Efficient Elimination of Multidrug-Resistant *Staphylococcus aureus* by Cloned Lysin Derived from Bacteriophage ϕMR11

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We report the successful purification of a cloned lysin encoded by the novel *Staphylococcus aureus* bacteriophage ϕMR11. The lysin, designated MV-L, rapidly and completely lysed cells of a number of *S. aureus* strains tested, including methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* and a subset of vancomycin-intermediate *S. aureus* (VISA) in growing conditions. MV-L–mediated killing is specific to *S. aureus* and not to other species, except for *S. simulans.* MV-L exerted its staphylocidal effect synergistically with glycopeptide antibiotics against VISA. MV-L efficiently eliminated MRSA that had been artificially inoculated into the nares of mice. The intraperitoneal administration of MV-L also protected mice against MRSA septic death, without any harmful effects. Although MV-L evoked detectable levels of a humoral response in mice, the antibodies did not abolish the bacteriolytic activity. These results indicate that MV-L might be useful as a powerful therapeutic agent against multidrug-resistant *S. aureus* infections.

Staphylococcus aureus is a common pathogen involved in various infectious diseases [1, 2]. Treatment of these infections has become more difficult with the emergence of multidrug-resistant strains, such as methicillin-resistant *S. aureus* (MRSA) [1–7]. Although MRSA has been reliably susceptible to vancomycin, MRSA strains with intermediate or extreme resistance to that drug (vancomycin-intermediate and -resistant *S. aureus* [VISA and VRSA], respectively) have aroused new medical concerns [8–12]. Furthermore, *S. aureus* is aggressively challenging the efficacy of all classes of antibiotics, as exemplified by the rapid evolution in MRSA of resistance to the new synthetic oxazolidinone, linezolid [13]. Thus, the epidemic of MRSA is growing, which demands novel therapeutic agents or alternatives to conventional antibiotic therapies directed against this formidable pathogen [2, 3, 14].

Bacteriophages (phages) with double-stranded (ds) DNA genomes, such as λ and T4, generally produce a bacterial cell-wall (peptidoglycan)–degrading enzyme, endolysin (lysin), at the end stage of infection, to release progeny virions [15–16]. Recent studies have provided substantial evidence that lysins from certain phages [17–20] are highly efficient in killing bacteria, even when applied exogenously. These findings strongly support the clinical usefulness of phage lysin in controlling virulent bacterial infections. However, to our knowledge, there have been no investigations into the scope of therapeutic applications of active purified (cloned) *S. aureus* phage lysin.

In the recent context of phage therapy [21], we pre-

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Table 1. Bacterial strains used in the study and their susceptibility to purified ϕMR11 lysin (MV-L).

Bacterial species, strains tested	Sensitivity to MV-L[a]	Source or ref.[b]
Staphylococcus aureus MSSA		
IID671 (209P)	++	I
SA37	++	[22]
TF1[RN4220 pGC2], TF8 [RN4220 pTFS6 (*epr*/pGC2)]	++	[23]
S. aureus MRSA		
MR1, MR3-MR30	++	[22]
N315, MR108, NCTC10442, 85/2082, 85/4547	++	[24]
TY21, Y721	++	II
S. aureus VISA		
MI, LIM2, 98141, 99/3759-V, BR3, Mu3 (hetero-VISA)	++	[25]
MR12047	++	II
Mu50, NJ, PC, IL, AMC11094, 99/3700-W, 28160	+	[25]
BR1, BR2, BR4, BR5	+	[25]
S. aureus VRSA VRS1, VRS2	++	III
S. epidermidis		
IID866 (ATCC12228)	–	IV
AF9, AF36, AF51, AF72, AF706, AF720, AF740	–	V
AF750, AF751, AF755, AF757, AF758, AF760	–	V
S. saprophyticus GIF3170 (ATCC15305)	–	IV
S. simulans GIFU9127 (ATCC27848)	++	IV
Streptococcus mutans, 1058	–	VI
Enterococcus faecalis ENT1	–	VII
Bacillus cereus IID1681	–	IV
Escherichia coli B (ATCC11303)	–	VIII
Vibrio parahaemolyticus RIMD2210001	–	IV
Chromobacterium violaceum GIFU1462	–	IV
Flavobacterium meningosepticum GIFU506	–	IV
Salmonella enteritidis IID604	–	IV

[a] Strains except for *E. coli* and *V. parahaemolyticus* were grown in tryptic soy broth at 37°C. *E. coli* or *V. parahaemolyticus* was grown in Luria-Bertani or yeast extract–peptone medium at 37°C, respectively. ++, complete bacteriolysis with 50 U of MV-L; +, lysis with ⩾250 U of MV-L and/or synergistic lysis with 50 U of MV-L and 4 μg/mL vancomycin; −, no bacteriolysis with 50 U of MV-L.

[b] I, provided by Research Center for Emerging Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; II, isolated in Department of Microbiology, Graduate School of Biomedical Sciences Hiroshima University, Hiroshima, Japan; III, provided by The Network on Antimicrobial Resistance in *S. aureus* (NARSA), Focus Technologies; IV, provided by Gifu University Culture Collection, Department of Microbiology, Regeneration and Advanced Medical Science, Gifu University Graduate School of Medicine, Gifu, Japan; V, isolated from healthy humans in Kochi Gakuen Junior College, Kochi, Japan; VI, provided by Department of Microbiology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan; VII, Kochi Medical School Hospital, Nankoku, Japan; VIII, purchased from American Type Culture Collection.

viously demonstrated, to our knowledge for the first time, the experimental protection of mice against lethal *S. aureus* infection using the novel phage ϕMR11 [22]. We subsequently sequenced the entire genome of ϕMR11; on the basis of those data, its putative lysin gene was identified and cloned, and the protein product was successfully purified on a large scale. We showed that the purified ϕMR11 lysin, designated "MV-L," exerts strong killing activity against multidrug-resistant *S. aureus* in vitro and in vivo. MV-L might provide an effective approach to the specific control and/or detection of pernicious *S. aureus* infections in humans and domestic animals.

MATERIALS AND METHODS

Bacterial strains and reagents. Details of bacterial strains used in the study are listed in table 1. All reagents used were purchased from Nacalai Tesque, unless otherwise specified. A streptomycin-resistant mutant of MRSA strain 85/2082, 85/2082-STR1, was isolated in the presence of 500 μg/mL streptomycin and was used in the animal experiments to distinguish the inoculated strain from normal flora–derived *S. aureus.* This mutant was confirmed to have the same growth properties and sensitivity to MV-L as the parental 85/2082. Terrific broth (TB)

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was purchased from Sigma-Aldrich. Tryptic soy broth (TSB) and brain-heart infusion broth (BHI) were purchased from Becton Dickinson (BD). Luria-Bertani medium consists of 1% tryptone (BD), 0.5% yeast extract (BD), and 1% NaCl (pH 7.0). Yeast extract–peptone medium consists of 0.3% yeast extract (BD), 1% polypepton (Nihon Seiyaku), and 3% NaCl (pH 8.0).

Pulsed-field gel electrophoresis (PFGE). PFGE was performed using the GenePath (Bio-Rad) apparatus, as described elsewhere [26], with simple modifications. After electrophoresis, the gels were stained with ethidium bromide, photographed, and then analyzed with Fingerprinting software (Japanese edition, version 3.0; Bio-Rad).

Cloning of the gene for MV-L. ϕMR11 phage genome DNA was purified and sequenced as described elsewhere [22]. On the basis of the sequenced data, putative open reading frames (ORFs) were annotated using GENETYX software, version 11.0.2 (Software Development). A homology search using BLAST at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) predicted that ϕMR11 gene *65* was its lysin (MV-L) gene (DNA Data Bank of Japan accession no. AB254389). Polymerase chain reaction amplification of ϕMR11 DNA with primers 5′-GGACGAATTCTAACAGGAAACAGACCATGCAAGCAAAATTAACTAAAAAAGAG-3′ (forward) and 5′-CGCATGGATCCCTAGTGATGGTGATGGTGATGACTGATTTCTCCCCATAAGTCACCTAATATC-3′ (reverse) yielded the full-length MV-L gene, with *Eco*RI and ribosomal binding sites at the 5′ end and a 6-histidine tag (6×His) and *Bam*HI site at the 3′ end. The product was ligated into the pTrc99A vector (Amersham Biosciences), to produce pTrc-65–6×His.

Purification of MV-L recombinant protein (Gp65-6×His). *Escherichia coli* BL-21 carrying pTrc-65–6×His was cultured overnight in TB medium that contained 100 μg/mL ampicillin (TB-amp) at 37°C and diluted with 50 vol of fresh TB-amp and further grown until the OD_{600} reached 1.2. The cells were induced for MV-L (Gp65-6×His) expression with 1 mmol/L isopropyl-β-D-thiogalactoside for 2 h, washed with PBS (pH 7.2), and disrupted with an ultrasonic disintegrator. MV-L protein was purified using TALON Metal Affinity Resin (BD) in accordance with the manufacturer's instructions (detailed procedures will be provided on request). The peak fractions were collected and dialyzed against enzyme buffer (150 mmol/L NaCl and 50 mmol/L NaPB [pH 6.1]).

Enzyme unit determination. The activity units for MV-L were determined using 85/2082 MRSA as described elsewhere [19] with simple modifications. 85/2082 was grown at 37°C to log phase, centrifuged at 9000 *g*, and resuspended in sterile saline to an absorbance at 600 nm of 0.5. The purified MV-L was serially 2-fold diluted, and 50 μL of each enzyme dilution or the enzyme buffer was mixed with 50 μL of bacterial suspension, and optical densities at 600 nm were measured with a plate reader right after exposure (ThermoMax; Molecular Devices). Next, the mixtures were incubated for 15 min at 37°C, and their optical densities at 600 nm were measured again. One enzyme unit was defined as the reciprocal of the highest dilution that caused a 50% decrease in absorbance after 15-min incubation at 37°C, compared with the control wells.

Bacterial growth and viability. Bacterial growth/viability was assessed by measuring optical densities at 600 nm with a microtiter plate reader or by a method involving the chemical reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) [27], and findings were confirmed by counting the colony-forming units in duplicate on BHI plates.

Electron microscopy. Next, 100 μL of MV-L (appropriate units for each experiment) or buffer was added to 100 μL of bacterial culture at the log phase. After incubation at 37°C for varying times, 2% glutaraldehyde (TAAB Laboratories Equipment) was added to stop the reaction. The cell pellet was coated with 1% agarose, rinsed with PBS that contained 5% sucrose, and postfixed with a 1.5% osmium tetraoxide/0.1 mol/L phosphate buffer/5% sucrose solution at 4°C for 1 h. After dehydration through an ethanol series, the fixative was substituted with propylene oxide, and the samples were embedded in epoxy resin (TAAB 812). Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and observed with an H7100 transmission electron microscope (Hitachi).

Mouse experiments. All mouse experiments were conducted with the approval of the Animal Experiment Committee of Kochi Medical School. Female BALB/c mice, 6–8 weeks old were fed with water that contained 200 μg/mL streptomycin for 24 h and inoculated in each nasal cavity with 2×10^9 85/2082-STR1 cells in 20 μL of sterile saline. Sixty hours after infection, they were randomly divided into 2 groups ($n = 9–10$) and were administered MV-L (310 U/40 μL) or the enzyme buffer intranasally. Six hours after treatment, the mice were killed, and their nasal cavities were aseptically dissected. They were processed basically as described elsewhere [28] and subjected to colony-forming unit titration on streptomycin-containing BHI plates. In another experiment, mice were inoculated intraperitoneally with 5×10^9 85/2082 cells, a preliminarily determined optimal dose with which to evaluate the efficacy of MV-L, and were randomly divided into 4 groups (5–10 mice each). Three groups received 500 U of MV-L via the same route at various intervals after challenge with bacteria, and the other group received the enzyme buffer. A mock-infected (saline) group was also given 500 U of MV-L. The survival rate was observed for all mouse groups for up to 60 days after infection.

To check the possible toxicity of MV-L itself, an additional

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3 groups of mice ($n = 5$) were injected with the enzyme buffer or with 1500 or 2000 U of MV-L and monitored for 1 year. Furthermore, 2000 U of MV-L was administrated into the peritoneal cavities 3 times at 3-day intervals ($n = 10$) and monitored for 2 weeks after the last injection.

Immune response to MV-L. MV-L (500 U) or the enzyme buffer was injected into the peritoneal cavities of mice 3 times at 10-day intervals. Serum was sampled 15 days after the last injection and checked for antibodies to MV-L by Western blotting and ELISA. A neutralization assay was performed by mixing 20 μL of MV-L solution (50 U) with 80 μL of MV-L–immunized or nonimmunized mouse serum, followed by incubation at 37°C for 15 min or 1 h. The mixture then was added to growing cultures (100 μL) of 85/2082 MRSA (1×10^7 cells) and further incubated at 37°C. An aliquot (5 μL) was taken from the cultures at various time points and subjected to colony-forming unit counting.

RESULTS

Identification and purification of ϕMR11-encoding lysin. Sequencing showed that the ϕMR11 phage has a 43,011-bp dsDNA genome [22] in which 67 putative ORFs were identified (data not shown). Of these ORFs, gene *65* was deduced, on the basis of a homology search, to encode a possible lysin (481 aa) of ϕMR11 phage. Gene *65* was cloned, expressed in *E. coli* as a tagged recombinant protein (Gp65-6×His), and affinity purified. SDS-PAGE analysis revealed that purified Gp65-6×His is a 54.8-kDa protein (figure 1*A*), as expected from the sequence data.

The staphylolytic activity of Gp65-6×His was examined using the epidemic MRSA strain, 85/2082 [24], as the representative test bacterium. Purified Gp65-6×His (5 μg) lysed the strain in 15 min as shown by cells that remained achromatic after the addition of TTC (figure 1*B, left wells*). By contrast, the buffer-treated control showed a color change to deep red over time, indicative of aggressive bacterial growth (figure 1*B, middle wells*). Thus, Gp65 proved to be the ϕMR11-encoded lysin (MV-L). No bacterial regrowth was observed in MV-L–treated cultures 48 h after its addition (data not shown). Complete lysis of the *S. aureus* strain was also confirmed by colony-forming unit counting (see below). The activity units of MV-L were determined using strain 85/2082 as the indicator bacterium. Approximately 0.1 μg of purified MV-L protein corresponds to 1 U of lytic activity. A BLAST search revealed that MV-L has 2 peptidoglycan hydrolase domains, homologous to CHAP and Ami-2 domains, which represent D-alanyl-glycyl endopeptidase and *N*-acetylmuramyl-L-alanyl amidase activity, respectively [29] (figure 1*C*).

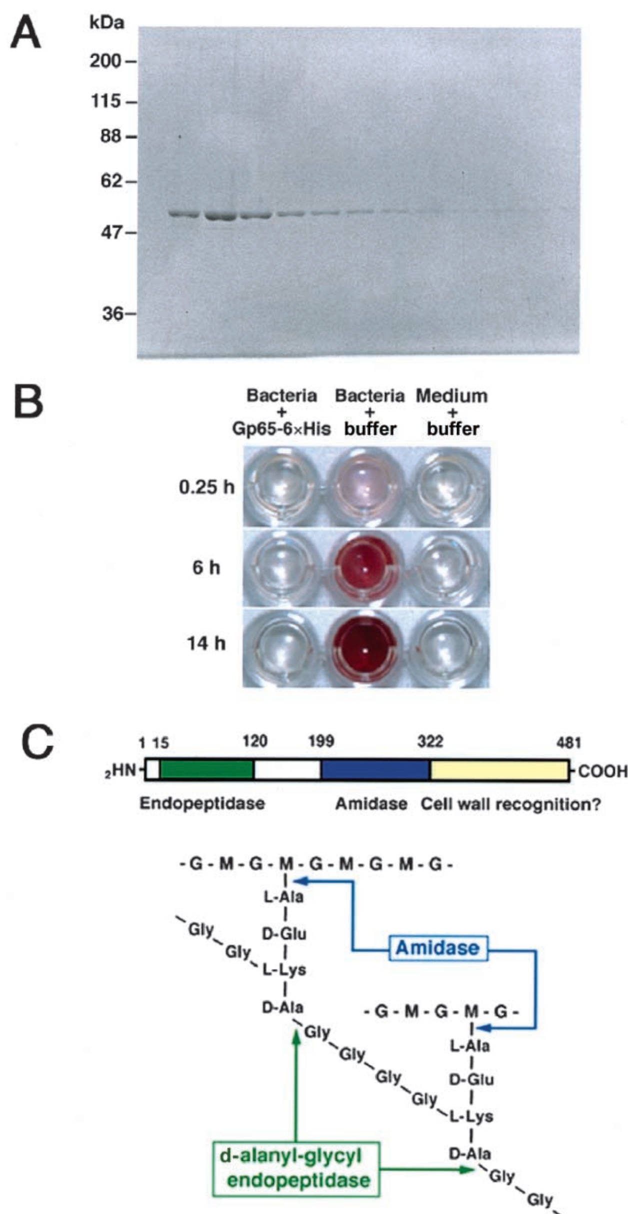

Figure 1. Characterization of the product of gene *65* of the ϕMR11 phage. *A,* Different fractions of purified recombinant protein (Gp65-6×His) analyzed by SDS-PAGE (10%) and stained with Coomassie brilliant blue R-250. *B,* Staphylolytic activity. After purified ϕMR11 lysin (MV-L) or buffer treatment, 2,3,5-triphenyl tetrazolium chloride was added to the medium to monitor bacterial growth. *C,* Schematic diagram of the putative functional domains of MV-L *(top)* and sites of predicted cleavage by MV-L in the staphylococcal peptidoglycan molecule *(bottom).* Amino acid coordinates are indicated above the limits *(vertical bars)* of each domain. The endopeptidase and amidase sites represent homologues of the known catalytic domains of CHAP and Ami-2, respectively.

Rapid and specific killing of* S. aureus *by MV-L. The bactericidal effect of MV-L was further investigated against a number of clinical MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates from different areas of Japan and other countries (table 1). PFGE analysis demonstrated that these MRSA strains are genetically different from each other (figure 2*D*). Purified MV-L (50 U) was added to liquid bacterial cultures

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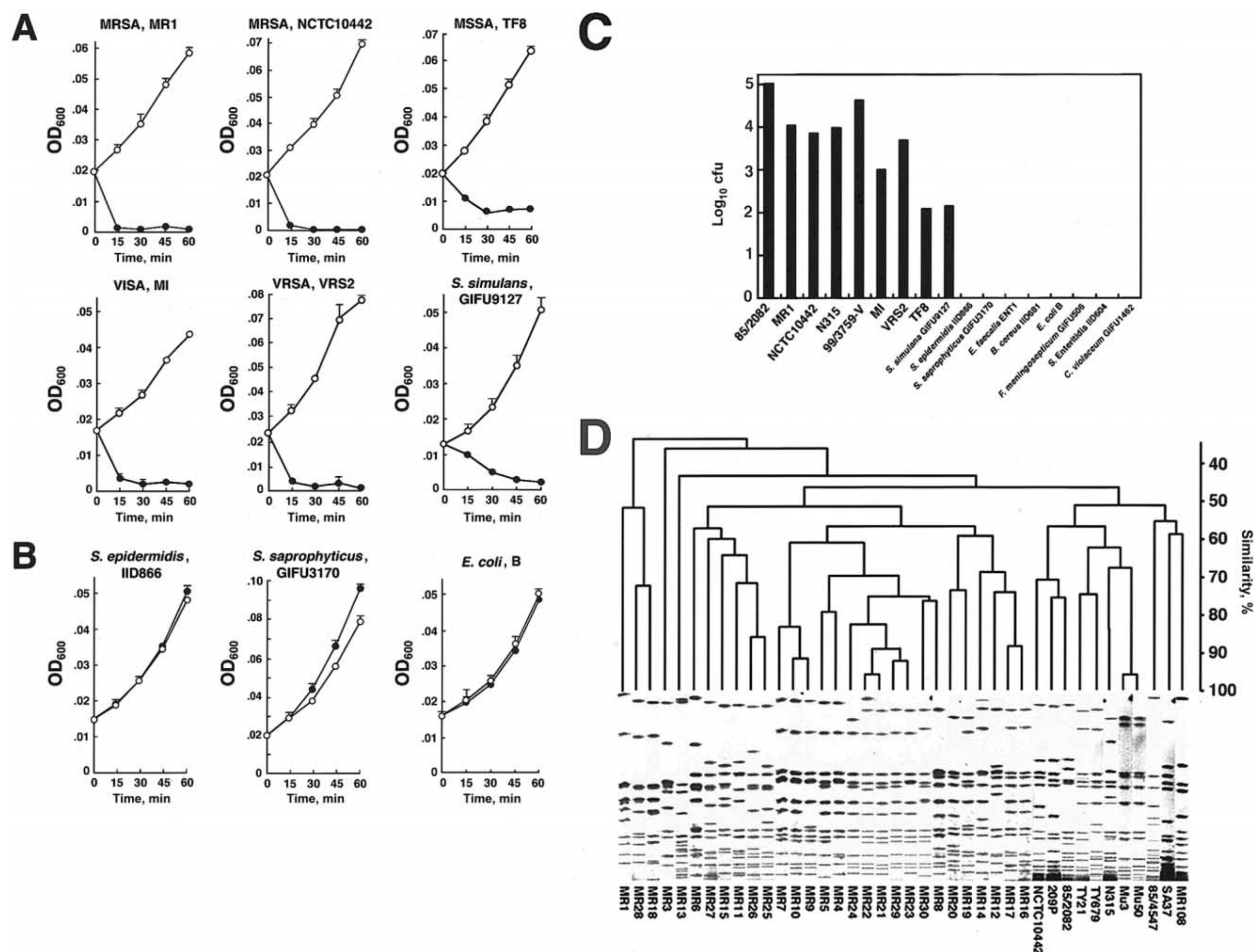

Figure 2. Spectrum of purified ϕMR11 lysin (MV-L)–mediated bacteriolysis. *A* and *B,* Time course of lytic activity of MV-L (50 U) against growing bacteria, shown by changes in optical density at 600 nm (OD_{600}). *Black circles,* MV-L–treated culture; *white circles,* buffer-treated culture. Each symbol with a bar represents the average ± SD of triplicate assays. *A,* Results for some strains of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-intermediate *S. aureus* (VISA), vancomycin-resistant *S. aureus* (VRSA), and methicillin-susceptible *S. aureus* (MSSA) carrying the endopeptidase resistance gene and *S. simulans* strain. *B,* Results for some bacterial species other than *S. aureus* (also see table 1). Assays were performed under growing liquid culture conditions. *C,* Viable counts. The decrease in colony-forming units by MV-L treatment was examined in representative strains shown in table 1. The results are shown as the no. of viable bacteria after buffer treatment divided by the no. of viable bacteria after MV-L treatment at 30 min, except for 85/2082, which was measured at 15 min. Averages of triplicate samples for each assay were used for calculations. *D,* Divergence of *S. aureus* strains used in the study. Dendogram of the different MSSA, MRSA, and VISA strains tested (table 1) based on pulsed-field gel electrophoresis pattern analysis.

($0.8–2.0 \times 10^7$ cells/0.1 mL) in the logarithmic growth phase, which mimicked the in vivo conditions of a serious infection. MV-L lysed all of the MRSA and MSSA strains examined, whereas the bacterial cultures exposed to the enzyme buffer alone continued to grow steadily (figure 2*A* and 2*C* and table 1). Complete killing of the bacteria was achieved within the initial 15 min of exposure in most cases (figure 2*A*). To assess the bacteriolytic spectrum of MV-L, the same assay was applied to bacterial species other than *S. aureus* (table 1). MV-L (50 U) lysed none of the strains of *S. epidermidis, S. saprophyticus,* or the other gram-positive or -negative species tested, with the exception of *S. simulans* (figure 2*B* and 2*C* and table 1). Interestingly, MV-L also destroyed TF8 (RN4220 [*epr*/pGC2]) (figure 2*A* and 2*C* and table 1), which resists both lysostaphin and Ale-1 [23]. Thus, MV-L might have the potential to kill all *S. aureus* strains, including MRSA and MSSA, without perturbing the commensal microflora.

To clarify the dynamics of the bactericidal effects of MV-L, the morphological changes in MRSA at the logarithmic growth phase were analyzed serially after treatment with the lysin. Electron microscopic observations revealed that cell lysis began within 10 s and was complete within 60 s of treatment with 50 U of MV-L (figure 3*A*). The enzymatic attack occurred simultaneously at multiple sites on the bacterial cell wall, and

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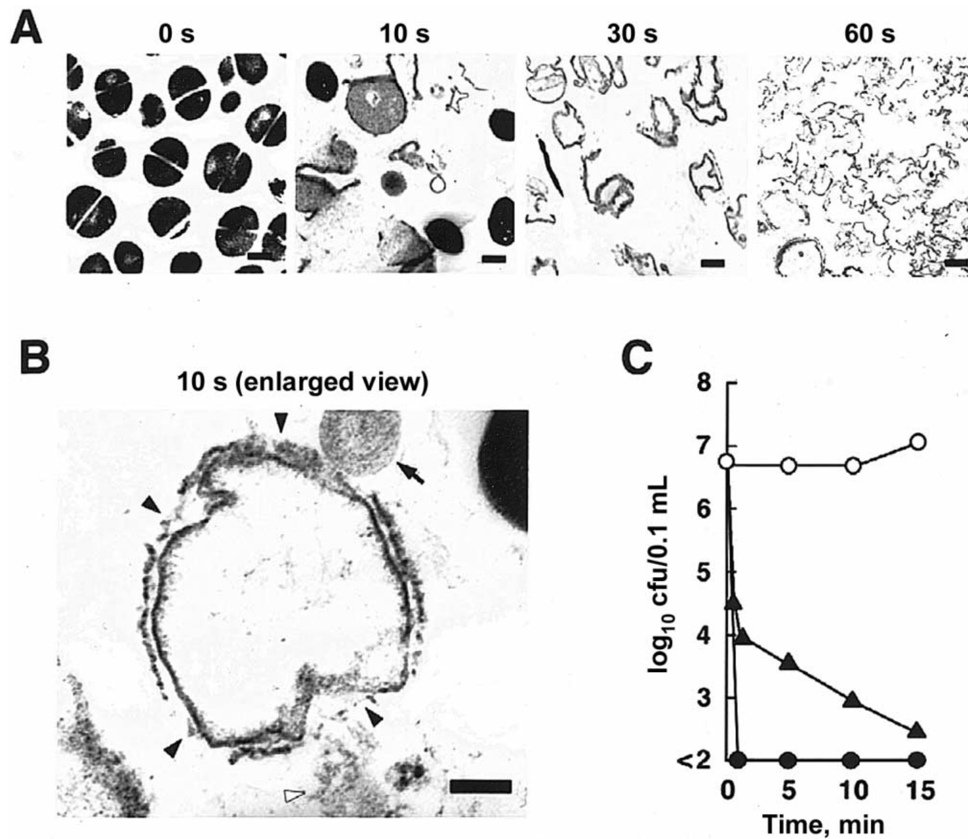

Figure 3. Morphological analysis of *Staphylococcus aureus* lysis by purified ϕMR11 lysin (MV-L). Data for 85/2082 methicillin-resistant *S. aureus* (MRSA) are representatively shown. *A,* Electron micrographs taken sequentially at the indicated time points after treatment with 50 U of MV-L. Scale bar, 500 nm. *B*, Enlarged image from an electron micrograph taken 10 s after the addition of 50 U of MV-L. The bacteriolytic process is reflected in the cell-wall degradation *(black arrowheads),* cell-membrane protrusion *(black arrow),* and cytoplasmic leakage through a cell membrane rupture *(white arrowhead).* Scale bar, 250 nm. *C*, MV-L dose-dependent killing of MRSA. Colony-forming unit counts in bacterial cultures treated with 25 U *(black triangles)* or 50 U *(black circles)* of MV-L are shown in comparison with those in the buffer-treated control culture *(white circles).* The average of duplicate samples for each assay is shown.

the resultant cell-membrane protrusions through the breaks in the cell wall were often observed 10 s after the addition of the lysin (figure 3*B*). After 30 s, most cells were destroyed, as indicated by their complete loss of cytoplasmic contents (figure 3*A*). Consistently, a follow-up of bacterial viability revealed a sharp dose-dependent decrease in colony-forming units: to 1×10^{-3} or 1×10^{-5} of the initial input 60 s after the addition of 25 or 50 U of MV-L, respectively (figure 3*C*).

Effects of MV-L on VRSA and VISA. Recently, VRSA and VISA strains [25] have been an increasing medical concern. Therefore, we examined MV-L activity against 2 VRSA strains, VRS1 and VRS2, and 18 VISA strains (table 1). The addition of MV-L (50 U) to growing cultures of VRS1, VRS2, and 7 VISA strains resulted in their immediate lysis (figure 2*A* and 2*C* and table 1), as was also seen in common MRSA strains. However, the other 11 VISA strains—for example, Mu50 (the thickest cell wall among VISA strains tested) [30]—were clearly not susceptible to 50 U of MV-L (table 1). Therefore, the subgroup of VISA that was slightly sensitive to the usual dose of MV-L was reexamined under different test conditions. When exponentially growing Mu50 cells were resuspended in saline (nongrowing conditions), cell lysis occurred dose dependently (6.25–200 U) (figure 4*A*). Mu3, the precursor strain of Mu50 and known as hetero-VISA [25], was rapidly lysed by 50 U of MV-L even under growing conditions (table 1).

Synergistic killing potential of a subgroup of VISA by MV-L and glycopeptides. We tested the joint action of MV-L and glycopeptide inhibitory to VISA strain Mu50. MV-L (50 U) or vancomycin (4 μg/mL) alone weakly suppressed the growth rate of Mu50 (figure 4*B*), but the simultaneous addition of 12.5–50 U of MV-L and 4 μg/mL vancomycin destroyed Mu50 cells (figure 4*B*) [31]. In an experiment similar to that shown in figure 4*B*, the reduction ratios in colony-forming units per milliliter after 2 h of growth for the MV-L, vancomycin, or combination treatment, compared with controls, were 1.2, 30, and 4200, respectively. According to the common synergy def-

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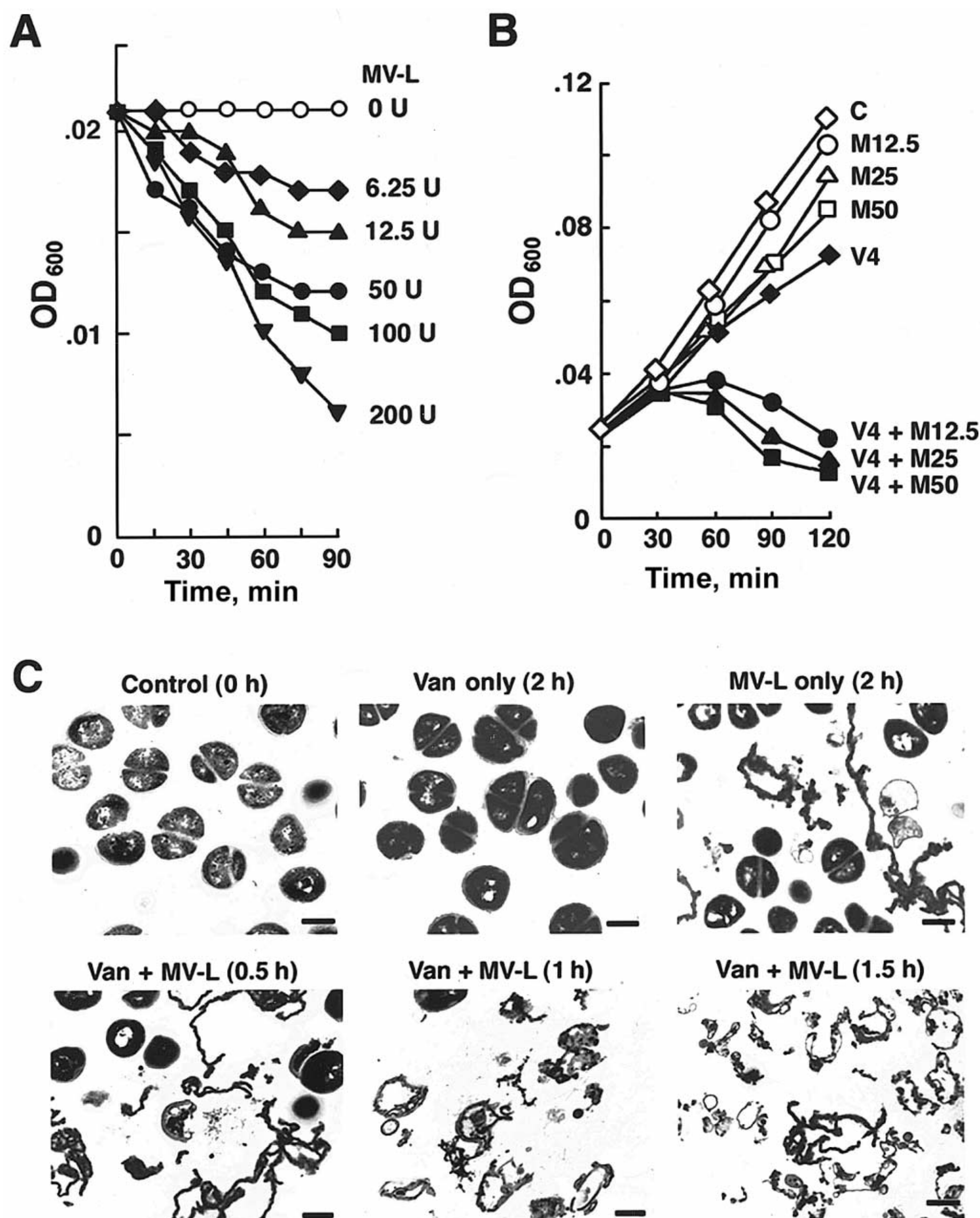

Figure 4. Bactericidal effects of purified ϕMR11 lysin (MV-L) on a subgroup of vancomycin (Van)–intermediate *Staphylococcus aureus* (VISA) strains less sensitive to MV-L. The results for the VISA strain Mu50 are representatively shown. *A,* Lysis of Mu50 cells by MV-L under nongrowing culture conditions (suspended in saline). The representative data among several similar results are shown. *B*, Synergistic effects of MV-L and glycopeptides. Bacteriolysis with the addition of varying doses of MV-L (12.5–50 U) was examined in the presence of a fixed concentration (4 μg/mL) of Van. The representative data among several similar results are shown. C, untreated control; M12.5, 12.5 U of MV-L; M25, 25 U of MV-L; M50, 50 U of MV-L; V4, 4 μg/mL of Van. *C,* Electron microscopic analysis. Morphology of Mu50 in the presence of 50 U of MV-L and/or 4 μg/mL Van was photographed at different time points indicated above each panel. Scale bar, 500 nm.

inition in broth culture, which predicts at least a 100-fold reduction in colony-forming units per milliliter for combined treatment, compared with the lowest counts when agents are used separately, the combination effect of MV-L and vancomycin was determined to be synergistic.

This synergy was also verified morphologically using electron microscopy (figure 4*C, bottom panels*). By contrast, treatment with 50 U of MV-L alone for 2 h lysed only a fraction of the Mu50 cells (figure 4*C, top right*), and lysis was negligible even 2 h after the application of 4 μg/mL vancomycin alone (figure 4*C, top center*). Similar results were obtained when teicoplanin was used instead of vancomycin (data not shown).

Elimination of MRSA by MV-L in animal models. *S. aureus* persistently or intermittently colonizes the nares of ~80%

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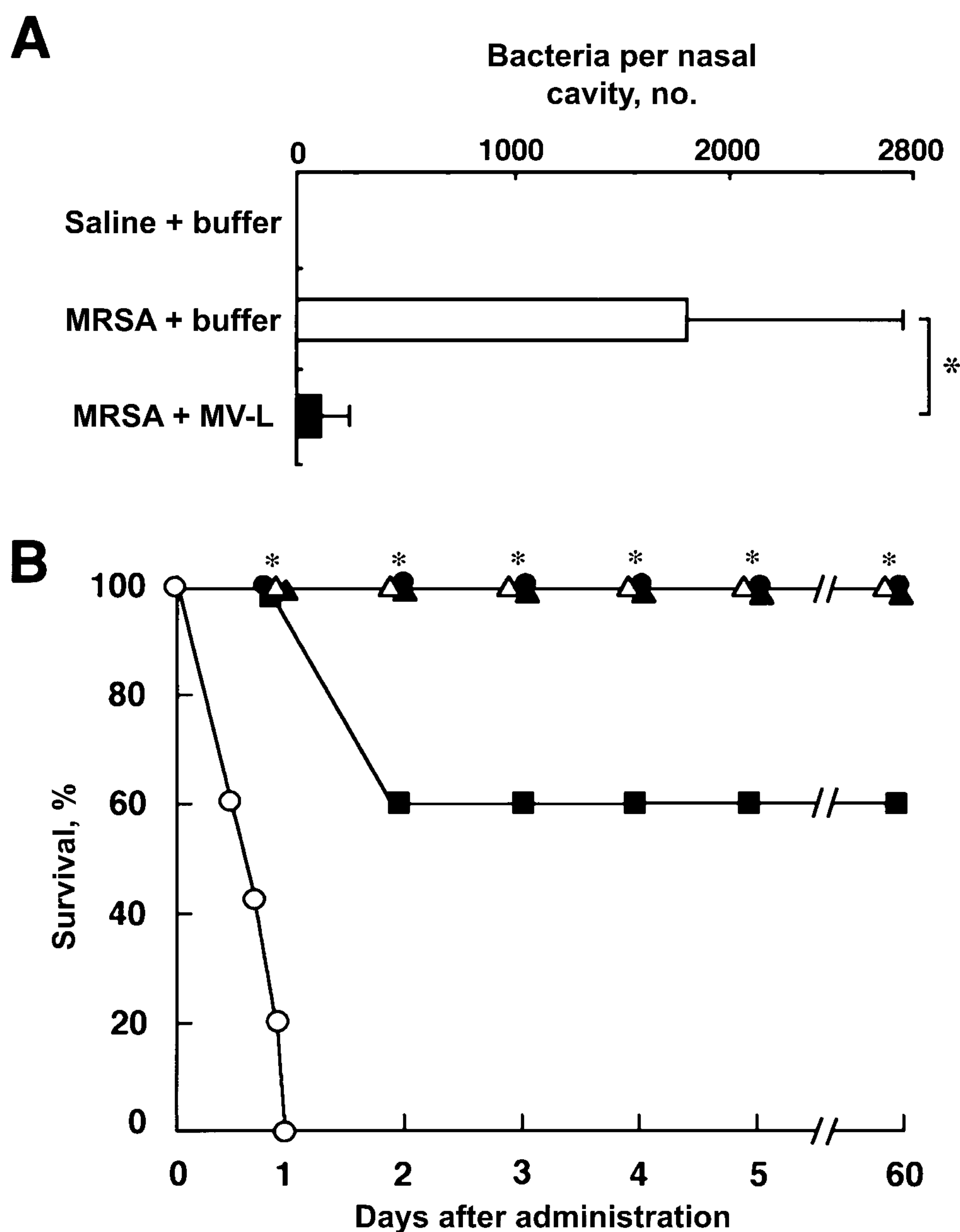

Figure 5. Anti–methicillin-resistant *Staphylococcus aureus* (MRSA) effects of purified ϕMR11 lysin (MV-L) in vivo. *A,* Elimination of nasally inoculated MRSA. The nasal cavities of mice were inoculated with an MRSA substrain 85/2082-STR1, and, after 60 h, either MV-L (310 U) or the enzyme buffer was administered intranasally to the mice ($n = 9$ and 10, respectively). Viable bacteria were then evaluated by colony-forming unit counting 6 h after administration of MV-L or the enzyme buffer. The saline group consisted of control mice not inoculated with 85/2082-STR1 ($n = 5$). The statistically significant difference between colony-forming units in mice treated with MV-L and untreated control mice was analyzed with the Mann-Whitney *U* test ($P < .01$). *B,* Curative effects on a model of systemic MRSA disease. MV-L (500 U) or buffer was administered into the peritoneal cavities of mice at the indicated time intervals after intraperitoneal challenge with 85/2082. MV-L was given at 0 min (*black circles,* $n = 10$), 30 min (*black triangles,* $n = 10$), or 60 min (*black squares,* $n = 5$) after infection. As controls, infected mice were treated with buffer (*white circles,* $n = 8$), and mock-infected mice were treated with 500 U of MV-L alone (*white triangles,* $n = 5$). The statistically significant difference between survival rates of mice treated with MV-L (0 and 30 min) and untreated control mice was analyzed with Fisher's exact test ($P < .01$).

of the general population [32] and is a major source of nosocomial infections [28]. We therefore assessed the clinical efficacy of MV-L against MRSA colonization and infection using mice models. Six hours after buffer or MV-L treatment, a number of bacterial colonies appeared in the samples of nasal tissues resected from the buffer-treated mouse group (average, 1818 cfu/nasal cavity), contrasting clearly with the fewer colonies grown from the nasal-tissue samples from the MV-L–treated

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group (average, 61 cfu/nasal cavity) (figure 5*A*). Complete elimination of the bacteria was achieved in 1 of 9 mice treated with MV-L.

An intraperitoneal injection of 85/2082 cells (5×10^9 cells/mouse) followed by the administration of enzyme buffer caused physical deterioration, including reduced activity and ruffled hair, within 1 h and septic death within 24 h of infection in 100% of mice (figure 5*B*). However, the administration of MV-L (500 U) into the peritoneal cavity 0 and 30 min after challenge with 85/2082 fully protected mice against lethality (figure 5*B*). A life-saving effect was observed when MV-L was administered 60 min after infection, although a few mice collapsed during the subsequent 6 days (figure 5*B*). Although 6.0×10^4 cfu/mL were detected in the blood at 30 min after the injection, immediate administration of MV-L (500 U) reduced colony-forming units to 8.0×10^3 cfu/mL (average, 5 mice). The single administration of excess doses (1500 and 2000 U) and the repeated administration (2000 U) of MV-L alone neither influenced the survival rate nor produced any adverse effects in mice.

Immunological and safety issues of MV-L. Finally, we tested whether MV-L elicited immune responses that could cause any therapeutically undesirable events. Western blotting detected antibodies reactive to MV-L in the serum of all 6 mice injected with the lysin but in the serum of none of the 6 buffer-injected mice (figure 6*A*). ELISA also demonstrated that MV-L–injected mice accumulated substantial levels of antibodies (average absorbance at 492 nm [A_{492}], 0.615) compared with those of the control mice (average A_{492}, 0.059), indicating the immunogenicity of MV-L (figure 6*B*). However, mice that were repeatedly given MV-L manifested no physical deterioration (data not shown), and the detected antibodies scarcely abrogated the MV-L activity (figure 6*C*).

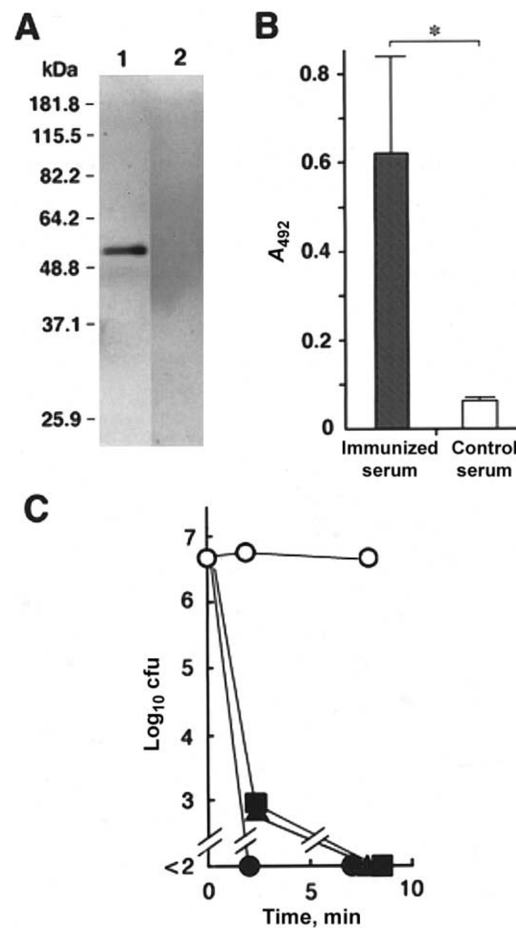

Figure 6. Immunogenicity of purified ϕMR11 lysin (MV-L). *A,* Humoral response. Serum antibodies to MV-L were examined by Western blotting in mouse groups repeatedly injected with a therapeutic dose (500 U) of MV-L or the enzyme buffer. The results for an MV-L–injected mouse *(lane 1)* and a control mouse *(lane 2)* are representatively shown. In each lane, 0.1 μg of purified MV-L protein was run. Serum dilution, 1:500. *B,* Levels of anti–MV-L antibody. Antibody titers for MV-L in mouse serum were determined by ELISA using 1000-fold diluted samples. Columns with bars represent means ± SDs of absorbance at 492 nm (A_{492}) values obtained from 6 serum samples from each mouse group. The statistically significant difference between serum immunized with MV-L and normal serum was analyzed with the Mann-Whitney *U* test ($P < .01$). *C,* Neutralization assay. MV-L solution was preincubated with MV-L–immunized mouse serum (positive for antibodies to MV-L) for 15 min *(black squares)* or for 1 h *(black triangles)* or with nonimmunized mouse serum for 15 min *(black circles).* As the assay control, buffer solution was preincubated with immunized serum for 15 min *(white circles).* Each mixture was then added to growing cultures of 85/2082 MRSA, and colony-forming units were counted at different time points during incubation, as indicated. The average of duplicate samples for each assay is shown.

DISCUSSION

In the present study, the full-length gene for a novel *S. aureus*–specific lysin, MV-L, which is encoded by the potent therapeutic phage ϕMR11 [22], was cloned and expressed, and the active recombinant enzyme was then successfully purified on a large scale. The usual dose of MV-L efficiently killed a wide array of *S. aureus* strains, including MRSA, VRSA, and a subgroup of VISA. Many groups have been unsuccessful in the purification of a staphylococcal phage lysin because of various unknown reasons.

Together with recent insightful research [17, 19, 20, 33], our results support the notion that a phage lysin might be relevant to therapies against *S. aureus* infectious diseases. MV-L–mediated killing is highly specific to *S. aureus* but not to other members of the indigenous bacterial flora. Unlike antibiotics, this "pinpoint attack" by MV-L is advantageous in the treatment of bacterial infections, because it might not cause an undesirable bacterial imbalance by disturbing microbial symbiosis.

The VRSA strains examined in the study were shown to be quite susceptible to MV-L. A group of VISA was highly sensitive to MV-L under growing conditions, but the other group was

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not (table 1). Importantly, we found that 50 U of MV-L acted synergistically with vancomycin or teicoplanin against such VISA strains with thickened cell walls. This effect was conceivably produced by the MV-L–mediated partial degradation of the thickened cell wall, thereby allowing the coexisting glycopeptide easy access to sites near the cell membrane.

Bacterial resistance is the most serious concern in antibacterial chemotherapy. In our experiments, no regrowth appeared from MV-L–treated MRSA cultures during longer observation periods. This finding, which is consistent with recent investigations by others [19, 20], implies that mutations associated with resistance to phage lysin are rare events. Phage lysin has an evolutionarily close relationship to the host lysin (also called autolysin) [34, 35]. It is likely that any bacterial changes made to evade an attack by phage lysin would also severely affect bacterial proliferation itself [20], and, even if mutants are resistant to the lysin, the bacteriolytic activity could be restored by domain interchange [36].

The nasal cavity is the major reservoir of *S. aureus,* including MRSA, in humans [32, 37]. Our study demonstrated that the topical use of MV-L efficiently eliminated MRSA and that systemic administration of MV-L also rescued mice from deadly MRSA infection. Importantly, intraperitoneal injection of MV-L alone, even at an excessive dose (2000 U), gave rise to no adverse effects in mice. Although repeated administration of MV-L elicited serum antibodies, those mice were physiologically intact, and, also important, immunized serum did not significantly inactivate MV-L. Thus, MV-L might be systemically administered with safety to assist the control and treatment of nosocomial infections of MRSA/VISA/VRSA in humans.

Attempts to utilize lysostaphin to treat *S. aureus* diseases [37–39] have been made. However, MV-L might be superior to lysostaphin for the following reasons. First, the lysostaphin gene is integrated into a plasmid in *S. simulans* biovar *staphylolyticus,* but the plasmid also carries the lysostaphin immunity factor *(lif)* gene [40, 41]. Similarly the expression of the endopeptidase resistance *(epr)* gene endows *S. aureus* with resistance not only to Ale-1 but also to lysostaphin [23]. If lysostaphin is frequently used, plasmids encoding *lif* and/or *epr* will be widely disseminated among *S. aureus,* reminiscent of the spread of plasmid-based antibiotic resistance. Interestingly, MV-L can efficiently kill *epr*-expressing *S. aureus* and probably also *lif*-expressing *S. aureus.* Second, unlike lysostaphin, MV-L does not kill *S. epidermidis.* Because *S. epidermidis* populates competitively with *S. aureus,* the coeradication of *S. epidermidis* by lysostaphin might cause an imbalance in the microflora and a predominance of lysostaphin-resistant *S. aureus* or other opportunistic pathogens. On the other hand, MV-L–mediated lysis specific to *S. aureus* (and also *S. simulans*), and not to *S. epidermidis,* might avoid such problems of lysostaphin. Also, the high specificity of MV-L suggests that it might also be useful as a laboratory diagnostic probe for *S. aureus* diseases, as has been suggested for other phage lysins [19, 20].

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