Synergism between a Novel Chimeric Lysin and Oxacillin Protects against Infection by Methicillin-Resistant *Staphylococcus aureus*^{†‡¶}

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Staphylococcus aureus is the causative agent of several serious infectious diseases. The emergence of antibiotic-resistant *S. aureus* strains has resulted in significant treatment difficulties, intensifying the need for new antimicrobial agents. Toward this end, we have developed a novel chimeric bacteriophage (phage) lysin that is active against staphylococci, including methicillin-resistant *S. aureus* (MRSA). The chimeric lysin (called ClyS) was obtained by fusing the N-terminal catalytic domain of the *S. aureus* Twort phage lysin with the C-terminal cell wall-targeting domain from another *S. aureus* phage lysin (phiNM3), which displayed *Staphylococcus*-specific binding. ClyS was expressed in *Escherichia coli*, and the purified protein lysed MRSA, vancomycin-intermediate strains of *S. aureus* (VISA), and methicillin-sensitive (MSSA) strains of *S. aureus* *in vitro*. In a mouse nasal decolonization model, a 2-log reduction in the viability of MRSA cells was seen 1 h following a single treatment with ClyS. One intraperitoneal dose of ClyS also protected against death by MRSA in a mouse septicemia model. ClyS showed a typical pattern of synergistic interactions with both vancomycin and oxacillin *in vitro*. More importantly, ClyS and oxacillin at doses that were not protective individually protected synergistically against MRSA septic death in a mouse model. These results strongly support the development of ClyS as an attractive addition to the current treatment options of multidrug-resistant *S. aureus* infections and would allow for the reinstatement of antibiotics shelved because of mounting resistance.

Staphylococcus aureus is an opportunistic pathogen inhabiting human skin and mucous membranes and is the causative agent of a variety of skin and soft-tissue infections as well as serious infections, such as pneumonia, meningitis, endocarditis, and osteomyelitis. *S. aureus* exotoxins also cause disease syndromes such as bullous impetigo, scalded skin syndrome, and toxic shock syndrome. While outbreaks of cutaneous infections in otherwise-healthy people may be managed well without antibiotics (34), in compromised individuals staphylococci are an important cause of life-threatening nosocomial infections, such as ventilator-acquired pneumonia (VAP) (20). The spread of methicillin-resistant *S. aureus* (MRSA) has been of critical concern to health care providers, and the further distribution of Panton-Valentine leukocidin (PVL) carrying community-acquired MRSA strains to the hospital (33) poses a threat that is even more serious than that of MRSA alone due to the striking pathogenicity of this toxin (2).

Currently, 40 to 60% of nosocomial infections of *S. aureus* are resistant to oxacillin (27), and greater than 60% of the isolates are resistant to methicillin (13). Treating infections

caused by drug-resistant *S. aureus* has become increasingly difficult and therefore is a major concern among health care professionals. To combat this challenge, the development of new and effective antibiotics belonging to different classes are being aggressively pursued. A number of new antimicrobial agents, such as linezolid, quinupristin-dalfopristin, daptomycin, telavancin, new glycopeptides, and ceftobiprole, have been introduced or are under clinical development (1). However, clinical isolates of MRSA with resistance to these new classes of antibiotics have been reported already (25, 38, 41). Consequently, there is an urgent need to develop novel therapeutic agents or antibiotic alternatives that are active against MRSA. As an option, current antibiotics to which MRSA strains are resistant may be resurrected as viable candidates in the treatment of MRSA when used in combination with other agents, offering a new dimension to a dwindling list of potential anti-infectives for this pathogen.

The carriage of both methicillin-susceptible *S. aureus* (MSSA) and MRSA in the human anterior nares is the major reservoir for *S. aureus* infection. Studies have shown that roughly 80% of the population could be nasally colonized by *S. aureus*, and that colonization increases the risk factor for developing other more serious *S. aureus* infections (19). The elimination of nasal carriage in the community or in the hospital setting could reduce the risk of infection and slow the spread of drug-resistant *S. aureus* (19). Only one agent, intranasal mupirocin, has been approved in this indication. Mupirocin ointment must be applied twice daily to the anterior nares for 3 to 5 days. When used in this manner, mupirocin ointment has been shown to significantly reduce the risk of postoperative staphylococcal infection in patients who were *S. aureus* carriers (32). However, the value of mupirocin is com-

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promised by a relatively high rate of resistance mutations (6) and by potential compliance issues associated with its method of use. Despite these issues, preoperative prophylaxis with mupirocin already is mandated for cardiovascular surgery (11) and also has been recommended for orthopedic surgery (43). Clearly a superior product for intranasal prophylaxis in at-risk patients would be valuable.

Bacteriophage endolysins (lysins) are a new class of antimicrobial agents that are emerging as effective agents for the prevention and treatment of bacterial infections. Lysins are cell wall hydrolases that are produced during the infection cycle of double-stranded DNA bacteriophages, enabling the release of progeny virions. When applied exogenously, native or recombinant lysins are able to cleave the integral peptidoglycan bonds of susceptible Gram-positive bacteria, resulting in rapid cell lysis (30). Lysins have been developed against a number of Gram-positive pathogens, including *Streptococcus pyogenes* (30), *Streptococcus pneumoniae* (22), *Bacillus anthracis* (36), enterococci (46), group B streptococci (5), and *Staphylococcus aureus* (31, 35). The efficacy of most of these lysins has been demonstrated in *in vivo* models. Several unique characteristics of lysins make them attractive antibacterial candidates against Gram-positive pathogens. These include (i) rapid antibacterial activity both *in vitro* and *in vivo*; (ii) narrow lytic spectrum (species specific); (iii) strong receptor-binding affinity, typically in the nanomolar range; (iv) very low probability of developing resistance, since the binding epitopes on the bacteria are essential for viability; (v) safety; and (vi) relative ease of modification by genetic engineering (12).

The development of a highly active *S. aureus*-specific lysin has been challenging either due to the lack of expression in a heterologous host, the insolubility of the expressed protein, or poor expression, except for the results of one report (to our knowledge) of a *S. aureus*-specific lysin from phage phiMR11 (35). To circumvent these issues, a few studies have reported the construction of truncated (16) or chimeric versions of lysins (26).

Typically, lysins have two distinct functional domains consisting of an N-terminal catalytic domain for peptidoglycan hydrolysis and a C-terminal binding domain for the recognition of surface moieties on the bacterial cell walls. The catalytic domains are relatively conserved among lysins, and the activities can be classified into three basic groups based on peptidoglycan bond specificity: (i) glycosidases that hydrolyze linkages within the amino sugar moieties; (ii) endopeptidases that cleave the peptide moiety; and (iii) amidases that hydrolyze the amide bond connecting the glycan strand and stem peptide. The binding domains, however, are not conserved among lysins. Hence, the binding domain often imparts species specificity, because the binding targets, typically carbohydrates associated with the peptidoglycan, display species-specific distribution (V. A. Fischetti, personal communication). The modular architecture of lysins is an important feature with respect to their development as antimicrobial agents. This enables the creation of chimeric enzymes by swapping lysin domains and thereby altering binding specificity, enzymatic activity, or both (7, 10, 23, 37).

In this paper, we describe the genetic engineering of a novel chimeric lysin constructed by fusing the catalytic domain of a *Staphylococcus*-specific phage lysin with a unique binding domain from a different *Staphylococcus* phage lysin. This engineered lysin, called ClyS (for chimeric lysin for staphylococci),

TABLE 1. Strains used in this study

Organism	Strain name	Source ^a
<i>Staphylococcus aureus</i>	8325-4	1
<i>Staphylococcus aureus</i>	RN4220	1
<i>Staphylococcus aureus</i>	191-SM ^R	1
<i>Staphylococcus aureus</i>	lyrA del2335	2
<i>Staphylococcus aureus</i>	Newman	2
MRSA	COL	4
MRSA	E2125	4
MRSA	HPV107	4
MRSA	HDE288	4
MRSA	BK2529	4
MRSA	HAR24	4
MRSA	MW2	5
VISA	HIP 11714	4
VISA	Mu50	4
VISA	JH9	4
VISA	PC3	4
VISA	MI	4
VISA	NJ	4
<i>Staphylococcus epidermidis</i>	RP62A	3
<i>Staphylococcus epidermidis</i>	HER1292	3
<i>Staphylococcus simulans</i>	TNK3	2
<i>Staphylococcus sciuri</i> subsp. <i>sciuri</i>		3
Group A streptococcus	D471	1
Group C streptococcus	2GRP66	1
Group E streptococcus	K131	1
Group B streptococcus	NCTC 11234	1
<i>Streptococcus gordonii</i>	PK488	1
<i>Streptococcus salivarius</i>	ATCC 9222	1
<i>Streptococcus uberis</i>		1
<i>Bacillus cereus</i>	ATCC 4342	1
<i>Pseudomonas aeruginosa</i>	RS1	1
<i>Escherichia coli</i>	Top10	1

^a 1, The Rockefeller University Collection; 2, Olaf Schneewind, University of Chicago, Chicago, IL; 3, Barry Kreiswirth, Public Health Research Institute, New Jersey, NJ; 4, Alexander Tomasz, The Rockefeller University; 5, ATCC.

was soluble and highly active, and it displayed rapid and specific lytic activity against susceptible and drug-resistant staphylococci. We demonstrate the protective activity of ClyS in *in vivo* colonization and septicemia models as well as its synergistic activity with oxacillin in *in vitro* and *in vivo* models. These results highlight the potential of ClyS as a novel therapeutic agent for the treatment of MRSA and other staphylococcal infections.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains (Table 1) were stored at -80°C and routinely grown at 37°C . Staphylococcal strains used in this study were grown in trypticase soy broth (TSB) medium, streptococcal strains were grown in THY (Todd-Hewitt broth, 1% [wt/vol] yeast extract) medium, *Bacillus cereus* and *Pseudomonas aeruginosa* were grown in BHI (brain heart infusion) medium, and *E. coli* was cultivated in LB (Luria-Bertani) medium. Unless stated otherwise, all media were supplied by Becton, Dickinson, and Company (Sparks, MD).

Construction of ClyS. The chimeric lysin was constructed by amplifying and ligating individual domains from respective genes. For this, the gene fragment encoding the Twort endopeptidase domain was PCR amplified from plasmid pCR2.1plyTW, which contains the entire lysin (*plyTW*) gene, using primers TW-Endo-NcoI-F (5'-CTAGCCATGGAAACCCGTGAAACAAGCAG-3') and TW-Endo-PstI-R (5'-ACATGCTGCAGAACCATATTGTAATTAATATTAGTTCTATC-3'). The gene fragment encoding the cell wall-targeting (CWT) domain was PCR amplified from *S. aureus* strain 8325 genomic DNA containing the phi 13 bacteriophage using primers NM3-CBD-PstI-F (5'-ACATGCTGCAGG GTAAATCTGCAAGTAAATAACAG-3') and NM3-CBD-Hind-R (5'-CCC AAGCTTAAACACTTCTTTTCAATCAATCTC-3'). The two PCR amplicons were ligated using the PstI restriction endonuclease site. The ligated

product was cloned into pBAD24 vector using the *Nco*I-*Hind*III cloning sites to generate recombinant plasmid pAD127. In the second step, the entire DNA fragment corresponding to *clpS* was PCR amplified from pAD127 using primers NM3-Lys-Xba-F (5'-CTAGTCTAGAGGTGGAATAATGAAAACATACAGTGAAGCAAG-3') and primer NM3-CBD-*Hind*-R. The PCR product was cloned into expression vector pJML6 to generate pAD138. The DNA sequence of *clpS* was confirmed through the DNA sequencing of the pAD138 plasmid (Genewiz, Inc., South Plainfield, NJ). The recombinant plasmid pAD138 was transformed into *E. coli* DH5 α cells (Invitrogen Corporation, Carlsbad, CA).

Overexpression and purification of ClyS. ClyS was induced overnight from *E. coli* DH5 α (pAD138) cells with lactose (final concentration, 10 g/500 ml) at 30°C. Cells were harvested by centrifugation, resuspended in buffer A (20 mM phosphate buffer [PB], 1 mM dithiothreitol [DTT]), and lysed at 4°C by an Emulsi Flex-C5 high-pressure homogenizer (Avestin) at 4°C. The lysates were cleared by centrifugation (two rounds of 50,000 $\times$ g) for 30 min at 4°C, and the supernatant was applied to a CM-Sepharose column (Amersham Pharmacia, Piscataway, NJ). ClyS was eluted with buffer A plus 1 M NaCl using a linear gradient of 0 to 50% B in 15 column volumes. Fractions were analyzed for lytic activity as previously described (30). Fractions displaying lytic activity were pooled and dialyzed overnight against buffer B (PB, 1 mM DTT, 50 mM NaCl). The dialyzed sample was applied to a hydroxylapatite (40 μ m; MacroPrep TypeII; Bio-Rad) column and eluted with elution buffer (500 mM PB, 50 mM NaCl, 1 mM DTT) using a linear gradient of 0 to 100% buffer B in 20 column volumes. The fractions were analyzed by SDS-PAGE and for lytic activity. Active and pure fractions of ClyS were pooled and dialyzed against buffer B. The protein concentration was determined with the bicinchoninic acid method (Sigma, St. Louis, MO). The final preparation was <90% pure (see Fig. S3A in the supplemental material).

Quantification of ClyS activity. ClyS activity was measured as previously described (30), with some modifications. Briefly, *S. aureus* strain 8325-4 was grown to an optical density at 600 nm (OD₆₀₀) of 0.25 to 0.3, centrifuged, and resuspended in PB to a final OD₆₀₀ of 0.8 to 1.0. Twofold serial dilutions of purified ClyS (100 μ l) were added to 100 μ l of bacterial suspension in 96-well plates (Costar), and the decrease in OD₆₀₀ was monitored by a Spectramax Plus 384 spectrophotometer (Molecular Devices) for 30 min at 37°C. A unit of ClyS activity per milliliter was defined as the reciprocal of the highest dilution of lysin that decreased the absorbance by 50% in 15 min (30). The specific activity of ClyS (molecular mass, 36 kDa) is 200 U/mg or 7.1 U/nM.

In vitro ClyS activity. The effect of ClyS on bacterial viability was tested as previously described (30). Briefly, log-phase cultures of *S. aureus* strain 8325-4 were resuspended in PB to an OD₆₀₀ of 0.8 to 1.0. Two hundred fifty micrograms of ClyS or the corresponding volume of PB was added to bacterial cells, and aliquots were removed, serially diluted, and plated at 1, 5, 10, 30, and 60 min to assess the viability of the treated and control cells. All experiments were performed in triplicate. The activity of ClyS also was tested on various bacterial strains as described previously (36). Briefly, log-phase bacterial cells were treated with 250 μ g of ClyS at 37°C for 15 min. The samples were serially diluted and plated. Control experiments with the addition of phosphate buffer (pH 7.0) were performed under the same conditions.

Electron microscopy. *S. aureus* strain 8325-4 was grown to log phase, centrifuged, and resuspended in phosphate-buffered saline (PBS) to an absorbance at 600 nm of 1.0. The bacterial suspension was incubated with 250 μ g of ClyS at room temperature. The lytic reaction was terminated after 1 and 5 min by adding glutaraldehyde (final concentration, 2.5%). The suspension was pelleted by centrifugation and overlaid with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The samples then were postfixed in 1% osmium tetroxide, block stained with uranyl acetate, and processed according to standard procedures by The Rockefeller University Electron Microscopy Service.

Fluorescent labeling and binding analyses of phiNM3 CWT. *S. aureus* strain 8325-4 genomic DNA was used to amplify the gene fragment encoding the putative CWT of phi 13 lysin using primers NM3-FWD (5'-CATGCCATGGG TAAATCTGCAAGTAAATAACAG-3') and NM3-REV (5'-CCCAAGCTT AAAACACTTCTTTCACATCAATCTC-3'). The resulting amplicon was cloned into the arabinose-inducible expression vector pBAD24 (ATCC, Manassas, VA). Positive clones containing the insert were confirmed by sequencing. The ~10-kDa phiNM3 CWT protein was expressed in *E. coli* DH5 α , which was lysed as described above, and the protein was purified in one step by cation-exchange chromatography (see Fig. S3B in the supplemental material). The purified protein (1 mg/ml) was incubated for 1 h with 10 μ l of fluorescein isothiocyanate (FITC) (1 mg/ml; Sigma). Excess FITC was removed on a desalting column. The labeled protein (50 μ g) was incubated with bacterial cells for 10 min, washed three times with PB (pH 7.4), and observed under an Eclipse E400 microscope (Nikon) using the QCapture Pro version 5.1 imaging software.

Immunological assays. To test whether ClyS-specific antibodies would neutralize the activity of ClyS, two rabbits were hyperimmunized with purified ClyS using a standard protocol: primary immunization with complete Freund's adjuvant and three monthly boosts in incomplete Freund's adjuvant. Enzyme-linked immunosorbent assay (ELISA) titers were >100,000 for each animal (reciprocal of the highest dilution with an OD₄₀₅ of ≥ 1.0). ClyS was diluted in 2-fold dilutions through 11 wells in a microtiter plate beginning at 800 μ g. Ten microliters of either hyperimmune rabbit serum, preimmune serum, or PBS was added to each well and incubated at 21°C for 15 min. An equal volume of staphylococci (prepared as described above) then was added to each well and immediately assayed as described above. The rate of OD₆₀₀ decrease by ClyS was measured in each well for the hyperimmune serum and compared to those of PBS and preimmune serum for the same dilution of ClyS.

Synergy testing of ClyS with oxacillin or vancomycin. ClyS and oxacillin-vancomycin interactions were assessed by the standard checkerboard broth microdilution assay as described before (10a). Briefly, ClyS and antibiotic were diluted 2-fold horizontally and vertically, respectively, in a final volume of 50 μ l with an inoculum of 3×10^5 to 5×10^5 CFU per well. MRSA strain COL was used to test ClyS-oxacillin interaction, while vancomycin-intermediate *S. aureus* (VISA) strain Mu50 was used to test ClyS-vancomycin interaction. The plates were incubated at 37°C with intermittent shaking in a spectrophotometer. Growth was determined by reading the OD₆₀₀ of the plates for 20 h. The fractional inhibitory concentrations (FICs) of ClyS and antibiotics were determined and plotted in an x/y plot called an isobologram. The FIC index (Σ) was calculated as the MIC of the drug when used in combination with ClyS divided by the MIC of the drug when used alone (10a). In all cases, the Σ FICs were found to be <0.5. Synergy was defined as a Σ FIC of ≤ 0.5 .

In vivo murine models. All *in vivo* protocols were approved by The Rockefeller University's Institutional Animal Care and Use Committee. A modified method from Kiser et al. (18) was used for the intranasal colonization model. Briefly, MRSA strain 191-SM^R was grown overnight at 37°C, with shaking at 250 rpm, in TSB medium plus 200 μ g/ml of streptomycin. The culture then was diluted 1:50 and grown as described above to mid-log phase (OD₆₀₀, 0.5), centrifuged, and resuspended in 0.9% saline for injection (Hospira, Inc., Lake Forest, IL) to a predefined titer ($\sim 5 \times 10^9$ CFU/ml) for mouse inoculation. Actual inoculum titers were derived from plating serial dilutions of each inoculum onto Spectra MRSA agar plates (a selective chromogenic medium developed to diagnostically detect MRSA nasal colonization; Remel, Lenexa, KS) and Columbia blood agar (Becton, Dickinson & Co., Sparks, MD). Six-week-old female C57BL/6J mice (weight range, 22 to 24 g; Charles River Laboratories, Wilmington, MA) were fed water that contained 5 g/liter of streptomycin for 48 h prior to infection. Mice were anesthetized with a mixture of ketamine (1.2 mg/animal; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (0.25 mg/animal; Miles Inc., Shawnee Mission, KS) and then inoculated with two consecutive doses of 15 μ l per nostril of the 191-SM^R bacterial suspensions. Twenty-four hours after treatment, the animals were divided into two groups ($n = 20$) and administered 60 μ l (20 μ l per nostril and 20 μ l orally) of either 20 mM PB or 960 μ g ClyS. Mice were sacrificed 1 h after treatment and subjected to nasal dissection. The excised nasal cavities were aseptically bisected, suspended in 500 μ l of PB, and vortexed for 60 s to suspend adherent bacteria, and dilutions of the bacterial suspensions were plated to Spectra MRSA agar plates and Columbia sheep's blood agar plates for CFU determination. No significant differences in CFU were obtained between plating to Spectra MRSA agar or Columbia blood agar (data not shown). Three independent experiments were performed to evaluate a total of 20 mice for each treatment group. Nasal colonization rates of the treatment groups were analyzed statistically by an analysis of variance (ANOVA) test (<http://www.danielsoper.com/statcalc/>) and Student's *t* test.

For the systemic infection model, 4- to 5-week-old female FVB/NJ mice (weight range, 15 to 20 g) were obtained from The Jackson Laboratory (Bar Harbor, ME). The PVL toxin encoding MRSA strain MW2 (oxacillin MIC = 24 μ g/ml) (ATCC, Manassas, VA) initially was passaged twice through the FVB/NJ mice to select for isolates that might be more capable of causing systematic infection in mice. Briefly, MW2 was grown in TSB medium to mid-log phase, centrifuged, and suspended in saline to a predefined titer of 1×10^9 CFU/ml. A volume of 0.5 ml of the bacterial suspension was injected intraperitoneally, and bacteria were isolated, on Spectra MRSA plates, from the spleens and hearts of the mice upon death 18 to 20 h later. The bacterial isolates then were grown in TSB overnight, and aliquots were frozen in 30% glycerol at -80°C. For intraperitoneal (i.p.) infection, a 10- μ l loop of MRSA from a frozen aliquot of mouse-passaged MW2 was inoculated into 10 ml of TSB medium and grown overnight at 37°C with shaking. This culture then was diluted 1:50 in TSB medium and grown for 2 h at 37°C to mid-log phase (OD₆₀₀, 0.5). The bacteria then were centrifuged, washed once with saline for injection, and resuspended in

saline to a predefined titer of 1×10^8 to 5×10^8 CFU/ml. This suspension was serially diluted with 5% hog gastric mucin (Sigma) in saline to a bacterial titer of 5×10^5 to 1×10^6 CFU/ml, and 0.5 ml of the bacterial suspension was injected intraperitoneally into each mouse. For each experiment, actual bacterial inoculation titers were calculated by serial dilution and plating to Columbia blood agar plates. For the ClyS *in vivo* efficacy experiments, 3 h postinfection the animals were divided into two treatment groups and were intraperitoneally administered 0.5 ml of either 20 mM PB ($n = 14$) or 2 mg/ml ClyS.

For the *in vivo* synergy experiments, 3 h after i.p. infection the animals were divided into three to eight treatment groups and were intraperitoneally administered a combination of 0.5 ml of 0.33-mg/ml ClyS (166 μ g) or 20 mM PB along with a 100- μ l intramuscular (i.m.) injection of 10 to 100 μ g oxacillin in saline or saline-alone control.

The survival rate for each experimental group was monitored every 4 h for the first 24 h and then every 12 to 24 h up to 10 days postinfection. The data were analyzed statistically by Kaplan-Meier survival curves, and a log rank test was performed for 95% confidence intervals using the Prism computer program (GraphPad Software, La Jolla, CA).

RESULTS

Identification of a unique CWT domain in *Staphylococcus* lysins. Previous studies have indicated that the pentaglycine peptide cross-bridge within the staphylococcal peptidoglycan functions as the receptor for the CWT domain of lysostaphin, a staphylolytic enzyme produced by *Staphylococcus simulans* (15). The CWT domain of lysostaphin has homology with the bacterial SH3-like (SH3b) domain, suggesting that lysins with SH3b domains also utilize the peptide cross-bridge as their receptor (24). Since resistance to lysostaphin can be due to small alterations within the peptide cross-bridges (15), we sought to identify a CWT domain within staphylococcal lysins that was unique and not homologous to SH3b domains. Our hypothesis was that such a CWT domain would bind to alternative epitope(s) such as cell wall-associated carbohydrates in the staphylococcal cell wall instead of the peptide cross-bridges, thereby reducing the likelihood of becoming the target of resistance.

Conserved domain searches of *Staphylococcus*-specific phage and prophage lysin sequences in the GenBank database (<http://www.ncbi.nlm.nih.gov/GenBank/index.html>) identified a few lysins that did not display homology to a C-terminal SH3b domain. These included the *S. aureus* phage phiNM3 lysin (protein accession no. YP_908849), *S. aureus* prophage phi13 amidase (accession no. NP_803402), and *S. aureus* prophage MW2 amidase (accession no. NP_646703.1). These three lysins share 100% sequence identity with each other (data not shown). We thus characterized the region corresponding to the putative CWT domain of the phiNM3 lysin with respect to its biochemical and functional properties.

The phiNM3 CWT binds specifically to staphylococci. The putative CWT domain corresponding to amino acid residues 158 to 251 of phiNM3 lysin (see Fig. S1 in the supplemental material) was cloned and expressed in *E. coli*. The ~10-kDa protein was highly soluble and was purified to homogeneity (see Fig. S3B in the supplemental material). To determine the binding specificity of this domain toward bacterial cells, the purified protein was labeled with FITC and exposed to log-phase *S. aureus*, *S. epidermidis*, and a mixed population of *S. aureus* and *Bacillus cereus*. *S. pyogenes*, *E. coli*, and *B. cereus* served as controls. The FITC-labeled phiNM3 CWT domain displayed species specificity by binding specifically to *S. aureus* (Fig. 1, image pair 1) and *S. epidermidis* (Fig. 1, image pair 3)

cells and was able to bind specifically to the staphylococcal cells in mixed populations (Fig. 1, image pair 6). No binding was observed to *Bacillus* (Fig. 1, image pair 2), *E. coli* (Fig. 1, image pair 4), or streptococci (Fig. 1, image pair 5), a characteristic of the specificity of many phage lysins. The binding of the labeled CWT domain generally was over the entire cell surface (Fig. 1, image pairs 1 and 3), with more intense localization near the polar and septal regions.

Function-guided construction of chimeric lysin ClyS. Previous attempts in our laboratory to clone and express a soluble and active native staphylococcus-specific phage lysin have been unsuccessful. Therefore, we attempted to circumvent these problems by taking advantage of the modular nature of lysins and developing lysin chimeras. Many inactive chimeras were generated in a series of logical but progressive steps (see the supplemental material), the most successful of which was chimera AD127, composed of the lysin endopeptidase domain of phage Twort fused amino terminally to the phiNM3 CWT domain. The AD127 chimera was soluble and highly active against staphylococcal cells but suffered from poor expression in *E. coli*. To overcome this, the open reading frame (ORF) encoding AD127 was cloned into expression plasmid pJML6 to get pAD138. Plasmid pJML6 was previously used to overexpress *S. pneumoniae* lysin Cpl-1 and PAL in *E. coli* (21). Chimera AD138 was named ClyS for chimeric lysin for *Staphylococcus*. ClyS is a 280-amino-acid protein with a deduced molecular mass of 31,956 Da and a theoretical isoelectric point of 9.17. It was purified by a two-step column chromatography method to >90% homogeneity (see Fig. S3A in the supplemental material).

***In vitro* activity of ClyS.** In preliminary experiments using various quantities of ClyS with staphylococci, it was determined that 250 μ g was an effective dose. For example, when 250 μ g of ClyS was added to the exponential phase of *S. aureus* 8325-4 cells, the OD₆₀₀ dropped 3- to 4-fold within ~15 min (Fig. 2). To confirm that the observed loss of turbidity corresponded to a decrease in viable cell counts, aliquots from the spectrophotometric lysis assay were plated to TSB agar at various time points, and CFU were enumerated. A decrease in viability of ~3 logs was observed in 30 min (Fig. 2).

The lytic effect on *S. aureus* 8325-4 cells exposed to 250 μ g of ClyS for 1 to 3 min was visualized by transmission electron microscopy (Fig. 3A and B). Cells exposed to ClyS showed the localized degradation of the cell wall at single or multiple sites, which is typical of lysin-mediated cell lysis. The sites of degradation on the cell were randomly distributed over the entire cell surface. This observation correlated with the binding of the FITC-labeled CWT domain to the whole surface of the staphylococcal cells. The localized weakening of the cell wall resulted in the extrusion and rupture of the cell membrane (Fig. 3A to C) and subsequent loss of cytoplasmic contents and formation of cell ghosts (Fig. 3D).

Specificity of ClyS. The muralytic activity of ClyS was tested on a number of bacterial strains, representing a variety of genera and species that were divided into several sets (Table 1 and Fig. 4). Set I consisted of methicillin-sensitive (MSSA) and methicillin-resistant strains (MRSA) of *S. aureus*. ClyS was very active against both MSSA and MRSA, although some differences were observed between *S. aureus* strains. ClyS lysin also effectively killed six VISA strains of staphylococci and the lysostaphin-resistant *S. aureus* strain LyrA (14) (data not

FIG. 1. phiNM3 CWT binds specifically to staphylococci. Purified phiNM3 CWT was labeled with FITC and exposed to *S. aureus* (image pair 1), *B. cereus* (image pair 2), *S. epidermidis* (image pair 3), *E. coli* (image pair 4), *S. pyogenes* (image pair 5), and a mixed suspension of *S. aureus* and *B. cereus* cells (image pair 6). P, phase-contrast image; F, fluorescent image.

shown). Set II consisted of different species of staphylococci, including *S. epidermidis*, *S. simulans*, and *S. sciuri* subsp. *sciuri*. ClyS was active not only against *S. epidermidis*, including the biofilm-forming strain RP62A (39), but also was active against *S. simulans* and *S. sciuri* subsp. *sciuri*. Set III consisted of a mix of Gram-positive and Gram-negative bacteria, including representatives of group A, B, C, and E streptococci, the oral

streptococcal species *S. gordonii*, and *S. salivarius*, as well as *Streptococcus uberis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *E. coli*. ClyS did not exhibit lytic activity against any of these bacteria (Fig. 4).

Immune response to ClyS. It was shown previously that lysins have the unique capacity to resist neutralization by antibodies in both *in vitro* and *in vivo* assays (21, 35). However, chimeric lysins have not evolved naturally, and as such they may not retain this unique capacity. To address this, rabbit hyperimmune serum raised against ClyS (ELISA titer of >100,000) was assayed for its effect on lytic activity. When the antibody was added to ClyS (100 μ g) at a 1:10 dilution (final ELISA titer of 10,000) and allowed to stand for 15 min before adding staphylococci, no effect on the lytic activity was observed (Fig. 5). A similar result was obtained with a second hyperimmune rabbit serum to ClyS (data not shown).

***In vivo* nasal decolonization of MRSA by ClyS.** To study the potential of ClyS to reduce the MRSA colonization of the nasal cavities, C57BL/6J mice were intranasally inoculated with $\sim 5 \times 10^9$ of a spontaneously streptomycin-resistant strain of MRSA (191-SM^R). Twenty-four hours postcolonization, mice were administered one dose of either buffer or ClyS into the nasal passages. An hour after the treatment, mice were sacrificed and bacterial colonies were enumerated on Spectra MRSA agar. Three independent experiments were performed to evaluate a

FIG. 2. Activity of ClyS against *S. aureus* *in vitro*. *S. aureus* strain 8325-4 cells were resuspended in 20 mM PB (pH 7.4) and incubated with 250 μ g of ClyS, and the OD₆₀₀ (filled circles) was monitored by a spectrophotometer. Control experiments (filled squares) were performed under the same conditions with buffer alone. Cell viability (filled triangles), measured as CFU/ml, was determined by serially diluting and plating the same cell suspensions to TSB agar plates.

FIG. 3. ClyS causes cell wall disruption and, ultimately, the lysis of 8325-4 cells. (A to C) Thin-section transmission electron micrographs (bars, 200 nm) of *S. aureus* 3 min after exposure to 250 μ g of ClyS. Ultimate lysis results in mostly cell ghosts (D) after the loss of cytoplasmic contents (bar, 500 nm).

total of 19 mice for each treatment group (Fig. 6). Compared to the buffer-alone control (average of 120,197 CFU/cavity), a single ClyS treatment significantly ($P < 0.002$) reduced the mean CFU on the nasal mucosa by greater than 2 logs (average of 731 CFU/cavity).

ClyS treatment of systemic MRSA infections. To assess whether ClyS treatment could prevent death resulting from systemic MRSA infections, 4-week-old FVB/NJ mice were intraperitoneally injected with $\sim 5 \times 10^5$ CFU of the community-acquired (PVL toxin-encoding) MRSA strain MW2 in 5% mucin. Preliminary experiments determined that 5×10^5 CFU was 10-fold greater than the 100% lethal dose (LD_{100}) for a 24-h period, and that within 1 to 3 h of i.p. injection the MRSA infection was systemic (i.e., $>1,000$ CFU of MRSA was recovered from heart, liver, spleen, and kidney) (data not shown), indicating that the infection had disseminated systemically at the time of treatment. Treatment occurred 3 h postinfection with either 20 mM PB or a single 1-mg dose of ClyS in 20 mM PB injected i.p. Mice then were monitored for survival for 10 days. The results from three independent experiments were combined (ClyS treatment, $n = 16$; buffer treatment, $n = 14$), and mouse survival data were plotted with a Kaplan-Meier survival curve (Fig. 7). Within 24 h of MRSA infection, all of the control mice died of bacterial sepsis, while only 2/16 of ClyS-treated mice died at 48 h. The remaining ClyS-treated mice (14/16; 88%) survived the 10-day course of the experiments (Fig. 7). Other experiments showed that treatment

with one dose of 1 mg of ClyS/mouse at 1 or 6 h postinfection also rescued mice from death (data not shown).

ClyS shows synergistic interaction with vancomycin and oxacillin. A standard checkerboard broth microdilution assay was used to test the interaction of ClyS with vancomycin and ClyS with oxacillin to determine synergy. The ClyS MIC was 30 to 40 μ g/ml for both strains tested, while the vancomycin MIC for VISA strain Mu50 was 16 μ g/ml and the oxacillin MIC for MRSA strain COL was 64 μ g/ml. Isobolograms were plotted for ClyS with vancomycin and with oxacillin by transcribing the enzyme concentrations along the inhibitory line on the microtiter plate in an x/y plot. The shape of the curves for both antibiotics were characteristic of highly synergistic interactions (Fig. 8) and were confirmed by calculating the ΣFIC for both interactions, which were <0.5 .

In vivo synergy of oxacillin and ClyS in treatment of systemic MRSA infections. To determine if the *in vitro* synergy observed between ClyS and oxacillin could be translated in our MRSA septicemia model, FVB/NJ mice were intraperitoneally injected with $\sim 5 \times 10^5$ CFU of MRSA strain MW2 as described above. At 3 h postinfection, the bacteremic mice were treated in parallel with a low i.p. dose of ClyS (166 μ g/mouse) and different concentrations of oxacillin i.m. (ranging from 10 to 100 μ g/mouse) or buffer controls by the same routes. Preliminary experiments determined that an 30% effective dose (ED_{30}) of ClyS (166 μ g/mouse) provided enough efficacy to

FIG. 4. ClyS exerted specific killing of antibiotic-susceptible and -resistant staphylococci. Log-phase cultures of different bacteria were exposed to 250 μ g of ClyS for 15 min. Fold killing was calculated by dividing the number (CFU) of viable bacteria after buffer treatment by the number (CFU) after exposure to ClyS enzyme. Several MSSA and MRSA strains, along with *S. epidermidis*, *S. simulans*, and *S. sciuri* strains were tested for susceptibility to ClyS killing. Control strains (GAS, group A streptococci; GBS, group B streptococci; GCS, group C streptococci; GES, group E streptococci; along with enterococci, other streptococci, bacilli, pseudomonas, and *E. coli*) also were tested.

offer partial protection to mice while being a low enough concentration to evaluate the effect of combinatorial treatment with oxacillin (data not shown). Mice were monitored for survival for 10 days, and the results of five independent experiments were combined and plotted in a Kaplan-Meier survival curve (Fig. 9). While only 30% (6/20) to 35% (8/23) of mice survived with individual treatments of either 166 μ g/mouse of ClyS or 100 μ g/mouse of oxacillin, respectively, neither differed significantly from the survival rate of the buffer-alone control (13%; 2/15). Conversely, a single dose of the combined treatment of intraperitoneally injected ClyS with either 50 or

100 μ g of oxacillin injected intramuscularly significantly increased mouse survival (80% [8/10] and 82% [18/22], respectively) compared to that of the individual treatments and buffer alone (Fig. 9).

DISCUSSION

ClyS, a staphylococcal-specific lysin, has demonstrated synergistic interaction *in vitro* (Σ FIC < 0.5) with two antibiotics commonly used to treat staphylococcal infections, namely, oxacillin and vancomycin. These results are in agreement with previous studies suggesting a synergistic interaction between phage lysins and different classes of antibiotics (26, 35). More importantly, however, we have demonstrated the synergistic interaction of ClyS with oxacillin *in vivo*, the first report to our knowledge. A suboptimal concentration of ClyS in combination with oxacillin was able to significantly increase the survival of mice infected with MRSA strain MW2 compared to survival rates for each compound individually. We speculate that the *in vivo* synergy between oxacillin and ClyS is due to the enhanced lysis of the bacteria, similarly to what is observed with the oxacillin treatment of the *mgrA/sarA* mutants of *S. aureus* (40). Oxacillin inhibits cell wall assembly enzymes, resulting in the increased expression of internal peptidoglycan hydrolases and autolysins, causing the weakening of the cell wall peptidoglycan. As an endopeptidase, adding ClyS would elevate the total concentration of wall-degrading enzymes, further shifting the balance of the cell wall repair and degrading machinery to

FIG. 5. Effect of hyperimmune rabbit sera on ClyS activity. Ten microliters of either hyperimmune rabbit serum (final ELISA titer of 10,000), preimmune rabbit serum, or PBS was added to 100 μ l of ClyS (100 μ g). After 15 min, staphylococci were added and the change in OD₆₀₀ was measured for 30 min.

FIG. 6. Effect of ClyS on nasal colonization by MRSA. Nasal passages of C57BL/6J mice were inoculated with $\sim 5 \times 10^9$ of MRSA strain 191-SM^R. After 24 h, mice were treated by a single intranasal administration of 1 mg of ClyS or 20 mM PB. One hour after treatment, the bacteria from the excised nasal cavities were plated on Spectra MRSA agar to enumerate CFU. Data from three independent experiments were combined (19 mice per treatment group) and analyzed for statistical significance with the Student's *t* test.

cause increased degradation and, thus, the lysis of the bacteria. These results could allow antibiotics now discontinued in the clinic due to increased resistance issues to be reinstated when combined with an appropriate dose of ClyS.

Using ClyS alone may prove to be a viable treatment for more serious *S. aureus* diseases, such as soft-tissue infections, bacteremia, infective endocarditis, and pneumonia. Unlike an-

FIG. 7. ClyS protected mice from death caused by MRSA septicemia. FVB/NJ mice were intraperitoneally injected with $\sim 5 \times 10^5$ CFU of MRSA strain MW2 in 5% mucin. Three hours postinfection, mice received one intraperitoneal injection of 20 mM PB control or 1 mg of ClyS. Mice were monitored for survival for 10 days. The results from three independent experiments were combined (ClyS treatment, $n = 16$; buffer treatment, $n = 14$) and the mice survival data plotted with a Kaplan-Meier survival curve.

FIG. 8. ClyS showed synergistic interaction with vancomycin or oxacillin. Interactions between ClyS and antibiotics were tested by the checkerboard broth microdilution assay. Fractional concentrations of MICs along the inhibitory line for enzyme or antibiotic were plotted on the *x/y* plot to generate an isobologram. Error bars show standard errors of the means.

tibiotics whose effects are seen only hours after administration, lysins work immediately, reducing the accumulation of toxic substances. This is illustrated in recent studies showing the effectiveness of phage lysins in treating systemic infections (21, 36) and, in one case, the successful treatment of an established pneumococcal pneumonia (45). In our study, a single i.p. dose of ClyS in mice septic with MRSA resulted in significantly higher survival rates (88%) than that for untreated septic mice (0%). It is likely that the survival rates of the treated mice would be increased further by changing the dosing to either a higher dose of ClyS or by repeat dosing.

To accomplish these protection experiments, it was necessary to develop a chimeric lysin to circumvent the solubility and expression problems inherent in staphylococcal phage lysins (29, 31, and our own unpublished data). We took advantage of the modular nature of lysins, which has been exploited by researchers to swap different catalytic and binding domains while retaining the activity and/or specificity, respectively, of the original lysin (10). Using biochemical and functional assays as guidance, more than 15 chimeras were strategically engineered to contain different combinations of catalytic and binding domains. From these constructs a novel lysin, ClyS, was engineered that had the desirable properties of a well-ex-

FIG. 9. Synergistic effects of ClyS and oxacillin protected mice from MRSA septicemia-induced death. FVB/NJ mice were intraperitoneally injected with $\sim 5 \times 10^5$ CFU of MRSA strain MW2 in 5% mucin. Three hours postinfection, mice received an i.p. injection of a suboptimal concentration of ClyS (166 μg) or 20 mM PB along with an i.m. injection of oxacillin (10 to 100 μg) or saline control. Mice were monitored for survival for 10 days, and the results of five independent experiments were combined and plotted in a Kaplan-Meier survival curve.

pressed and highly soluble lysin that was active against staphylococci.

ClyS is a chimera consisting of the N-terminal endopeptidase domain of the lysin from phage Twort fused with the C-terminal CWT domain of phage phiNM3 lysin. The phiNM3 CWT domain by itself was highly soluble and displayed binding specificity toward staphylococcal cells. Additionally, the CWT domain met a second criterion wherein it did not have homology to the SH3b-like binding domain associated with lysostaphin and some lysins. SH3b binding domains are presumed to bind the peptide cross-bridges in the bacterial cell wall peptidoglycan (15). These peptide bridges can be altered readily by the bacteria, rendering them resistant to these lytic enzymes, including lysostaphin. This mechanism of resistance presumably could be circumvented by engineering a chimeric lysin that contained a novel CWT domain. As such, the phiNM3 CWT domain of ClyS does not share homology to any known domains in the GenBank database. The absence of homology in the binding domain is a common feature for lysins such as PlyG and Cpl-1 (22, 36). These lysins use carbohydrate and choline moieties, respectively, in the bacterial cell wall as their receptors. The uniqueness of the binding domains of such lysins complements the unique structures present on the bacterial cell walls, thereby imparting to lysins their characteristically exquisite specificity. The binding domain of ClyS also presumably recognizes a cell wall-associated carbohydrate or another moiety. This in turn may make ClyS an attractive target for development as an alternative therapeutic because of the lack of resistance, as seen for PlyG (36), Cpl-1, and other lysins (unpublished data).

The activity of ClyS against *S. simulans* indicates that the binding domain is unaffected by the presence of a serine residue in the peptide cross-bridges of *S. simulans* peptidoglycan, the reason for its resistance to lysostaphin. ClyS was also active against several *Staphylococcus* species, including all tested MRSA and VISA strains, as well as *S. sciuri* and biofilm-producing and nonproducing strains of *S. epidermidis*, suggesting that the binding moiety of ClyS is a common cell wall component found in all *Staphylococcus* species. Such broad-spectrum activity of ClyS seen against all staphylococci tested is uncommon among native phage lysins, which typically display species specificity (22, 36). However, the lack of activity of ClyS against other Gram-positive or Gram-negative bacteria resembles the characteristic specificity seen in native phage lysins. The broad antistaphylococcal activity of ClyS and its ability to kill multidrug-resistant as well as biofilm-producing staphylococci *in vitro* makes ClyS a valuable treatment option for staphylococcal infection or the decolonization of skin and mucous membranes.

In humans, the nasal mucous membranes are the major reservoir of staphylococci, including MRSA, and are an endemic risk factor for skin and soft-tissue infections as well as bacteremia in the patient population (6). A decline in nosocomial infection is reported with the reduction or elimination of this reservoir. Lysins have been used successfully in several different animal models of mucosal colonization to decolonize a wide range of pathogenic bacteria, including staphylococci, pneumococci, and group A and group B streptococci (5, 22, 30, 35, 36). When administered intranasally to mice, ClyS efficiently eliminated MRSA by decolonizing the nasal passages. A greater-than 2-log drop was seen 1 h after a single treatment

compared to the level of buffer alone. The currently accepted *S. aureus* decolonization regimen includes treatment with mupirocin ointment nasally for 5 days, but recolonization and inadequate clearance often result because of increasing resistance to the antibiotic or poor patient compliance (6, 42). The fact that ClyS works as a potent decolonizing agent suggests that lysin therapy is a viable treatment option in specific high-risk populations, such as in hospitals and in nursing homes, etc., and may aid in decreasing primary and secondary infection rates. Furthermore, unlike antibiotics, the specificity of ClyS to staphylococci may allow it to be used prophylactically to reduce further carriage of *S. aureus* and MRSA in health care employees or in community settings where MRSA resistance is becoming an increasing problem, such as military bases, prisons, and sports teams (6). Finally, recent evidence indicates that >90% of deaths as a result of influenza pandemics resulted from secondary infections caused by *S. aureus*, *S. pneumoniae*, and *S. pyogenes* (3, 28), and at least 29% of deaths from the ongoing H1N1 pandemic were complicated by the same causes (4). Thus, ClyS and other lysins may be used during flu season to reduce colonization and/or treat secondary infections by these pathogens in high-risk individuals.

The current standard of care for the treatment of serious bacterial infections, pending the identification of the organism, is to cover the most likely organisms based on the suspected site of infection, the patient's clinical condition, and any relevant environmental factors (8, 9). Since staphylococci are ubiquitous organisms, antistaphylococcal coverage is included in many empirical regimens. The treatment of serious infections caused by *S. aureus* has been a challenge for decades, because the bacteria rapidly develop resistance to novel antibacterials. Beta-lactam antibiotics are among the most widely used for the treatment of serious infection, but no currently available beta-lactam agent is effective against MRSA. Furthermore, MRSA strains have developed resistance to newer agents, including glycopeptides (17) and oxazolidinones (44). Lysins, because of their low probability of bacterial resistance plus rapid activity and high specificity, could be an important addition to the medical armamentarium.

In summary, we report the development of a novel chimeric lysin with improved biochemical properties and excellent lytic activity against all staphylococci, including MRSA and VISA strains. We have demonstrated the effectiveness of ClyS when used alone or in combination with existing antibiotics for the treatment of serious staphylococcal infections. This alternative therapeutic option will provide a viable tool to combat the increasing problem of infections caused by multidrug-resistant *S. aureus*.

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