

# Novel Bacteriophage Lysin with Broad Lytic Activity Protects against Mixed Infection by *Streptococcus pyogenes* and Methicillin-Resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pyogenes* (group A streptococcus [GrAS]) cause serious and sometimes fatal human diseases. They are among the many Gram-positive pathogens for which resistance to leading antibiotics has emerged. As a result, alternative therapies need to be developed to combat these pathogens. We have identified a novel bacteriophage lysin (PlySs2), derived from a *Streptococcus suis* phage, with broad lytic activity against MRSA, vancomycin-intermediate *S. aureus* (VISA), *Streptococcus suis*, *Listeria*, *Staphylococcus simulans*, *Staphylococcus epidermidis*, *Streptococcus equi*, *Streptococcus agalactiae* (group B streptococcus [GBS]), *S. pyogenes*, *Streptococcus sanguinis*, group G streptococci (GGS), group E streptococci (GES), and *Streptococcus pneumoniae*. PlySs2 has an N-terminal cysteine-histidine aminopeptidase (CHAP) catalytic domain and a C-terminal SH3b binding domain. It is stable at 50°C for 30 min, 37°C for >24 h, 4°C for 15 days, and –80°C for >7 months; it maintained full activity after 10 freeze-thaw cycles. PlySs2 at 128 µg/ml *in vitro* reduced MRSA and *S. pyogenes* growth by 5 logs and 3 logs within 1 h, respectively, and exhibited a MIC of 16 µg/ml for MRSA. A single, 2-mg dose of PlySs2 protected 92% (22/24) of the mice in a bacteremia model of mixed MRSA and *S. pyogenes* infection. Serially increasing exposure of MRSA and *S. pyogenes* to PlySs2 or mupirocin resulted in no observed resistance to PlySs2 and resistance to mupirocin. To date, no other lysin has shown such notable broad lytic activity, stability, and efficacy against multiple, leading, human bacterial pathogens; as such, PlySs2 has all the characteristics to be an effective therapeutic.

Gram-positive pathogens such as *Streptococcus pyogenes* (group A streptococci [GrAS]), *Staphylococcus aureus*, *Streptococcus agalactiae* (group B streptococci [GBS]), and *Listeria monocytogenes* are responsible for millions of serious and sometimes fatal infections worldwide. Additionally, resistance to conventional antibiotics has been on the rise, resulting in increased infection rates, morbidity, mortality, and treatment costs. Consequently, new therapeutic methods need to be developed to reduce the antibiotic pressure on these pathogens.

*S. pyogenes* annually infects over 750 million people (1), resulting in 25% mortality among the ~650,000 cases that progress to severe infection (1). This pathogen is responsible for a broad range of infections, such as pharyngitis, impetigo, scarlet fever, erysipelas, cellulitis, toxic shock syndrome, and necrotizing fasciitis; it can lead to serious sequelae, such as rheumatic fever and acute glomerulonephritis (2–4). *S. aureus* is capable of producing severe, secondary infections in immunocompromised individuals, as well as causing disease in otherwise-healthy people. Besides skin and soft tissue infections (SSTIs), *S. aureus* can cause sepsis, pneumonia, necrotizing fasciitis, pyomyositis, endocarditis, toxic shock syndrome, and scalded skin syndrome (5, 6). Unfortunately, many *S. aureus* strains, such as methicillin-resistant *S. aureus* (MRSA) and (less often) vancomycin-resistant *S. aureus* (VRSA), have acquired resistance to one or more antibiotics used as standard therapy. MRSA account for more than 50% of hospital isolates causing pneumonia and septicemia (7), particularly in intensive care units, resulting in 30 to 40% mortality (8, 9). While health care-associated MRSA strains usually infect susceptible patients, community-associated MRSA (CA-MRSA) strains can infect healthy individuals (10–13). CA-MRSA strains are often more virulent and are capable of causing more severe diseases (14, 15).

A novel antimicrobial strategy to control resistant bacterial pathogens involves the use of lytic enzymes (also known as endolysins, or lysins) whose genes are carried by bacteriophages (or phages) (reviewed in references 16 and 17). At the end of phage replication and assembly inside a host bacterium, the progeny phage must escape. To accomplish this, phage produce lysins—peptidoglycan hydrolases that degrade the bacterial cell wall—that result in hypotonic lysis of the bacterium and release of phage progeny. When applied exogenously, these enzymes are likewise able to access the peptidoglycan layer in the Gram-positive cell envelope (due to its lack of an outer membrane) and produce the same lytic effect. While no lysin has yet been FDA approved, these enzymes could be used to treat antibiotic-resistant bacteria. Unlike antibiotics, an important feature of phage lysins is their rapid, lethal effect on bacteria (18–20). Lysins are notable for the potencies and specificities they demonstrate, generally toward the species that the phage carrying the lysin gene infects or closely related

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organisms (17–19, 21, 22). As such, they presumably exert a less dramatic effect on the normal flora than conventional antibiotics.

Several lysins have been developed against MRSA (21, 23, 24) and *S. pyogenes* (25); to date, however, no lysin has shown high lytic activity against multiple species of different bacterial pathogens. While developing a lysin with activity against the zoonotic pathogen *Streptococcus suis*, we discovered an enzyme (PlySs2) with activity against a wide range of Gram-positive pathogens and *in vivo* efficacy against MRSA and *S. pyogenes*. In this report, we describe the initial characterization of the first broadly acting lysin that could be used against multiple Gram-positive pathogens.

## MATERIALS AND METHODS

**Bacterial strains.** All strains were stored at  $-80^{\circ}\text{C}$  (see Table S1 in the supplemental material). *Staphylococcus*, *Streptococcus*, *Listeria*, *Enterococcus*, *Pseudomonas*, and *Bacillus* strains were cultivated in brain heart infusion (BHI) broth, unless the medium was replaced with Mueller-Hinton (MH) medium for MIC determinations, as described below. *Lactobacillus* strains were cultivated in de Man, Rogosa, and Sharpe (MRS) broth (Sigma). *Escherichia coli* was grown in Luria-Bertani (LB) broth. All media were acquired from Becton, Dickinson, and Company (Sparks, MD), unless otherwise stated. Bacteria were propagated at  $37^{\circ}\text{C}$  and shaken at 200 rpm, if necessary.

**Genomic sequence analysis and cloning of PlySs2.** The sequenced genomes of 8 *S. suis* isolates in GenBank were manually inspected for the presence of integrated prophage regions. If a prophage was suspected, the theoretical translations of each open reading frame (ORF) in that region were subjected to BLASTP and Pfam analyses to locate potential lysin-encoding genes.

A candidate lysin gene (*PlySs2* from *S. suis* strain 89/1591) was PCR cloned from genomic DNA with the following primers: AATGCTAGCC TGATACACAGTTAGAGACC (forward) and CCTAAGCTTCTTTTCA CAAATCATAATCCCCAG (reverse). The underlined nucleotides represent engineered restriction sites (NheI and HindIII), which were cut with the corresponding enzymes (NEB, Ipswich, MA) to clone *PlySs2* into the pBAD24 expression plasmid (pBAD24\_*PlySs2*) carrying genes for ampicillin selection and arabinose induction. The pBAD24\_*PlySs2* vector was transformed into *E. coli* TOP10 cells (Invitrogen).

**Recombinant expression and purification of PlySs2.** The aforementioned clone was grown as a patch on LB agar supplemented with 0.2% arabinose, permeabilized by a 10-min exposure to chloroform vapor, and overlaid with soft agar containing heat-killed *S. suis* bacteria. A streptococcal clearing zone around the *E. coli* patch confirmed active recombinant expression of PlySs2 (26).

For PlySs2 purification, the above clone was propagated in LB broth ( $37^{\circ}\text{C}$ , 220 rpm aeration) with 100  $\mu\text{g}/\text{ml}$  ampicillin. Recombinant expression was induced at an optical density at 600 nm ( $\text{OD}_{600}$ ) of  $\sim 0.8$  by addition of arabinose (0.2%, final concentration). Following overnight incubation, the cells were pelleted and resuspended in 15 mM phosphate buffer (PB; pH 8.0; buffer A) supplemented with protease inhibitor cocktail tablets (Roche). Cells were lysed with an EmulsiFlex C-5 homogenizer. After debris removal via ultracentrifugation ( $35,000 \times g$ , 1 h), the supernatant was adjusted to pH 7.4 with the addition of  $\sim 4$  volumes of buffer A.

The sample was passed through a HiTrap fast flow DEAE anion-exchange column (General Electric), and the flowthrough (which contained the desired PlySs2) was subjected to ammonium sulfate precipitation at 225 g/liter (40% saturation). The precipitated protein was resolubilized in 40 ml of 15 mM PB, pH 6.7 (buffer B) for every liter of initial *E. coli* culture. This solution was dialyzed extensively against buffer B. Finally, the dialysate was passed through a HiTrap fast flow carboxymethyl (CM) cation-exchange column (General Electric). The CM column was washed in buffer B plus 17 mM NaCl, which resulted in gradual, pure elution of PlySs2.

The presence of PlySs2 was confirmed based on lytic activity (clearing

zones on agar plates containing embedded, autoclaved *Pseudomonas aeruginosa* [26]) and verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with Coomassie stain. All fractions containing PlySs2 were pooled and stored at  $-80^{\circ}\text{C}$ . For *in vivo* tests, purified PlySs2 was dialyzed (into 15 mM NaCl, 5 mM PB; pH 7.4), frozen to  $-80^{\circ}\text{C}$ , lyophilized overnight, and resuspended in approximately 1/10 of the initial volume before lyophilization. A bicinchoninic acid (BCA) assay (Sigma) was used to determine the protein concentration.

**PlySs2 specificity.** The  $\text{OD}_{600}$  of log-phase bacterial cultures was adjusted with buffer A to  $\sim 1.0$  in 96-well microtiter plates (Falcon). PlySs2, at 32  $\mu\text{g}/\text{ml}$ , or buffer B control vehicle was added to each sample well. In each run, *S. suis* 7997 was included as a positive control. Spectrophotometric readings ( $\text{OD}_{600}$ ) of each well were taken by using a Spectramax Plus 384 apparatus (Molecular Devices) every minute over 60 min at room temperature. Lysin activity was gauged by the degree of turbidity reduction (based on the  $\text{OD}_{600}$ ) following enzyme addition.

**Bactericidal assay.** Log-phase bacteria were resuspended in buffer A to an  $\text{OD}_{600}$  of 0.1 (0.5 McFarland;  $\sim 10^8$  CFU/ml), and aliquots were added to wells of a polypropylene microtiter plate (Costar). Actual inoculum titers for each experiment were derived from plating serial dilutions of each inoculum. For each organism, buffer B control vehicle or PlySs2 was added at 128  $\mu\text{g}/\text{ml}$  to wells in triplicate. Plates were sealed and incubated at  $37^{\circ}\text{C}$  with agitation every 5 min for 1 h. After incubation, cells were serially diluted in 10-fold increments and plated on BHI agar. Death was calculated as follows:  $-\log[(\text{number of cells surviving under test condition})/(\text{number of cells surviving under control condition})]$ .

**MIC analysis.** The protocol described by Wiegand et al. (27) was followed to determine MICs, with adjustments as detailed below. Briefly, a final suspension of  $\sim 5 \times 10^5$  cells/ml in MHB (or BHI for *S. pyogenes*) plus sterile-filtered lysin or control vehicle was distributed within a 96-well microtiter plate in triplicate (27). Cells were challenged with 0.5 to 1,024  $\mu\text{g}/\text{ml}$  PlySs2 in triplicate. MICs were determined by detection of cell pellet formation in the bottom of rounded wells of polystyrene plates; they were corroborated colorimetrically with alamarBlue vital dye (Invitrogen), following the manufacturer's protocol.

**In vitro resistance studies.** According to an established protocol for the *in vitro* development of mupirocin resistance (28, 29), *S. aureus* CA-MRSA MW2, *S. aureus* MSSA 8325, and *S. pyogenes* MGAS 5005 were grown in the presence of PlySs2 in liquid culture. Initially, bacterial cells at  $5 \times 10^8$  CFU/ml were grown overnight in the presence of  $1/32 \times$  the MIC of PlySs2 against each strain ( $37^{\circ}\text{C}$ ; BHI broth for *S. pyogenes* with cap secured during gentle shaking; MHB for *S. aureus* with 220 rpm aeration). The cells were pelleted and subdivided into two aliquots.

One aliquot was diluted 10-fold into fresh MHB medium with double the concentration of PlySs2; a portion of the other was spread onto the surface of MHA containing the PlySs2 MIC for that species. The MIC of 4 resultant colonies was recalculated to determine if a resistant clone had emerged (defined as a 4-fold increase in the MIC). The above procedure was repeated over an 8-day period, and the concentration of PlySs2 in the liquid culture was serially doubled from  $1/32 \times$  to  $4 \times$  the original MIC. This process was also conducted with mupirocin for each MRSA strain, to serve as an antibiotic resistance positive control.

**In vitro characterization of PlySs2.** The optimal biochemical conditions for PlySs2 enzymatic activity against log-phase pathogenic *S. suis* 7997 were screened using the same spectrophotometric analysis for evaluating PlySs2 specificity, as described above. The pH dependence of the enzyme was first addressed using two buffer sets with overlapping pH ranges: citrate/phosphate (pH 4.6 to 8.0) and bis-Tris propane (pH 7.0 to 9.7). Concentrations of NaCl, EDTA, and dithiothreitol (DTT) were also varied.

**PlySs2 stability.** The thermal stability of PlySs2 was studied by preexposing the enzyme to temperature conditions for defined scales of time: various high temperatures for 30 min,  $37^{\circ}\text{C}$  for hours,  $4^{\circ}\text{C}$  for days, and  $-80^{\circ}\text{C}$  for months. The activity of each aliquot against *S. suis* 7997 was determined spectrophotometrically as described above. PlySs2 activity

was also tested after consecutive freeze-thaw cycles between  $-80^{\circ}\text{C}$  and room temperature.

**In vivo murine model.** The Rockefeller University's Institutional Animal Care and Use Committee approved all *in vivo* protocols. A systemic infection model described by Daniel et al. (21) was used to test the *in vivo* efficacy of PlySs2 against multiple Gram-positive bacteria. Briefly, 4- to 5-week-old female FVB/NJ mice (weight range, 15 to 20 g) were obtained from The Jackson Laboratory (Bar Harbor, ME). After a period of acclimation, mice were injected intraperitoneally (i.p.) with 0.5 ml of mid-log-phase ( $\text{OD}_{600}$ , 0.5) bacteria diluted with 5% hog gastric mucin (Sigma) in saline. Bacterial suspensions contained  $\sim 5 \times 10^5$  CFU/ml of MW2,  $\sim 1 \times 10^7$  of *S. pyogenes* MGAS 5005, or a simultaneous combination of both bacteria at the above concentrations for the mixed infection experiments. Actual bacterial inoculation titers were calculated by serial dilution and plating to Columbia blood agar plates for each experiment.

Mice became bacteremic within 1 to 3 h and contained MRSA and/or *S. pyogenes* within multiple organs, including spleen, liver, kidney, and heart/blood (reference 21 and unpublished observations). Three hours postinfection, the animals were divided into 4 to 5 treatment groups per infection type and were administered i.p. 0.5 ml of either 20 mM phosphate buffer, 2 mg/ml of the streptococcal-specific lysin PlyC (25), 2 mg/ml of the staphylococcal-specific lysin ClyS (21), 2 to 4 mg/ml PlySs2, or a combination of 2 mg/ml PlyC and 2 mg/ml ClyS. A PlySs2 stock of 4 mg/ml was used for *S. pyogenes*-infected mice to increase survival over an initial 70% survival rate with treatment of 2 mg/ml mg of PlySs2 (data not shown). While this dosage was possible with PlySs2, it was above our obtainable PlyC or ClyS stock concentrations at the time.

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

## RESULTS

**Identification of PlySs2.** PlySs2 was identified in a prophage region of a serotype 2 strain of *S. suis* 89/1591 (the ORF was originally annotated in GenBank as SH3 type 5 domain protein; ZP\_03625529 [S. Lucas, A. Copeland, A. Lapidus, et al., unpublished data]). The putative lysin sequence corresponding to PlySs2 has the greatest homology among *S. suis* sequences to a surface antigen, but this sequence has only 35% identity over 53% coverage, with an E value of  $<10^{-7}$ . On *S. suis* overlay plates, clearing zones formed around an *E. coli* strain transformed with an expression plasmid for PlySs2 (pBAD24\_PlySs2), confirming the successful cloning and soluble expression of the lysin (26). Computational sequence alignment indicated that PlySs2 encodes a predicted N-terminal CHAP catalytic domain (cysteine-histidine amidohydrolase/peptidase; PF05257) and a C-terminal SH3 type 5 binding domain (PF08460) (see Fig. S1 in the supplemental material). CHAP domains are catalytically diverse and can possess either alanine-amidase activity (18) or cross-bridge endopeptidase activity (21). Based on its primary sequence, the PlySs2 CHAP domain is divergent from other database CHAP domains (all pairwise E values were  $<10^{-15}$ ), including those in characterized streptococcal (25) and staphylococcal (21) phage lysins (data not shown, but see Fig. S2 in the supplemental material).

**Purification and yield of PlySs2.** With a predicted pI of 9.01, PlySs2 flowed directly through a DEAE column at pH 7.4 (see Fig. S3, lane 4, in the supplemental material), and (following an ammonium sulfate-precipitation step) eluted cleanly both in the shoulder of the flowthrough peak of a CM column and in the 17 mM NaCl wash (see Materials and Methods for details). The prep-

aration yielded  $\sim 60$  mg of protein per liter of *E. coli* culture, with  $>99\%$  purity (see Fig. S3, lane 6). All experiments were performed with this preparation. Concentrating PlySs2 to 20 mg/ml had no deleterious effect on solubility or activity.

**Broad lytic activity.** Purified PlySs2 was tested against a wide range of bacterial species and strains to determine the range of lytic activity. Starting at an  $\text{OD}_{600}$  of  $\sim 1.0$ , all tested strains of *S. aureus*, including strains resistant to methicillin, vancomycin, daptomycin, mupirocin, and lysostaphin, were reduced to an  $\text{OD}_{600}$  ratio  $\leq 0.3$  after lysis by PlySs2 over 30 min of exposure (Fig. 1). Readings were also taken after 60 min (see Fig. S4 in the supplemental material). The  $\text{OD}_{600}$  ratios of other staphylococci, including *Staphylococcus simulans* and *Staphylococcus epidermidis*, were reduced to  $\sim 0.2$ .

With streptococci, PlySs2 lysed most tested M serotypes of *S. pyogenes*, including M1, M3, M4, M6, M18, M49, and an M-negative variant, as well as unencapsulated and highly encapsulated strains, decreasing their  $\text{OD}_{600}$  ratio to  $\leq 0.4$ . PlySs2 also exhibited strong lytic activity against *S. suis*, *Streptococcus equi zooepidemicus*, *Streptococcus equi*, *S. agalactiae* type II (encapsulated), and *S. agalactiae* 090R. The pathogenic *Streptococcus sanguinis* and group G and E streptococci were moderately sensitive to PlySs2. For *Streptococcus mutans*, group C streptococci, *Streptococcus oralis*, *Streptococcus rattus*, and *Streptococcus sobrinus*, the  $\text{OD}_{600}$  ratio was only reduced to between 0.7 and 0.9. PlySs2 did not reduce the  $\text{OD}_{600}$  ratio below 0.5 for any *Streptococcus pneumoniae* strains. *Streptococcus gordonii* was the only commensal against which PlySs2 exhibited activity (Fig. 1).

PlySs2 showed some activity against genera outside *Staphylococcus* and *Streptococcus*. While two strains of *Listeria* sp. were sensitive to PlySs2, other strains were not. In the *Enterococcus* genus, which is associated with high levels of antibiotic resistance, *E. faecalis* was sensitive to PlySs2 (although less so than staphylococci or streptococci), but *E. faecium* was not. No activity was seen against any of the different species of *Bacillus*, strains of lactobacilli, or Gram-negative organisms.

**Efficacy of PlySs2 against Gram-positive pathogens.** PlySs2 was tested for the log fold killing of several species and strains of susceptible organisms that were tested based on the  $\text{OD}_{600}$  decrease (see above). At 128  $\mu\text{g/ml}$  PlySs2 and with a 60-min exposure, the VISA strain was only reduced by 2 logs. However, PlySs2 reduced the viability of *L. monocytogenes*, *S. agalactiae*, *S. aureus*, and *S. pyogenes* from  $\sim 3$  to  $>6$  logs (Fig. 2).

When the MIC of PlySs2 was tested against these strains, most of the values qualitatively correlated directly to the lytic and killing activities. MICs ranged from 8 to 256  $\mu\text{g/ml}$  for all strains, except the VISA strain, which was not inhibited at  $>1,024$   $\mu\text{g/ml}$  (Table 1).

**Resistance to PlySs2.** By using a published, standardized method for calculating resistance, both staphylococcal and streptococcal strains were analyzed for the development of resistance against PlySs2 by serial exposure to incrementally doubling concentrations of the lysin. Under these testing conditions, none of the *S. aureus* or *S. pyogenes* strains exposed to PlySs2 over 8 days developed resistance (defined as a 4-fold increase from the original MIC) (Fig. 3). Following the same procedure, both *S. aureus* strains MW2 and 8325 developed resistance to the antibiotic mupirocin (Fig. 3).

**Biochemical characterization and stability.** PlySs2 activity was tested through a range of pH values to determine its optimum



TABLE 1 MIC of PlySs2 for various Gram-positive species<sup>a</sup>

Species	Strain	MIC ( $\mu\text{g/ml}$ )	
		Visual	Colorimetric
<i>L. monocytogenes</i>	HER 1184	8	16
	HER 1083	8	16
<i>S. aureus</i>	MSSA 8325	16	16
	MRSA MW2	16	32
	LyrA	32	32
	VISA III	32	64
	GrAS	SF370	128
GBS	MGAS 5005	128	256
	090R	256	256
<i>E. coli</i>	Type II	512	512
	TOP10	>1,024	>1,024

<sup>a</sup> All MICs were evaluated visually for bacterial growth and with alamarBlue vital dye (colorimetrically) at concentrations from 0.5 to 1,024  $\mu\text{g/ml}$  of PlySs2 for each strain of each species listed. There was a low MIC for MRSA MW2, as expected, and a higher MIC for GrAS strain MGAS 5005. The MIC of PlySs2 for the negative control, *E. coli*, was above the limits of the assay. For a reference, ClyS, LysK, and CHAP<sub>K</sub> lysins have registered MICs against *S. aureus* strains from 30 to 80  $\mu\text{g/ml}$ .

tion (see Fig. S7 in the supplemental material). Treatment with >4  $\mu\text{M}$  EDTA had an inhibitory effect on PlySs2-induced lysis of *S. suis* (see Fig. S8 in the supplemental material).

When PlySs2 was incubated at different temperatures from 22°C to 85°C for 30 min, its activity was principally unaffected until 60°C (see Fig. S9A in the supplemental material). Activity was retained during incubation at 37°C for 24 h, with activity starting to diminish after 48 h (Fig. 9B). There was no observable decrease in activity after 15 days at 4°C (see Fig. S9C) or storage at -80°C for >7 months (see Fig. S9D). The lysin also endured 10 consecutive freeze-thaws cycles (-80°C to room temperature) without any observable effects (see Fig. S10 in the supplemental material).

**Protection from a mixed bacterial infection.** To determine if the broad lytic activity of PlySs2 could provide *in vivo* protection from Gram-positive pathogens, mice were infected i.p. with either MRSA (MW2) or *S. pyogenes* (MGAS 5005) independently or si-

multaneously and subsequently treated with lysin(s). The results from 4 separate experiments were combined, and mouse survival data were plotted on a Kaplan-Meier survival curve (Fig. 4).

Mice infected with MRSA alone were protected from death with either PlySs2 (89%; 16/18) or ClyS (86%; 24/28) lysins, but not the *Streptococcus*-specific PlyC (17%; 2/12) (Fig. 4A), while mice infected with *S. pyogenes* alone could only be protected with either PlySs2 (94%; 15/16) or PlyC (100%; 12/12) but not the *Staphylococcus*-specific ClyS (0%; 0/12) (Fig. 4B). All deaths from the MRSA infection occurred within the first 2 days; deaths from the *S. pyogenes* infection continued into the fourth day. In the first 24 h of infection, 4/18 (22%) control mice survived MRSA, and 11/15 (73%) control mice survived *S. pyogenes*, with only 1/18 (6%) and 1/15 (7%) surviving to the end of the experiment, respectively. Likewise, only 1/23 (4%) of the buffer-treated control mice survived when simultaneously infected with both MRSA and *S. pyogenes*. In these mixed infection experiments, single-agent therapy with either ClyS (11%; 2/18) or PlyC (17%; 3/18) was also not significantly protective. However, mice were protected from death with either a combination of ClyS and PlyC (80%; 16/20) or PlySs2 alone (92%; 22/24) over the 10-day course of the experiment (Fig. 4C).

## DISCUSSION

A novel *S. suis* lysin, PlySs2, has demonstrated broad lytic activity against multiple Gram-positive pathogens, including *S. pyogenes* and *S. aureus*, *in vitro*. All previously characterized lysins, by contrast, have demonstrated activity against a narrow spectrum of species. Likewise, no lysin has been used to clear a bacteremic infection *in vivo* by more than one pathogenic organism; mixed infections, to our knowledge, have not been previously tested.

A noted strength of many lysins is their target specificity. Antibiotics may kill commensal organisms along with target pathogens, potentially leading to adverse sequelae (e.g., diarrhea or more serious *Clostridium difficile* complications). In contrast, lysins might be used to treat a single pathogen without disrupting the normal bacterial flora (17), although this specificity could admittedly be a limitation in treating multiple pathogens. Our re-

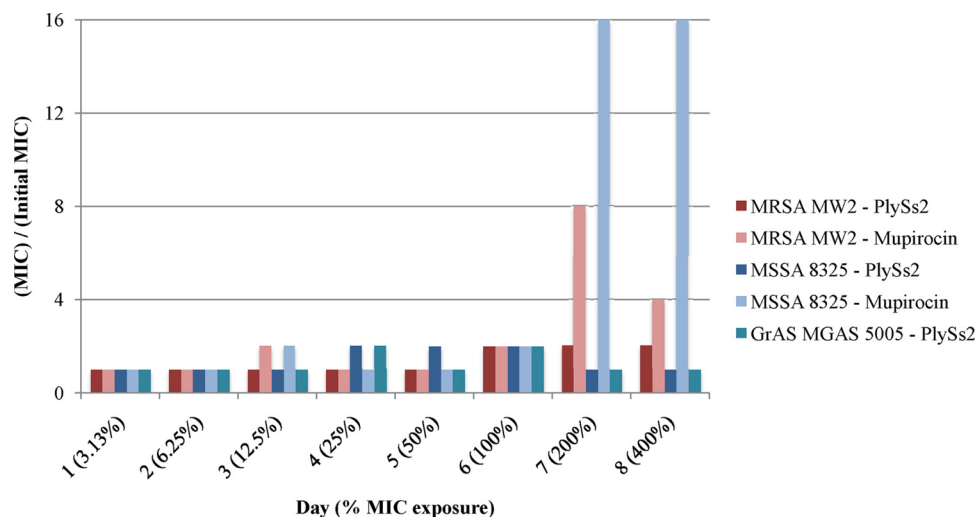
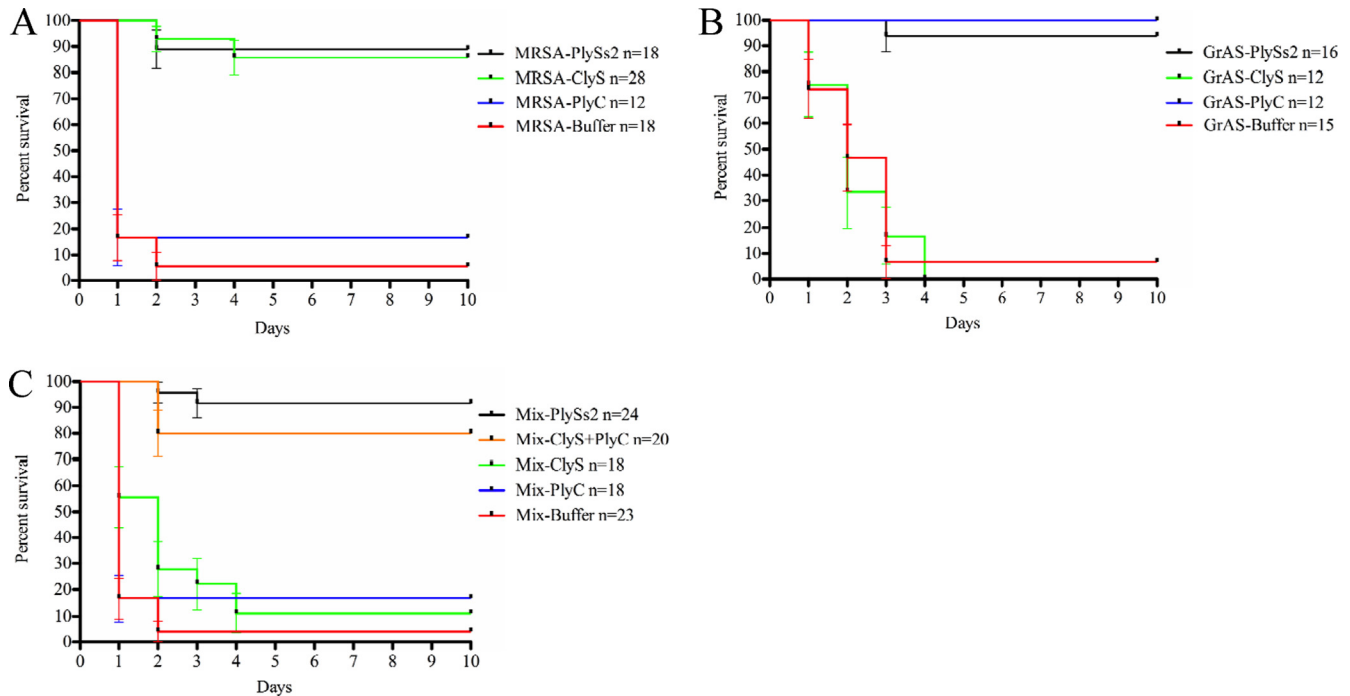


FIG 3 MRSA, MSSA, and GrAS did not acquire resistance to PlySs2 *in vitro*. MRSA strain MW2, MSSA strain 8325, and GrAS strain MGAS 5005 were exposed to 1/32 $\times$  to 4 $\times$  the MIC of PlySs2 and mupirocin (*S. aureus* strains) over 8 days. The daily MICs of PlySs2 were compared to the starting MIC of PlySs2 for each strain of bacteria to ascertain resistance. None developed resistance to PlySs2. Both MW2 and 8325 developed resistance to the positive control, mupirocin.



**FIG 4** PlySs2 protected mice from death caused by mixed MRSA and GrAS infection. FVB/NJ mice were injected i.p. with 5% mucin containing the pathogen of interest. Three hours postinfection, mice received one i.p. injection of either 20 mM phosphate buffer (control) or lysin treatment. (A) Survival data for the MRSA infection. Mice were infected with  $\sim 5 \times 10^5$  CFU of MRSA strain MW2 and treated with either 1 mg of ClyS, 1 mg of PlyC, or 1 mg of PlySs2. (B) Survival data for the GrAS infection. Mice were infected with  $\sim 1 \times 10^7$  GrAS strain MGAS 5005 and treated with either 1 mg of ClyS, 1 mg of PlyC, or 2 mg of PlySs2. (C) Survival data for the mixed MRSA and GrAS infection. Mice were infected with a combination of both bacteria from the above inoculums at the same concentrations. Mice were treated with either 1 mg of ClyS, 1 mg of PlyC, a combination of 1 mg of ClyS plus 1 mg of PlyC, or 2 mg of PlySs2. In all tests (A to C), mice were monitored for survival over 10 days. The results from 4 independent experiments were combined, and the data are plotted as a Kaplan-Meier survival curve; bars indicate standard errors. All the PlySs2 treatment groups (A to C) showed statistically significant differences ( $P < 0.0001$ ) compared to the nonspecific single lysin or buffer controls, based on the log rank (Mantel-Cox) test.

sults show it is possible that a single lysin like PlySs2 could be used to treat multiple Gram-positive pathogens, leaving many Gram-positive and all Gram-negative commensals unaffected. Furthermore, this study also demonstrated that a combination of lysins (PlyC and ClyS) with different specificities could be used effectively to treat mixed infections (Fig. 4C).

PlySs2 exhibits activity against members of two distinct phylogenetic orders: *Bacillales* (*Staphylococcus*, *Listeria*, etc.) and *Lactobacillales* (*Streptococcus*, *Enterococcus*, etc.). The peptidoglycan structures of these two orders are quite similar except for their cross-bridges, which vary widely in composition and length (30). Phage lysins have not previously displayed activity on different families or genera (and rarely on different species) (21). Furthermore, other lysins usually retain greater activity against the species infected by the phage from which the lysin was cloned, whereas PlySs2 demonstrated more activity against *S. aureus* than against *S. suis*.

All tested strains of *S. aureus* were highly susceptible to lysis by PlySs2 (Fig. 1), including strains resistant to methicillin, vancomycin, daptomycin, mupirocin, and lysostaphin. Its lytic activity against VISA and Newman strains was somewhat less than its lytic activity against other staphylococcal strains. The reduced activity against the VISA strains could be the result of the thicker cell wall in these organisms (31), which would increase the time necessary to result in lysis. The strong activity of PlySs2 against *S. simulans* and *S. epidermidis* supports its use in treatment of a wide array of other staphylococcal infections.

Many streptococci were susceptible to *in vitro* PlySs2 lysis. PlySs2 exhibited potent lytic activity against its native species, *S. suis*, as well as *S. equi*, *S. equi zooepidemicus*, and *S. pyogenes*. Of note, there was no difference in PlySs2 activity against unencapsulated or highly encapsulated variants of *S. pyogenes*. PlySs2 also demonstrated comparable activity against strains of GBS with or without a virulence-enhancing capsule (*S. agalactiae* type II and *S. agalactiae* 090R, respectively). Moderate activity was observed against group C, E, and G streptococci, suggesting *in vivo* experiments will be necessary to determine if PlySs2 can be used to treat infections by these organisms. It is unlikely that PlySs2 could be used therapeutically for *S. mutans*, *S. oralis*, *S. rattus*, and *S. sobrinus* infections, or for some strains of *S. pneumoniae*, because of its low activity against these pathogens. PlySs2 did not lyse any commensal lactobacilli. PlySs2 showed significant activity against other genera, killing some strains of *Listeria* and *E. faecalis*, but not *E. faecium* or strains of bacilli. The ability of PlySs2 to lyse a wide range of pathogens, without lysing many commensals, suggests that certain pathogens *per se* share a binding receptor for this lysin.

We found that the bactericidal assays quantitatively confirmed PlySs2's ability to effectively kill strains that vary in drug resistance and encapsulation, with correlations between the lytic, bactericidal, and MIC assays. Thus, even though they were not tested, it is likely that pathogens that were found to be more sensitive to PlySs2 lysis than *S. pyogenes* MGAS 5005 *in vitro* may also be sensitive to PlySs2 *in vivo*. The MIC of PlySs2 against several *S.*

*aureus* strains was less than or equal to the MICs of other staphylococcal lysins, including ClyS, LysK, and CHAP<sub>K</sub> (21, 29, 32, 33).

The binding domain of PlySs2 may contribute to its unique activity profile. Phage lysin binding domains have been shown to determine lysin specificity (34). As such, an SH3b (bacterial homolog of SH3) domain of a *Bacillus cereus* endopeptidase has been shown to bind the free amino group of the N-terminal alanine in the stem peptide of the peptidoglycan (35). Because of its ubiquity in peptidoglycans, this amine could also be the substrate for the PlySs2 SH3b domain. Accordingly, experiments are in progress to determine the binding and cleavage substrates for PlySs2.

An important finding in this study was our ability to clear mixed MRSA and *S. pyogenes* bacteremic infections from 92% of mice with a single dose of PlySs2. This survival rate was better than the 80% survival of mice treated with the combination of ClyS and PlyC (Fig. 4C). In a mixed infection, use of either PlyC or ClyS alone eliminated only one of the infecting pathogens, resulting in death by the other; this death occurred within the same time frame and rate as the corresponding singly infected, nontreated controls, i.e., MRSA-infected animals died within 24 h and *S. pyogenes*-infected animals died within 3 days. These results strongly support the idea that PlySs2 cures the animals of their infection by simultaneously killing both pathogens.

Recent studies have indicated that secondary infections caused by colonizing MRSA, *S. pyogenes*, or *S. pneumococcus* account for up to 90% of deaths from influenza pandemics (36–39). Mupirocin and polysporin are the only anti-infectives approved to reduce colonizing pathogenic bacteria on mucous membranes, but *S. aureus* can develop resistance to each (40). Lysins with specific activities against either staphylococci, streptococci, or pneumococci have each been shown to decolonize these pathogens in animal models of oral and nasal mucosal colonization (18, 19, 21, 22, 24, 41); however, a mixture of the three enzymes would need to be used to remove these pathogens. PlySs2 alone could be used to decolonize susceptible populations of staphylococci, streptococci, and perhaps certain pneumococcal isolates during flu season to reduce the possibility of a secondary infection.

The inability of pathogenic targets (MRSA and *S. pyogenes*) to establish resistance to PlySs2 (under conditions leading to mupirocin resistance) is consistent with findings for other lysins, such as PlyG (41). Antibiotic resistance may occur when bacteria either inactivate the drug or alter the target site. An extracellular lysin protease has yet to be identified, and it is less likely that a PlySs2-susceptible pathogen could easily alter the PlySs2 peptidoglycan target. To date, the only known resistance to a lysin-like molecule involves the insertion of a serine residue into an *S. aureus* pentaglycine cross-bridge to establish resistance to lysostaphin (a nonphage endopeptidase from *S. simulans*). However, it is unlikely that PlySs2 is a canonical cross-bridge endopeptidase, because of its activity against disparate bacterial species with diverse cross-bridge structures (42), including lysostaphin-resistant staphylococci.

PlySs2 is more tractable and stable than previously reported lysins (21, 25, 26). Its preparation is straightforward, yielding very pure, high yields of product in just a few steps. It remains soluble in concentrations exceeding 20 mg/ml (data not shown) and can be subjected to high or low temperatures for prolonged periods with little effect on its activity, even when repeatedly freeze-thawed. These features support PlySs2 as a suitable lysin for further development.

PlySs2 represents a novel breakthrough in the field of bacteriophage lysin technology. It is now possible to envision other lysins with broad therapeutic activities that retain specificity to a subset of mostly pathogenic Gram-positive organisms. For PlySs2, this novel capability possibly lies in the divergent PlySs2 CHAP domain and unique SH3 binding domain. PlySs2 occupies a vital space along the spectrum between strict lysin specificity and unselective antibiotic activity. Ideally, a therapeutic agent should have activity against major pathogens without affecting commensals; this report of PlySs2 is the first to indicate that a lysin could serve that function.

In summary, while pursuing a novel treatment for *S. suis* infection, we discovered a lysin with broad lytic activity against strains of MRSA, VISA, *S. suis*, *Listeria*, *S. simulans*, *S. equi zooepidemicus*, *S. equi*, *S. agalactiae*, *S. pyogenes*, *S. sanguinis*, *S. gordonii*, group G streptococci, group E streptococci, *E. faecalis*, and *S. pneumoniae*. PlySs2 was relatively simple to produce, tractable, and very stable. We demonstrated here the ability of PlySs2 to protect mice with mixed infection by MRSA and *S. pyogenes*. Neither of these pathogens was observed to develop resistance to PlySs2 *in vitro*. PlySs2 could therefore become a vital addition to the armamentarium against multidrug-resistant *S. aureus*, *S. pyogenes*, and various other Gram-positive pathogens.

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