaces and their disappearing after incubation with antimicrobials were easy to follow (Fig. 4 a-c).

The findings of this *in vitro* study suggest that lysostaphin might be considered for treatment of implant or catheter-associated infections, caused by staphylococci, mainly *S. aureus*. However, it should be stressed that still we are far from being able to use lysostaphin in clinics, although many studies revealed that lysostaphin possesses a potent anti-staphylococcal activity, for example in *in vivo* models of rabbit aortic valve endocarditis and nasal colonization in a cotton rat model or *in vitro* against bacteria isolated from anterior nares and blood (Climo *et al.*, 1998; Patron *et al*., 1999; Kokai-Kun *et al*., 2003; von Eiff *et al*., 2003). The promising results published by Wu *et al*., (2003) and the results of our study on the lysostaphin activity (alone or in combination with antibiotics) against the staphylococcal biofilms, should be considered with some caution due to: the unpredictable lysostaphin susceptibility of a given strain, which has to be established experimentally, the possibility of generation of lysostaphin-resistant strains when too low concentrations are used, or development of anti-lysostaphin antibodies when prolonged and repeated therapy with high lysostaphin doses is introduced (Boyle-Vavra *et al*., 2001; Climo *et al*., 2001; Dajcs *et al*., 2002; Kiri *et al.*, 2002). Nevertheless, enzymatic detachment of medical biofilms seems to be a new way to increase or replace the ineffective in many cases antibiotic therapy (Barequet *et al.*, 2004; Kaplan *et al*., 2004).

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