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In Vitro Activity of Recombinant Lysostaphin against *Staphylococcus aureus* Isolates from Anterior Nares and Blood

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The in vitro activity of recombinant lysostaphin was tested against a collection of well-characterized clinical *Staphylococcus aureus* isolates by disk diffusion (429 isolates) and minimum bactericidal concentration (10 isolates) assays. Minimum bactericidal concentrations of 0.16 μg/ml and zones of inhibition ranging between 15 and 21 mm in diameter demonstrate that lysostaphin was highly active against all isolates tested.

Oxacillin-resistant *Staphylococcus aureus* (ORSA) strains have become a widespread problem in many countries (7), which has become more complicated by the presence of vancomycin-intermediate *S. aureus* (VISA) (15) and, more recently, vancomycin-resistant (2, 3) strains. This latest development raises fears of increased therapeutic failures. Therefore, prevention of infections due to *S. aureus* and new treatments for *S. aureus* infections are of utmost importance not only for the safety of patients but also in terms of treating an infection by cost-effective means.

Nasal carriage has been identified as a risk factor for the pathogenesis of *S. aureus* infections and as a source of *S. aureus* bacteremia (10, 14, 17, 18). For patients with *S. aureus* bacteremia, a strong correlation between strains colonizing the anterior nares and strains isolated from blood was described, supporting strategies to prevent systemic *S. aureus* infections by eliminating nasal carriage (17). Indeed, suppression or eradication of *S. aureus* in a patient's nose has been associated with reduced rates of endogenous *S. aureus* infection in multiple studies (1, 8, 13, 16).

Lysostaphin, an endopeptidase produced by *Staphylococcus simulans* biovar Staphylolyticus cleaves the polyglycine interpeptide bridges of staphylococcal cell walls (4). With the recent formulation of lysostaphin in a nasal cream consisting of fatty acid esters and lysostaphin mixed in as an aqueous suspension (5%), a new product might be available for the nasal eradication of *S. aureus* (9).

Here, we tested the in vitro activity of recombinant lysostaphin against a well-characterized *S. aureus* strain collection using (i) a disk diffusion method and (ii) a minimum bactericidal concentration (MBC) assay with selected strains.

All together, 429 well-characterized *S. aureus* isolates were tested. All isolates were collected in Germany during the course of a multicenter study including 32 university and community hospitals (17); only one isolate per patient was included. Two hundred ten *S. aureus* isolates were obtained from nasal swabs during routine surveillance; the other 219 isolates were collected from the blood of patients with *S. aureus* bacteremia. The strains were collected in a setting with a low level of ORSA (between 3.5 and 9.1% of isolated *S. aureus* strains during the study period), with only 23 methicillin-resistant strains being part of the strain collection. These 23 isolates were confirmed to be methicillin resistant by using disks with 5 μg of oxacillin. Confirmation was accomplished by supplementation of the Mueller-Hinton agar with 2% NaCl; zones of inhibition were read after incubation at 30°C for 48 h. Isolates were considered resistant if they had a zone of inhibition of ≤15 mm according to Deutsches Institut für Normung document 58940-3 (6) and by detection of the *mecA* gene as previously reported (11). Results for the reference strains ATCC 33592 and ATCC 29213 were within acceptable limits throughout testing.

To determine the activity of recombinant lysostaphin versus the *S. aureus* strain collection, the following disk diffusion method was used. Six-millimeter-diameter disks were cut out of filter paper and then autoclaved. Sterile disks were impregnated with 50 μg of mature recombinant lysostaphin in 7 μl of phosphate-buffered saline (PBS) (a 7.1-μg/μl stock solution of lysostaphin). Disks were dried at room temperature for 30 min and stored at 20°C in a sealed container until used. *S. aureus* strains (with an inoculum at a McFarland standard of 0.5, corresponding to cell counts ranging between 5×10^7 and 1×10^8) were plated on cation-adjusted Mueller-Hinton agar supplemented with 2% NaCl, and 50-μg lysostaphin disks were added.

To quantitate lysostaphin in solution, 8 mg of powder (95% lysostaphin by weight) was dissolved in 1 ml of PBS. The concentration was determined by measuring the optical density at 280 nm of two dilutions: 1:40 and 1:80. The resulting optical density was multiplied by the dilution factor and by 0.49, which is the reciprocal of the extinction coefficient. The concentration given (in milligrams per milliliter) was adjusted to the desired final concentration for the production of disks.

The MBC assay was performed with selected strains isolated from blood ($n = 5$) and anterior nares ($n = 5$) exhibiting various zones of inhibition by the disk diffusion method and was carried out as follows. Diluted *S. aureus* cultures (grown overnight in Trypticase soy broth) were added to twofold serial dilutions of lysostaphin (ranging from 10 to 0.16 μg/ml) in PBS—with added bovine serum albumin (0.1%) to prevent

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TABLE 1. Activity of lysostaphin against selected *S. aureus* strains of different origins

Strain	Origin	ORSA	Disk diffusion method inhibition zone (mm)	MBC assay		
				Starting titer	Final titer (at MBC)	MBC (μg/ml)
1	Blood	No	15	4.69×10^6	7.70×10^2	0.16
2		No	16	7.72×10^5	1.85×10^2	0.16
3		Yes	17	7.32×10^5	0.05×10^2	0.16
4		No	19	1.63×10^6	2.95×10^2	0.16
5		Yes	21	2.58×10^6	0.80×10^2	0.16
6	Anterior nares	No	15	1.22×10^6	5.70×10^2	0.16
7		No	17	1.50×10^6	1.95×10^2	0.16
8		No	18	1.59×10^6	0.65×10^2	0.16
9		Yes	19	7.07×10^5	2.20×10^2	0.16
10		No	20	1.06×10^6	1.35×10^2	0.16

nonspecific sticking of lysostaphin to plastic (5)—to a final concentration of approximately 10^6 CFU/ml (maximum range, from 5×10^5 to 5×10^6). A dilution series of untreated *S. aureus* samples was also used to determine the starting titer of bacteria (Table 1). *S. aureus* was incubated with lysostaphin for 30 min at room temperature with vigorous shaking. Subsequently, proteinase K (10 mg/ml) was added to each tube to neutralize the remaining lysostaphin (9) and 100 μl of each tube was plated on Trypticase soy agar. After overnight incubation, colonies were counted and the MBC was determined as the concentration of lysostaphin required to cause a 3-log or greater drop from the initial bacterial titer.

All together, the in vitro activity of lysostaphin was tested against 429 well-characterized *S. aureus* strains collected during a multicenter study. Following incubation for 24 h, disks with 50 μg of lysostaphin had zones of inhibition ranging between 15 and 21 mm in diameter, without there being any significant differences between those of strains isolated from *S. aureus* nasal carriers and those of strains obtained from patients with *S. aureus* bacteremia (Fig. 1). For all strains tested, there was no growth of single colonies at 24 h within the zone of inhibition. Differences between methicillin-susceptible and methicillin-resistant strains were also not observed. Following further incubation (up to 48 h), the sizes of zones of inhibition increased, representing a "second zone" of inhibition (up to 31 mm when cultures were incubated for 48 h) probably due to continued diffusion of lysostaphin through the agar. In this second zone of inhibition, growth was strongly reduced. How-

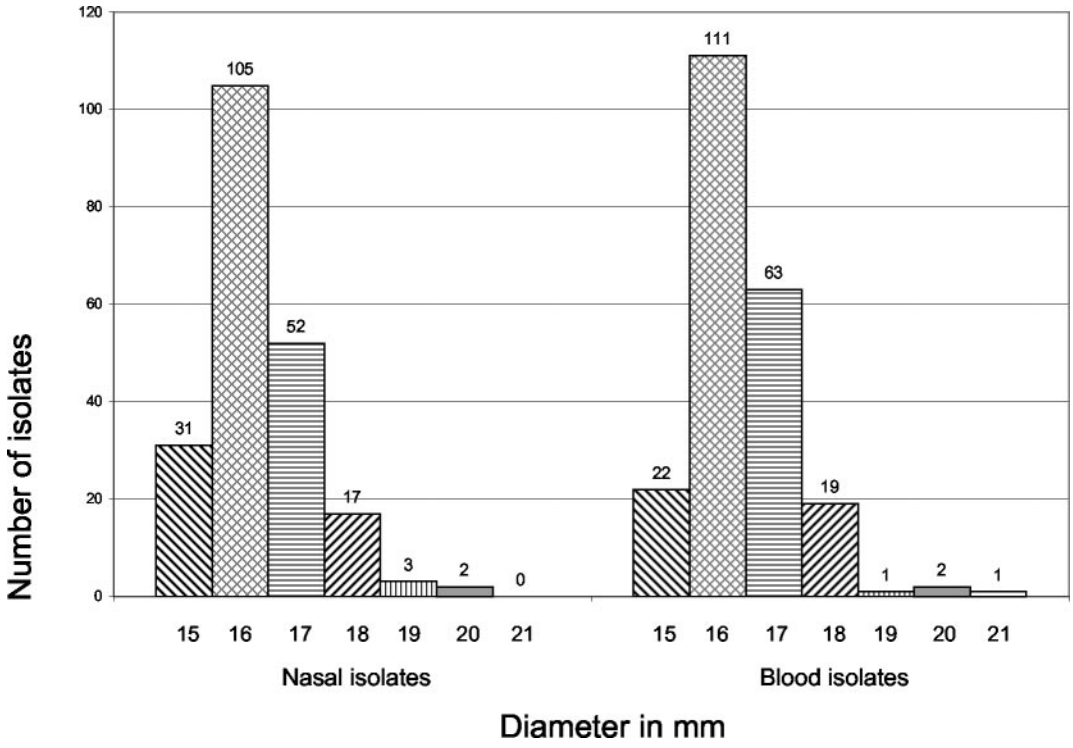

FIG. 1. Distribution of zone diameters around lysostaphin disks following incubation for 24 h. Zones of inhibition are given for isolates recovered from nasal carriers ($n = 210$) and from blood ($n = 219$) of patients with *S. aureus* bacteremia.

ever, following incubation for 48 h, the primary zones of inhibition remained at a diameter of >14 mm, without any colonies being detectable within this zone. Additional testing of selected colonies from the second zone of inhibition showed again zones of inhibition with diameters of >14 mm following incubation for 24 h, indicating that lysostaphin had the same activity against these colonies.

Three in vitro-isolated lysostaphin-resistant strains served as controls and did not reveal any zone of inhibition, while four mupirocin-resistant strains—two with low-level resistance (MICs, 8 and 32 μg/ml) and two with high-level resistance (MICs, >512 μg/ml; kindly provided by W. Witte and G. L. Archer)—showed zones of inhibition similar in size to those of mupirocin-susceptible strains.

Ten selected strains exhibiting zones of inhibition of various sizes (ranging from 15 to 21 mm in diameter) were further tested in an MBC assay. For all strains tested by MBC assay, including three ORSA strains, MBCs were 0.16 μg/ml. Following incubation of *S. aureus* with lysostaphin for only 30 min, we observed a 3-log or a greater drop from the initial bacterial titer (99.9% reduction in the original number of CFU) for all strains, irrespective of the methicillin resistance phenotype of the isolates or of the zones of inhibition observed with the disk diffusion method (Table 1).

Previous studies revealed that lysostaphin has potent antistaphylococcal activity, including against both ORSA and VISA strains (5, 12). In a rabbit model of endocarditis caused either by ORSA or by VISA, treatment with lysostaphin reduced mean aortic valve vegetation counts by >8.0 $\log_{10}$ CFU/g compared to those for untreated controls (5, 12).

Most recently, *S. aureus* colonies isolated from the noses of nasally colonized cotton rats treated with either 0.5% (actual dose, ~150 μg of lysostaphin) or 0.125% lysostaphin formulated in cream were tested by either the MIC assay or the disk diffusion method to determine whether lysostaphin resistance could emerge in the nares of treated cotton rats (9). In these assays, none of the isolates from cotton rat noses treated with lysostaphin formulated in cream was found to be lysostaphin resistant. The MICs of lysostaphin for the isolated colonies matched those for the parental strains, or the diameters of the zones of inhibition in the disk diffusion method were all >12 mm. In that study, *S. aureus* isolates were considered lysostaphin resistant if the diameter of the zone of inhibition was less than 12 mm (9).

Lysostaphin formulated in cream, allowing increased residence time and preserved enzyme activity, may prove to be a superior alternative to other antimicrobial agents for the clearance of *S. aureus* nasal colonization. In our study, lysostaphin revealed high in vitro activity against *S. aureus* strains obtained during the course of a German multicenter study of patients nasally colonized with *S. aureus* as well as of patients with *S. aureus* bacteremia, irrespective of the oxacillin resistance phenotype of the strains. Lysostaphin as formulated in the present study would be highly active at concentrations that can easily be achieved with topical application, indicating that this agent should be effective for topical decolonization of *S. aureus.*

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