Lysostaphin eradicates established *Staphylococcus aureus* biofilms in jugular vein catheterized mice

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**Objectives:** *Staphylococcus aureus* infections associated with indwelling devices can be very difficult to treat due to the recalcitrant nature of bacterial biofilms to conventional antibiotics. Lysostaphin has been shown to clear *S. aureus* biofilms in vitro, and in this study we determined whether lysostaphin could also eradicate established *S. aureus* biofilms on implanted jugular vein catheters in mice.

**Methods:** Jugular vein catheterized mice (four to six per group) challenged with *S. aureus* developed multiorgan infection and biofilm infections on the catheters. The infected mice with established biofilms received various doses of recombinant lysostaphin through the catheters, administered up to three times daily for up to 4 days. Some mice also received lysostaphin combined with nafcillin. Following treatment, mice were sacrificed and cfu on the catheter and in the liver and heart were determined. In another set of experiments, implanted jugular vein catheters in mice were pre-instilled with lysostaphin to determine whether this pre-treatment would protect the mice from biofilm infection.

**Results and conclusions:** Lysostaphin administered at 15 mg/kg in combination with 50 mg/kg nafcillin three times per day for 4 days eradicated established *S. aureus*, including methicillin-resistant *S. aureus*, biofilms from implanted catheters and sterilized heart and liver infections of *S. aureus*-infected mice. Furthermore, a single pre-instillation of 10 mg/kg lysostaphin in catheters completely protected catheterized mice from a subsequent biofilm infection. These results demonstrate that lysostaphin is an effective treatment as well as prophylaxis for *S. aureus* biofilms on indwelling catheters.

Keywords: MRSA, nafcillin, heart, liver, dosing regimen, pre-instillation

**Introduction**

Infections caused by *Staphylococcus aureus* continue to be a problem not only in healthcare environments but, with the emergence of community-associated methicillin-resistant *S. aureus* (CA-MRSA), as a public health problem as well.† The decreasing susceptibility of *S. aureus* to commonly prescribed antibiotics like vancomycin has been well documented, and some of the newer antibiotics like daptomycin are also reporting clinical failures.‡,§ A disturbing trend in CA-MRSA is the frequent progression from uncomplicated and treatable skin and soft tissue infections to disseminated multifocal infections often striking infants and young children.¶ These disseminated infections can be difficult to treat and may require prolonged courses of antibiotics and surgical intervention. One of the many factors that may contribute to the recalcitrant nature of *S. aureus* infections is the capacity of *S. aureus* to form biofilms on indwelling devices and damaged tissue.¶

Biofilms are an organized community of sessile cells that are less easily treated by antibiotics than their planktonic counterparts.¶,§ Much research has focused on understanding the nature of biofilms and their reduced antibiotic susceptibility.¶ *Staphylococcal* biofilm infections can be particularly difficult to treat, generally requiring removal of infected devices like catheters if possible.¶,§ Courses of antibiotics can be administered with apparent success as they eliminate most of the bacteria, only to have a recurrence of infection within a few days. This likely results from residual persistor staphylococci in the biofilm outgrowing, repopulating the biofilm and reseeding the infection.¶ A treatment that would rapidly eliminate free-living staphylococci as well as staphylococci in biofilms would be of great benefit.

Recently, we demonstrated that the staphylocidal enzyme lysostaphin can clear established *S. aureus* and *Staphylococcus epidermidis* biofilms from artificial surfaces *in vitro*.¶ Lysostaphin is a 27 kDa glycol-glycine endopeptidase that

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cleaves the pentaglycine cross-bridges in the staphylococcal cell wall rapidly lysing the bacteria. This potent antistaphylococcal not only killed the staphylococci in the biofilm, but also appeared to strip the extracellular glycocalyx from the artificial surface. In this study, we sought to determine whether lysostaphin could clear *S. aureus* biofilm infections *in vivo*, in a mouse jugular vein catheter infection model that includes biofilms and organ infections. We also determined whether pre-instillation of lysostaphin in catheters could protect the mice from subsequent biofilm infection.

Materials and methods

**Materials**

Recombinant mature lysostaphin was produced by fermentation in *Escherichia coli* and purified to homogeneity by Avicea (Stanstead, UK) under contract to Biosynexus Incorporated (Gaithersburg, MD, USA). Purified lysostaphin was formulated in pH 6.5 PBS buffer for storage at −70°C until used. Nafcillin was purchased from Sigma (St Louis, MO, USA).

**Jugular vein catheterized mouse model**

All animal procedures were conducted in accordance with the guidelines of the Biosynexus Institutional Animal Care and Use Committee. Six-week-old female, jugular vein catheterized CF-1 mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in individual cages upon receipt. Overnight cultures of *S. aureus* strain SAS-Lab [a capsular type 5 clinical, methicillin-susceptible *S. aureus* (MSSA) isolate from the Biosynexus strain library, lysostaphin MIC = 0.004 mg/L] or MRSA strain MBT 5040 (lysostaphin MIC = 0.004 mg/L) were washed and then diluted to a final challenge dose of $5 \times 10^5$ cfu/mouse in PBS (Cambrex, Walkersville, MD, USA). Jugal vein catheterized mice (typically 4–6 per group and 12 mice per experiment due to animal costs and housing requirements) were challenged by intravenous tail vein injection with 200 μL of *S. aureus* in PBS. Biofilm infections of the catheters were then allowed to become established for 5 days prior to commencing treatment.

**Treatment of established biofilms**

Treatment of mice with PBS (control group) or various doses of lysostaphin from 10 to 40 mg/kg or lysostaphin in combination with nafcillin (50 mg/kg) in PBS were administered through the jugular vein catheter by a slow push (over ~3 min) in a total volume of 200 μL per treatment using a blunt syringe needle. The entire catheter had a void volume of ~20 μL. Mice received treatments two (8 h apart) or three (4 h apart) times per day for 3 or 4 days. Twenty-four hours after the final treatment, the mice were sacrificed and the distal 1.5 cm portion of the catheter beyond the second cannula (void volume <5 μL) as well as the heart and liver were recovered from each mouse. The recovered catheter portion was placed in 1 mL of PBS and sonicated for 1 min on ice to dislodge adherent bacteria. Additional sonication failed to remove additional bacteria. The heart and liver were also placed in 1 mL of PBS and mechanically disrupted as previously described. Supernatant from each recovered catheter or organ was serially diluted in PBS and then a 100 μL aliquot of each dilution was plated on blood agar to quantify infecting bacteria. The limit of detection for this procedure was 10 cfu/organ or catheter, or one colony recovered per plate from 100 μL of the undiluted supernatant.

**Pre-instillation of lysostaphin**

In another set of experiments, the catheters of jugular vein catheterized mice were pre-treated with a single 200 μL instillation of lysostaphin (5–40 mg/kg) in PBS 24 h prior to the *S. aureus* challenge. Control animals received PBS alone. On the day of the challenge, 2 h prior to the challenge, all catheters were flushed with 200 μL of PBS to remove excess unbound lysostaphin, and then the mice were challenged with $\sim 5 \times 10^4$ *S. aureus* via the tail vein as described above. The challenged animals were sacrificed 5 days after the bacterial challenge and the catheters and organs recovered as described above.

**Determination of potential lysostaphin resistance**

Lysostaphin-resistant colonies are often phenotypically smaller than their lysostaphin-susceptible counterparts, so all *S. aureus* colonies recovered from lysostaphin-treated mice were first observed for phenotypic differences. To ensure that lysostaphin-resistant variants were not overlooked if no phenotypically different colonies were observed, the potential for lysostaphin resistance was also assessed by plating a subset of isolated *S. aureus* colonies (~10%) recovered from lysostaphin-treated mice on tryptic soy agar (BD, Sparks, MD, USA) containing 10 μg/mL lysostaphin as previously described. *S. aureus* colonies recovered from control animals were included as a positive control for lysostaphin susceptibility. Strong growth on 10 μg/mL lysostaphin-containing agar was considered to be lysostaphin resistance. Any questionable growth of *S. aureus* on lysostaphin-containing agar (i.e. weak growth) was followed-up with a lysostaphin disc diffusion assay as previously described.

**Scanning electron microscopy (SEM)**

SEM was performed under contract by Dr Mark Shirtliff, Center for Biofilm Engineering, Montana State University (current address University of Maryland Dental School) as previously described.

**Results**

Untreated jugular vein catheterized mice develop reproducible catheter, heart and liver infections following *S. aureus* challenge

A challenge dose of $5 \times 10^3$ was required to reproducibly infect jugular vein catheterized mice, which is a lower dose than is required for uncatheterized CF-1 mice that require $\geq 10^7$ *S. aureus* to reproducibly infect the mice. When control jugular vein catheterized mice (four per group) were challenged with $5 \times 10^5$ *S. aureus* and then sacrificed 7 days after the challenge, reproducible infections of the catheters, hearts and livers were found. As shown in Figures 1 and 2, all control mice challenged with MRSA strain MBT 5040 had infected catheters and all but two of these control mice also had infections of the heart and liver. In this animal model, kidney infections were found to be sporadic with low cfu (<60) recovery if any. Similar results were also seen when mice were challenged with MSSA strain SA5-Lab [Figure S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. When the catheters
recovered from several infected mice were observed by SEM, adherent cocci consistent with \textit{S. aureus} growing in biofilms were observed both in the catheter lumen as well as on the external surface of all the recovered catheters (Figure 3a).

\textit{Lysostaphin eradicates established biofilm infections on catheters}

In our previous work, doses of lysostaphin as low as 5 mg/kg administered once per day for 3 days were sufficient to clear both MSSA and MRSA infections in a mouse systemic infection model.\textsuperscript{15} In the current catheter model, however, we found that a dose of 10 mg/kg lysostaphin administered three times per day (4 h between doses) for 4 days significantly reduced the mean recovered MRSA from the catheters, hearts and livers of infected mice, and actually cleared infections in some of those organs (Figure 1). However, this dose did not eradicate the infection in all of the treated mice. We and others have previously reported a synergistic effect between lysostaphin and \&-lactam antibiotics against staphylococci, even MRSA.\textsuperscript{15,17,18} Addition of 50 mg/kg nafcillin to each of the 10 mg/kg lysostaphin treatments significantly improved MRSA clearance of the catheters and also improved clearance of the hearts and livers (Figure 1). This additional treatment still failed to eradicate the \textit{S. aureus} infections in all of the treated mice, however.

When the dose of lysostaphin was increased to 40 mg/kg delivered three times per day for 4 days, this regimen eradicated MRSA infection from the catheters, hearts and livers of all treated mice (Figure 2), leaving only small numbers of residual cfu recovered in the heart and liver of one mouse. When the lysostaphin treatment dose was reduced to 20 mg/kg three times per day for 4 days, a similar result was also seen (Figure 2). Further reducing the lysostaphin dose to 15 mg/kg three times per day for 4 days, however, left most of the mice with infected catheters, hearts and livers (Figure 2). Combining 50 mg/kg nafcillin treatment with the 15 mg/kg lysostaphin treatments resulted in eradication of infection from the catheters and hearts of infected mice and nearly complete clearance from the livers (Figure 2). As expected, nafcillin treatment by itself had no effect on infection in these mice challenged with MRSA with cfu recovered from the nafcillin-treated animals similar to those seen in control mice. When catheters from lysostaphin-treated mice were examined by SEM, no adherent embedded cocci were seen on either surface of the catheter, a result consistent with the quantitative results of such treatments (Figure 3b).

Similar results were also obtained when catheterized mice were challenged with an MSSA strain and then treated with

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\textbf{Figure 1.} cfu recovered from catheters and organs of MRSA-challenged mice following 10 mg/kg lysostaphin treatments. Infected mice were treated with PBS alone (Control), lysostaphin in PBS (Lyso, 10 mg/kg) or lysostaphin plus nafcillin in PBS (Lyso + Nafcillin, 10 and 50 mg/kg, respectively) three times per day for 4 days. The figure combines the results of five separate experiments. Symbols indicate cfu recovered from individual catheters or organs, while horizontal lines indicate geometric means for each group. Symbols on the x-axis indicate that no S. aureus were recovered from that catheter or organ. The asterisks indicate significance (\(P \leq 0.05\)): *the result was significantly different from the control group; **the result was significantly different from the lysostaphin-alone group.

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**Figure 2.** cfu recovered from catheters and organs of MRSA-challenged mice following increased-dose lysostaphin treatments. Infected mice were treated with PBS alone (Control), lysostaphin in PBS (40, 20 or 15 mg/kg) or 15 mg/kg lysostaphin plus 50 mg/kg nafcillin in PBS (15 mg/kg + Nafcillin) three times per day for 4 days. The figure combines the results of at least two separate experiments. Symbols indicate cfu recovered from individual catheters or organs, while horizontal lines indicate geometric means for each group. Symbols on the x-axis indicate that no *S. aureus* were recovered from that catheter or organ. The asterisks indicate significance (*P ≤ 0.05*: * the result was significantly different from the control group; ** the result was significantly different from the 15 mg/kg lysostaphin-alone group.

Lysozystaphin or nafcillin. A dose of 15 mg/kg lysostaphin delivered three times per day for 4 days was similar in effectiveness for the treatment of MSSA infection and for the treatment of MRSA infection in this model (Figure S1). Nafcillin alone (50 mg/kg) delivered three times per day for 4 days was also fairly effective against MSSA infection in this model, clearing 37.5% of the catheters and all of the hearts and half of the livers of the treated mice (Figure S1).

**Strategies to reduce the overall lysostaphin treatment regimen prove unsuccessful**

In an effort to reduce the total dosage of lysostaphin necessary to eradicate infection in these catheterized mice, a number of different strategies were pursued. The first strategy was to treat the mice with 15 mg/kg lysostaphin plus 50 mg/kg nafcillin three times per day for only 3 days or to treat the mice with 15 mg/kg lysostaphin plus 50 mg/kg nafcillin twice a day for 4 days, but while both of these treatment regimens reduced the infections, they failed to eradicate MRSA infection in all mice [Figure S2, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. In another strategy, MRSA-infected catheterized mice were dosed with a single large bolus of 40 mg/kg lysostaphin plus 50 mg/kg nafcillin followed by two more doses of 5 mg/kg lysostaphin plus 50 mg/kg nafcillin on day 1 and then three additional days of 5 mg/kg lysostaphin plus 50 mg/kg nafcillin three times per day. Again, this treatment course failed to eradicate MRSA infection in all mice (Figure S2). The final strategy pursued was to dose MRSA-infected mice with 15 mg/kg lysostaphin plus 50 mg/kg nafcillin three times on the first day of treatment and then follow this with three times per day dosing of 5 mg/kg lysostaphin plus 50 mg/kg nafcillin for the next 3 days. This regimen only eradicated the infection in one of the mice (Figure S2).

**Pre-instillation of lysostaphin into catheters protects mice from MRSA infection**

We have previously demonstrated that lysostaphin can coat plastic catheters in vitro and prevent adherence of the *S. aureus* to the catheters. In order to determine whether lysostaphin could similarly protect catheters in vivo from *S. aureus* challenge, jugular vein catheterized mice were pre-instilled with a single dose of lysostaphin 24 h prior to bacterial challenge. The catheters were then rinsed, and 2 h later the mice were challenged with MRSA. The catheters and organs were recovered.
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(no bacteria were recovered from the organs of pre-treated mice) from MRSA infection. This result does not reflect the activity of systemic lysostaphin inasmuch as a comparable dose of lysostaphin given intravenously through a peripheral vein 24 h prior to S. aureus challenge was not protective, resulting in a similar recovery of S. aureus to control animals. A single pre-treatment instillation of 5 mg/kg lysostaphin, however, only protected 66% of the mice from catheter infection (Figure 4). The catheter-infected, 5 mg/kg lysostaphin pre-treated mice also had low-level MRSA infection of the hearts and livers.

Lysostaphin-resistant S. aureus were not recovered from lysostaphin-treated mice

None of the S. aureus (MRSA or MSSA) isolated from lysostaphin-treated mice and tested for lysostaphin resistance was found to be resistant to lysostaphin by growth on lysostaphin agar or by lysostaphin disc diffusion assay.

Discussion

It is standard practice in many institutions to remove catheters and begin antibiotic treatment as soon as a staphylococcal infection is suspected.9 While this intervention may be possible for many catheters, this is much more problematic for other indwelling devices like artificial heart valves or prosthetic joints. Physicians often treat infections of these devices with long-term antibiotics in an attempt to eradicate infection and thereby avoid additional surgery.9 These prolonged antibiotic treatments expose the patients to the risks of longer hospital stays and the possibility of development of antibiotic resistance.9 An agent such as lysostaphin that can eradicate established biofilm infections from indwelling devices in as little as 4 days of intensive treatment would be extremely beneficial for preserving indwelling devices, avoiding costly hospitalizations and preventing additional surgeries.

We have previously demonstrated that lysostaphin is very effective for the treatment of systemic S. aureus infection in a mouse model.15 In keeping with the recalcitrant nature of biofilms to antibiotic treatment, however, we have now shown that greatly increased doses of lysostaphin are required to clear established S. aureus biofilm infections from an indwelling device.6 In this study, the minimum effective dose of lysostaphin alone to clear established biofilm infections was 20 mg/kg administered three times per day for 4 days. The dose of lysostaphin effective for clearance of MRSA could be reduced to 15 mg/kg three times per day for 4 days with the addition of 50 mg/kg nafcillin to each lysostaphin treatment. This supports previously published data demonstrating the synergistic effect of lysostaphin with β-lactam antibiotics.15,17,18 Addition of nafcillin to the lysostaphin treatments would also have the added benefit of preventing emergence of lysostaphin resistance, as lysostaphin resistance and β-lactam antibiotic resistance have been demonstrated to be mutually exclusive.17,18,20 Further, no lysostaphin-resistant S. aureus were found from animals treated with lysostaphin alone in this study, perhaps due to the high doses of lysostaphin required for the treatment of these animals, or the unfit nature of lysostaphin-resistant S. aureus variants.14

Assuming that one could extrapolate from this animal model, a dose of 15 mg/kg lysostaphin three times per day in a 70 kg
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adult would translate into administration of ≥12 g of lysostaphin over 4 days. Efforts to reduce the overall dosage of lysostaphin required to eradicate catheter infections in this model were examined, but none was able to eradicate infection in all animals. When compared with standard vancomycin treatment of 15 mg/kg every 12 h for 6 weeks to treat complicated endocarditis without the assurance of success, a 4 day treatment with lysostaphin has distinct advantages by greatly shortening the overall treatment duration and possibly preventing the removal of infected indwelling devices. With the recent advances in the production of recombinant lysostaphin in large amounts, this approach is feasible. Lysostaphin does not have a direct enzymatic effect on eukaryotic cells and has little toxicity as well. The main concern with lysostaphin toxicity would likely involve either possible allergic reactions or the potential for the formation of lysostaphin antigen/antibody complexes if an immune response was mounted to lysostaphin. This would not be expected to be a concern with only 4 days of treatment, but could become a concern if additional courses of treatment were required in the future.

One of the shortcomings of the present mouse model is that the indwelling catheters in the mice can become blocked or be pulled out by the mice. Two weeks is the longest period of time that catheters remain functional. This prevented comparison of 4 days of lysostaphin treatment of catheter infections with long-term treatment using conventional antibiotics such as vancomycin. In some experiments with MSSA, however, 50 mg/kg nafcillin treatment alone three times per day for 4 days appeared to be almost as effective as 15 mg/kg lysostaphin alone for clearance of catheter infections.

The *S. aureus* biofilms in this model were found both in the lumen and on the external surface of the catheters. The lysostaphin treatments were delivered through the catheter and thus immediately came into contact with the bacteria in the lumen, but these treatments also cleared the biofilms on the external surface of the catheters as well as the infections of the hearts and liver, thus clearance of the catheter infections cannot simply be explained by contact between the lysostaphin and the *S. aureus* biofilms in the lumen of the catheter. Circulating lysostaphin appears to have the capacity to diffuse into the tissue and eliminate *S. aureus*, and in the present study, lysostaphin treatment was able to clear all *S. aureus* both in the lumen as well as on the external surface of the catheters. Future tissue distribution studies with lysostaphin will further examine this activity.

As an alternative to the treatment of established *S. aureus* biofilm infections of catheters and other indwelling devices, pre-instillation or pre-treatment with lysostaphin may serve to protect indwelling devices from the establishment of staphylococcal biofilms. It has previously been demonstrated that when catheters are incubated with lysostaphin *in vitro*, the lysostaphin retains its staphylococcal activity. To determine whether lysostaphin would protect catheters *in vivo*, we pre-treated the catheters of the implanted mice with lysostaphin by administering a single instillation of lysostaphin at various concentrations through the catheters and allowing this to remain in the catheters overnight. The catheters were then rinsed with PBS 2 h prior to the bacterial challenge, which is sufficient time to remove unbound lysostaphin from the catheter and the system since lysostaphin has a short systemic half-life. These catheters, as well as the other organs, in animals pre-treated with as little at 10 mg/kg lysostaphin were protected from the development of subsequent *S. aureus* biofilm infection. This approach provides a possible alternative use for lysostaphin as a prophylaxis against the establishment of staphylococcal biofilms as well as a treatment for established biofilms of indwelling devices.

In this manuscript, we have demonstrated that lysostaphin may be useful for both treating established *S. aureus* biofilm infections of indwelling devices as well as for protecting such devices from the establishment of a biofilm infection. Even though the lysostaphin treatment regimen suggested by these animal studies might require large amounts of lysostaphin in humans, there are clinical situations where such treatment of staphylococcal infections in as few as 4 days without resorting to surgery would be highly desirable. This is especially true when treatments with conventional antibiotics may be required for many weeks with only sporadic success. Preventing infections of indwelling devices may be even more effective than treating existing infections as it may require far less lysostaphin and a single administration. Prevention may not be feasible in all instances, however, so having the potential for both prevention and treatment of biofilm infections expands the utility of lysostaphin.

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Transparency declarations

All authors are, or formerly were, employees and stock holders of Biosynexus Incorporated, and Biosynexus is in the process of developing lysostaphin for commercial use.

Supplementary data

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

References