

## Prevention of diseases caused by *Staphylococcus aureus* using the peptide RIP

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### Abstract

*Staphylococcus aureus* causes many diseases including cellulitis, keratitis, osteomyelitis, septic arthritis and mastitis. The heptapeptide RIP has been shown to prevent cellulitis in mice, which was induced by *S. aureus* strain Smith diffuse. Here we show that RIP can also significantly reduce the overall pathology and delay the onset of disease symptoms in several other models of *S. aureus* infections, including: keratitis (tested in rabbits against *S. aureus* 8325–4), osteomyelitis (tested in rabbits against *S. aureus* MS), mastitis (tested in cows against *S. aureus* Newbould 305, AE-1, and environmental infections) and septic arthritis (tested in mice against *S. aureus* LS-1). These findings substantiate that RIP is not strain specific in its inhibitory activity and that RIP is an effective inhibitor of bacterial pathology at multiple body sites following diverse routes and doses of administration. These findings strongly evidence the potential value of RIP as a chemotherapeutic agent. © 2000 Elsevier Science Inc. All rights reserved.

### 1. Introduction

#### 1.1. Drug resistant *S. aureus* is a major medical problem

*S. aureus* is a major human pathogen and is the most common cause of nosocomial infections, increasing hospital death rate by 35%. *S. aureus* is the leading cause of nosocomial pneumonia, surgical site and bloodstream infections, as well as community-acquired infections such as osteomyelitis and septic arthritis, skin infections, endocarditis and meningitis. Currently, more than 95% of patients with *S. aureus* infections worldwide do not respond to first-line antibiotics such as penicillin or ampicillin. Methicillin-resistant strains of *S. aureus* (MRSA) were first reported in the

1960s, but have become increasingly prevalent with resistance in approximately 30% of all *S. aureus* infections [1,2,3]. The emergence and spread of drug-resistant staphylococci underscores the need to find new modes of prevention and alternative antibiotic treatment.

#### 1.2. *S. aureus* cause diseases by producing toxic exomolecules

Many of the diseases caused by *S. aureus* have been associated with the toxins the bacteria produce [4,5,6]. The toxins include toxic-shock syndrome toxin (TSST-1), enterotoxins, proteases and hemolysins [2]. A novel approach for therapy development would be to interfere with *S. aureus* virulence. Eliminating the production of toxins not only makes the bacteria far less pathogenic, but would also make the bacteria more susceptible to host immune defenses.

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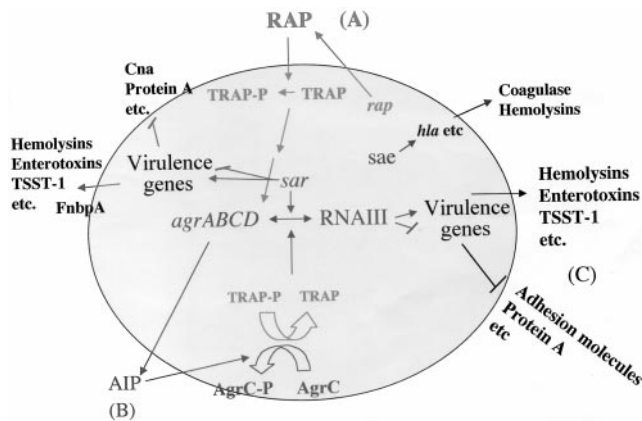


Fig. 1. Proposed regulatory pathways of virulence: As the colony multiplies, the autoinducer RAP accumulates and phosphorylates its target molecule TRAP (A), resulting in the production of RNAII. Once *agr* is activated (in the midexponential phase of growth), AIP and its receptor AgrC are produced. AIP downregulates TRAP phosphorylation and upregulates the phosphorylation of its receptor, AgrC (B), which is hypothesized to phosphorylate AgrA, which then acts as a transcription activator to activate P3, leading to the production of RNAIII (C). Production of RNAIII, in parallel with upregulation of *sar* and *sae*, causes the expression of toxic exomolecules and the suppression of adhesion molecules (C), resulting in dissemination and in disease. In the presence of RIP, RAP does not phosphorylate TRAP, RNAII and RNAIII are not produced, and RNAIII-activated proteins are not made. RAP; RNAIII activating protein. AIP; autoinducing peptide. TSST-1; toxic shock syndrome toxin. TRAP; target of RAP. Cna; collagen binding protein. FnbpA; fibronectin binding protein.

### 1.3. Regulation of toxin production

In culture, the bacteria produce toxins only when in higher densities, at the post exponential phase of growth. In the early exponential phase, when in lower densities, it expresses surface molecules such as fibronectin and fibrinogen binding-proteins that allow the bacteria to adhere to and colonize host cells [2]. The ability of the bacteria to switch between expression of surface adhesion molecules and toxin exomolecules [2] is highly regulated, and is hypothesized to enable the bacteria to adhere to host cells when in low numbers, but to disengage and spread when too crowded, thus allowing dissemination and establishment of the infection. *S. aureus* that cannot produce toxins do not survive long term in the host and is considered to be non-pathogenic [7].

The control of virulence in *S. aureus* is a complex process involving global regulatory systems such as *agr*, *sar* and *sae*, as well as quorum sensing mechanisms where proteins (RAP) or peptides (AIPs or RIP) produced by the bacteria autoregulate global regulatory systems, to activate or inhibit virulence.

The *agr* locus regulates at least 15 genes coding for potential virulence factors. *agr* mutants show a decreased synthesis of extracellular toxins and enzymes, such as  $\alpha$ -,  $\beta$ -, and  $\delta$ -hemolysin, leucocidin, lipase, hyaluronate lyase, and proteases, and at the same time an increased synthesis of adhesion molecules, coagulase and protein A [5]. *agr* is

partially under the influence of *sar*, an unlinked regulatory locus. *sar* mutants express increased levels of collagen binding protein, autolysins, protein A and V8 protease, and reduced levels of  $\alpha$ - and  $\beta$ - hemolysins, fibronectin binding protein, coagulase, TSST-1 and enterotoxin B [8]. A third regulatory system, *sae*, has been shown to upregulate the expression of  $\alpha$ - and  $\beta$ -hemolysins and coagulase and to slightly upregulate the expression of protein A [9]. The production of virulence factors has also been shown to be effected by environmental and growth conditions, such as sodium chloride or sucrose levels [10], growth rate [11] and microaerobic conditions [12].

Although *S. aureus* pathogenesis is regulated by multiple regulatory systems, interference with any one of these systems reduces virulence and disease symptoms (see for example [4,5,6]). Here we describe our studies aimed at interfering with *agr* function using the peptide RIP.

The *agr* locus contains two divergent transcription units, RNAII and RNAIII, driven by the promoters P2 and P3, both of which are active only from the midexponential phase of growth [13]. RNAII contains four open reading frames (ORFs): *agrA*, *agrB*, *agrC*, and *agrD*. The *agrA* and *agrC* genes encode a classic two-component signal transduction pathway composed of the AgrC signal receptor and the AgrA response regulator. The *agrD* gene product is a propeptide that is processed and secreted through AgrB. The resultant mature autoinducing peptide (AIP) [7] is the ligand that binds to and activates the phosphorylation of AgrC [14], which in turn is thought to phosphorylate AgrA, leading to upregulation of RNAIII synthesis. The RNAIII transcript acts as a regulatory RNA molecule and stimulates the transcription of genes encoding secreted toxins and enzymes, including *hla* ( $\alpha$ -toxin), *saeB* (enterotoxin B), *tst* (TSST 1), and *ssp* (serine protease), while repressing the transcription of genes encoding cell surface proteins, like *spa* (protein A) and *fnb* (fibronectin binding proteins) [13, 15].

### 1.4. Autoinduction of RNAIII synthesis

The synthesis of RNAIII is regulated by a quorum sensing mechanism [16]. Molecules produced and secreted by the bacteria (autoinducers) accumulate, and when they reach a threshold concentration, RNAIII is synthesized. The autoinducers of RNAIII that have been described to-date are the RNAIII activating protein (RAP) [17,18,19] and the *agr*-encoded cyclical autoinducing peptides (AIPs) containing a thiolactone structure [7,20,21]. RAP is a ~38kDa protein containing a putative NH<sub>2</sub>-terminal sequence IKKYPITN, and activates the synthesis of RNAII and RNAIII by phosphorylating TRAP [19]. The AIPs are encoded by the *agr*, and activate RNAIII by phosphorylating their receptor AgrC [14]. AIPs produced by some *S. aureus* strains inhibit the expression of *agr* in some other strains, and the amino acid sequences of peptide and receptor

(AgrC) are markedly different between such strains, indicating genetic hypervariability [21].

### 1.5. Novel therapy to *S. aureus* infections by suppressing RNAIII synthesis

Antibodies directed against RAP can inhibit RNAIII synthesis. Mice vaccinated with RAP are protected from a *S. aureus* infection, where protection level correlates with the titer of anti-RAP antibodies, suggesting that RAP is a promising vaccine candidate [18].

RNAIII synthesis can also be inhibited by AIPs of non-self [7,21], and by RNAIII inhibiting peptide (RIP) [17,18,19]. The RIP peptide is produced by coagulase negative staphylococcus (suggested to be *S. warnerii* or *S. xylosus*) [19,22] and has the sequence YSPXTNF, where X can be a cysteine, a tryptophan, or a modified amino acid. Both native RIP and a synthetic analog YSPWTNF are extremely effective in inhibiting RNAIII synthesis in vitro and in suppressing *S. aureus* infections in vivo [18,19]. The therapeutic potential of inhibiting RNAIII synthesis was confirmed by Mayville et al. [7], where it was demonstrated that peptides (AIPs) that inhibit RNAIII synthesis in vitro do in fact inhibit *S. aureus* infections in vivo. These peptides however, can only be used to prevent certain strains of *S. aureus* infections because while they inhibit toxin production in certain strains, they activate toxin production in other *S. aureus* strains [7].

### 1.6. The mode by which RIP inhibits RNAIII synthesis

Because of the sequence similarity between the NH<sub>2</sub>-terminal sequence of RAP and RIP (YKPITN as compared to YSPXTN) and because RIP has been shown to compete with RAP on the activation of RNAIII synthesis, it was hypothesized that RAP and RIP may bind to the same receptor, one as an agonist and the other as an antagonist. This hypothesis was supported by the fact that while RAP activates the phosphorylation of its target protein TRAP, RIP inhibits its phosphorylation, and because RNAIII gene of strains defective in TRAP production cannot be activated by RAP or inhibited by RIP (Balaban et al., unpublished). RIP derivatives were synthesized according to the putative NH<sub>2</sub>-terminal sequence of RAP. These peptides were tested for their ability to inhibit RNAIII synthesis in vitro and for their ability to prevent cellulitis in vivo. The results of these experiments indicate that the peptides most successful in inhibiting cellulitis were in fact the peptides that most resembled the NH<sub>2</sub>-terminal of RAP and contained the sequence YKPITN [19], further suggesting that RAP and RIP can bind to the same receptor, one as an agonist, the other as an antagonist.

It has been suggested that RIP is part of the AIP family of peptides [22]. While this may be the case, several experimental data do not support this hypothesis. 1. The AIPs must contain a thiolactone structure to be active [7] while

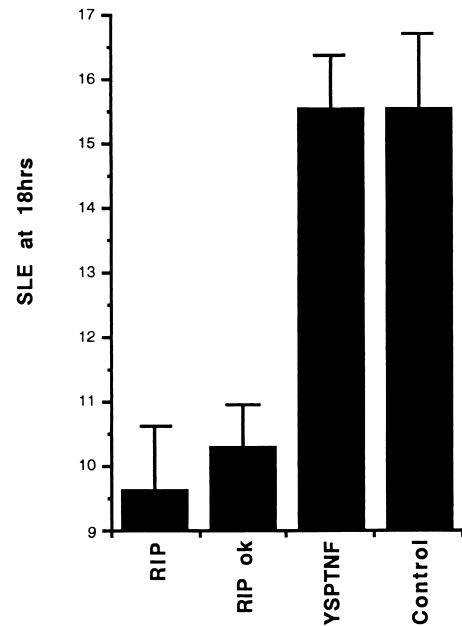


Fig. 2. The effect of RIP on *S. aureus* keratitis. Rabbits were intrastromally injected with 100 CFU of wild type *S. aureus* 8325–4 combined with 0.5% DMSO with or without one of the synthesized peptides (RIP, RIP ok, or YSPTNF). RIP (YSPWTNF) was synthesized by the Fmoc chemistry and was soluble in DMSO. “RIP ok” has the same sequence (YSPWTNF), was synthesized by the Fmoc chemistry and was further purified by reverse phase chromatography. The peptide YSPTNF (synthesized by the Fmoc chemistry) was used as a control peptide. Post challenge, rabbits were slit lamp examined (SLE). The SLE scores 18hrs post challenge are presented.

the RIP peptides are synthesized without a cysteine and a thiolactone structure and are active as linear peptides [18,19, current work]. 2. RIP and AIP use different signal transduction systems to activate or inhibit RNAIII synthesis [19]. 3. Unlike AIPs that inhibit RNAIII only in some strains of *S. aureus* while activating RNAIII in others, RIP is not strain specific in its inhibitory activity and can prevent infections caused by various strains of *S. aureus* [current work].

On the basis of these studies we hypothesize that auto-induction of *S. aureus* virulence occurs in a two step process (Fig. 1). In the first step (A), RAP accumulates in the culture media as the bacterial colony grows. RAP phosphorylates TRAP [19], leading to upregulation of the P2 *agr* promoter and to the production of RNAII (B). Once *agr* is activated (in the mid exponential phase of growth), the autoinducing octapeptide (AIP) and its receptor AgrC are produced [23]. In the second step (B) AIP causes the phosphorylation of AgrC [14], which is hypothesized to phosphorylate AgrA, which then acts as a transcription activator to activate P3 (C), leading to the production of RNAIII [23]. AIP also downregulates TRAP phosphorylation [19], thus enabling TRAP to be re-phosphorylated. Downstream, RNAIII, in parallel with upregulation of *sar* [24] and *sae* [9], induces the production of toxins and the suppression of adhesion molecules [2], resulting in dissemination and disease. In the presence of anti-RAP antibodies, RIP or inhibitory AIPs,

Table 1

Infection	Model	<i>S. aureus</i> strain	Animals (n)		% reduction in disease	<i>P</i>
			–RIP	+RIP		
Osteomyelitis	rabbit	MS	7	8	58	0.02
Sepsis	mouse	LS-1	10	11	44	0.04
Arthritis	mouse	LS-1	10	10	60	0.006
Keratitis	rabbit	8325-4	8	8	40	0.015
Mastitis	cow	Newbould/ AE-1	6	7	70-100	ND

ND, not determined

RNAIII is not produced, and the pathogenic potential of the bacteria is greatly reduced [7,18,19].

*S. aureus* is the most common cause of adults acute osteomyelitis [25,26] and of septic arthritis [27,28], is a frequent cause of contagious mastitis in dairy cows [29], and is the leading cause of bacterial infection of the human cornea [30]. The incidence and severity of these infections has been shown to be associated with toxin production. For example, *S. aureus* strains that were mutated within the *agr* locus were able to colonize the bone but caused a much lower incidence and severity of osteomyelitis [31]. The *agr* system has also been shown to be an important virulence determinant in the induction and progression of septic arthritis [5,32,33]. *S. aureus* producing toxins have been shown to have a higher pathogenic potential in *S. aureus* mastitis [34,35], and  $\alpha$ -toxin has been shown to be the major virulence factor responsible for corneal tissue damage [6,36,37].

Because the severity of infections have been shown to be correlated with the ability of the bacteria to produce toxins, and because RIP has been shown to be a suppressor of RNAIII synthesis and of toxin production and to be an effective suppressor of cellulitis [18,19], we tested whether RIP could also prevent other diseases caused by various strains of *S. aureus*. The present study demonstrates that RIP significantly reduced the overall pathology and delayed the onset of disease symptoms in several models of *S. aureus* infection including keratitis, osteomyelitis, mastitis, and septic arthritis. These findings, summarized in Table 1, strongly evidence the potential value of RIP as a chemotherapeutic agent.

## 2. Materials and methods

### 2.1. Peptide synthesis

The peptides YSPWTNF (RIP) and YSPTNF were synthesized by the Fmoc chemistry as described below. YSPWTNF was soluble in dimethyl sulfoxide (DMSO). YSPTNF was soluble both in water and in DMSO. The synthesis was performed with an automatic peptide synthesizer (PS3, Rainin-Protein Technologies, Emeryville, CA),

and all the procedures and programming followed the manufacturer's instructions as previously described [38]. The peptide was synthesized utilizing 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and high capacity (0.7–1.1 mmol/g) Knorr resin, Fmoc-2,4-dimethoxy-4'-(carboxymethoxy)-benzhydrylamine resin with 1% divinylbenzene crosslinker (100–200 mesh, Advanced Chemtech, Louisville, KY). The first amino acid was allowed to couple for two hours and the remainder amino acids (Advanced Chemtech, Louisville, KY) were coupled for twenty minutes at room temperature. The peptide was cleaved and deprotected by the addition of 90% trifluoroacetic acid (TFA), 5% 1,2-ethanediol, and 5% water solution to the resin. The resin was incubated at room temperature for fourteen hours and then washed several times with TFA. The peptide was extracted with cold ether. The peptide/TFA solution was reduced to a volume of 1.0 ml with nitrogen gas. After adding 25 ml ether, the peptide solution was mixed and incubated on dry ice for five minutes. Samples were then centrifuged at 1000 g for five minutes, the ether removed, and the extraction with ethyl acetate: ether (1.5:1 v/v) on dry ice was repeated three times. Finally 1.0 ml water and 25 ml ether were added to the peptide followed by another incubation on dry ice and centrifugation. The top layer was removed, the ether evaporated with nitrogen gas, and the peptide resuspended in water and dialyzed. Following dialysis, the peptide was lyophilized and stored at room temperature in a desiccator under vacuum. Proper molar ratios of the amino acids in the peptide were confirmed by amino acid analysis.

The peptide RIP ok (YSPWTNF) was synthesized by the Fmoc chemistry using FMOC-amino acyl Wang resin followed by reverse phase chromatography (Courtesy of Mary Booth) at the Molecular Biology Research Facility, William K. Warren Medical Research Institute, University of Oklahoma Health Science Center, Oklahoma City, OK. The peptide that was synthesized by the Fmoc chemistry was extensively dialyzed in water, evaporated, and resulted in a yellowish powder. The powder was resuspended in 30% acetic acid, extensively vortexed and sonicated, and was further diluted to a final concentration of 6% acetic acid and 0.083% TFA. Soluble material was applied to a C8 Dynamax 300A HPLC column (Rainin Inc, Woburn, MA) and

Table 2

Infection	Model	<i>S. aureus</i> strain	Peptide used	Amount injected $\mu\text{g}$ RIP/bacteria	% DMSO
Osteomyelitis	rabbit	MS	RIP	$40/1 \times 10^6$	2.0
Sepsis	mouse	LS-1	RIP	$216/1.2 \times 10^7$	3.0
Arthritis	mouse	LS-1	RIP	$88/8 \times 10^6$	3.0
Keratitis	rabbit	8325-4	RIP	$5/1 \times 10^2$	0.1
Keratitis	rabbit	8325-4	RIP ok	$5/1 \times 10^2$	0.1
Mastitis	cow	Newbould	RIP	$5/2 \times 10^2$	0.5
Mastitis	cow	AE-1	RIP	$150/1 \times 10^7$	3.0
Mastitis	cow	AE-1	RIPb	$500/1 \times 10^3$	3.0

eluted by the following gradient: 0–21% acetonitrile in 0.083% TFA for 6 min, 21–27% acetonitrile in 0.083% for 18 min, followed by 27–60% acetonitrile in 0.083% TFA. Peptide was eluted within 21–27% acetonitrile gradient. Resulting peptide was white and could be solubilized in water or in DMSO.

RIPb (YSPWTNF) was synthesized and purified as RIP ok by Genemed Synthesis, Inc., San Francisco, CA).

All peptides were solubilized in DMSO (approximately 20 mg/ml) and kept at  $-70^\circ\text{C}$  in aliquots.

## 2.2. Preparation of bacteria used for challenge

Unless stated otherwise, before challenge, bacteria were grown overnight at  $37^\circ\text{C}$  on sheep blood agar plates (Remel, Lenexa, Kansas) and resuspended in phosphate-buffered saline (PBS) or in saline to the desired concentration. RIP or DMSO was added, and the bacteria were further incubated for 30 min at room temperature. A summary of the type and number of bacteria used in each infection model and the amount of RIP or DMSO it was incubated with, is summarized in Table 2.

## 2.3. Bacteria used for challenge

*S. aureus* MS, which was isolated from a patient with osteomyelitis at the University of Texas Medical Branch, was used in the osteomyelitis model [39]. Arthritogenic *S. aureus* strain LS-1, which was originally isolated from a swollen mouse joint, was used in the sepsis arthritis model [40,43]. Strain 8325–4, a wild type *S. aureus*, is an  $\alpha$ -toxin-producing strain analyzed previously in the rabbit keratitis model [6,36]. *S. aureus* Strain Newbould 305, which produces contagious mastitis [41], was kindly provided by Dr. Bill Owens at Hills Farm, Louisiana State University, and used in the mastitis model. *S. aureus* “Gemada” AE-1 clone which was isolated from a naturally infected cow that was diagnosed with subclinical mastitis from within a rural herd (Travessão, Campos dos Goytacazes-RJ), was used in the environmental mastitis model.

## 2.4. Animals used in the infection models

New Zealand white female rabbits, eight to 12 weeks old and weighing 2.0 to 3.5 kg were used in the osteomyelitis and keratitis models. Female (NMRI) mice, 6–7 weeks old were used in the septic arthritis model. Mature lactating Holstein dairy cows were used in the mastitis models.

## 2.5. Induction of tibial osteomyelitis (rabbit osteomyelitis model)

A localized *S. aureus* osteomyelitis was surgically induced in the left lateral tibial metaphysis of according to Mader and Shirtliff [39]. Briefly, rabbits were anesthetized, an 18-gauge needle was inserted percutaneously through the lateral aspect of the left tibial metaphysis into the intramedullary cavity.  $150\mu\text{l}$  of 5% sodium morrhuate (Eli Lilly, Indianapolis, Indiana),  $100\mu\text{l}$  inoculum ( $10^6$  CFU MS *S. aureus* pre incubated for 30 min with 2% DMSO with or without  $40\mu\text{g}$  RIP), and  $200\mu\text{l}$  saline were injected. The infection was allowed to progress and the severity of osteomyelitis was determined radiographically at ten, twenty and thirty days following infection. Radiographic scores (0–4) were assigned as described [39].

## 2.6. Induction of sepsis or septic arthritis (murine septic arthritis model)

$1.2 \times 10^7$  CFU pre incubated with 3% DMSO with or without  $216\mu\text{g}$  RIP, were used for sepsis experiments and  $8 \times 10^6$  CFU pre incubated with 3% DMSO with or without  $88\mu\text{g}$  RIP were used for arthritis experiments. The addition of RIP and DMSO to strain LS-1 at these concentrations had no effect on bacterial viability counts. Bacteria that were pre incubated in RIP or in DMSO were injected into the tail vein as described [40]. Mice were observed daily for mortality and swelling of joints. Joint swelling was scored in a double blind fashion as described [40,42]. Each joint was given a score (1 point for mild swelling or erythema or both, 2 points for moderate swelling and erythema, and 3 points for marked swelling and erythema and possible ankylosis).

Each animal was given a total score, depending on how many joints were infected.

### 2.7. Induction of keratitis (rabbit keratitis model)

Wild type *S. aureus* 8325–4 ( $10^2$  CFU) grown to log phase in tryptic soy broth were pre incubated for 30 min at room temperature with 0.1% DMSO with or without  $5\mu\text{g}$  RIP or RIP ok (in a total volume of  $10\mu\text{l}$ ). Rabbits were challenged intrastromally as described [6,36]. Post challenge, rabbits were slit lamp examined (SLE) every six hours. Slit lamp examination involved two masked observers grading seven parameters. These parameters were scored on a scale of 0 (absent) to 4 (sever), including conjunctival injection, conjunctival chemosis, corneal infiltrate, corneal edema, fibrin in the anterior chamber, hypopyon, and iritis. The parameters were summed to give a final SLE score as described [6,36].

### 2.8. Induction of mastitis (cow mastitis model)

2.8.1. *Inoculum*: *S. aureus* Newbould 305 (25–200 CFU) pre incubated for 30 min with 0.5% DMSO with or without  $5\mu\text{g}$  RIP per quarter were used in the experimental mastitis model. AE-1 *S. aureus* cells were used at  $150\mu\text{g}$  RIP/ $10^7$  CFU per quarter or  $500\mu\text{g}$  RIP b/ $10^3$  CFU per quarter in the environmental mastitis experiments.

2.8.2. *Induction of S. aureus Newbould 305 mastitis*: Mature lactating Holstein dairy cows in their third lactation or greater were purchased from a local dairy producer. Before purchase, milk from individual udder quarters was sampled twice, 21 days apart and submitted for routine microbiology screening in the Mastitis Lab, School of Veterinary Medicine, University of California at Davis. Cows were enrolled in the study if both pre-enrollment samples were negative for *S. aureus* and the total bacterial count in the culture was 10 CFU or less. Cows were housed in a dry lot with a shade structure. Feed consisted of at least 50 lbs. per day of premium alfalfa hay and 15 lbs. of a lactating dairy cow grain mix. Cows were milked twice per day with automated milking equipment.

2.8.3. *Induction of infection*: Before challenge, the cow was milked, quarters dipped, and teat end was swabbed with 70% alcohol. 5 ml of the bacteria/RIP or bacteria/DMSO were injected into a mammary quarter through the teat end with a syringe fitted with a one-inch intra mammary canula.

2.8.4. *Sampling and testing for S. aureus*: Individual quarters were dipped, cleaned, dried and sampled before each morning's milking. Milk samples ( $10\mu\text{l}$ ) were swabbed onto sheep blood agar plates, and plates incubated overnight at  $37^\circ\text{C}$ . The presence of *S. aureus* was determined by a positive catalase reaction, hemolysis pattern, and a positive test for coagulase on rabbit plasma.

2.8.5. *Induction of S. aureus AE-1 mastitis or treatment of naturally occurring S. aureus infections*: Free stall-housed dairy cows of the Holstein breed from a dairy herd were selected for experimentation after a five-day serial in vitro culture screening for the absence or presence of *S. aureus* infection. Daily hand-milked animals were housed in an opened free-stall barn with poor sanitation conditions, to allow natural infections to occur. Calf suckling was permitted after milking. Once the first streams of milk were discarded, teat ends were disinfected, and quarter foremilk samples were aseptically collected into sterile tubes and cool-transported for bacteriologic analysis. All milk samples were incubated overnight at  $37^\circ\text{C}$  before plating  $10^{-3}$ – $10^{-8}$  dilutions on blood agar plates in triplicates. CFU were counted at 24 and 48h, respectively.

2.8.6. *Induction of infection*: AE-1 *S. aureus* cells bacteria were pre-incubated for 30 min at room temperature either with DMSO or RIP ( $150\mu\text{g}$  RIP/ $10^7$  bacteria or  $500\mu\text{g}$  RIP b/ $10^3$  bacteria). Immediately before injection via intracisternal inoculation into separate quarters, the experimental bacteria/RIP or the control bacteria/DMSO mixtures were diluted into 15 ml PBS as carrier. Experimentally infected animals were segregated into a culture positive management group. Treated animals were hand-milked once daily throughout the experiment. Milk samples for bacteriologic tests were collected up to 23 days. Clinical mastitis was determined by one or more of the following criteria: the presence of flakes or garget in the milk and/or its discoloration, inflammation of the udder as indicated by hardness, tenderness or elevated temperature, and/or calf rejection.

### 2.9. Statistical analysis

Data were analyzed using the Statistical Analysis System program for personal computers. For keratitis SLE scores, nonparametric one-way analysis of variance (Kruskal-Wallis test) and Wilcoxon's test were used for comparison among groups. All other analyses were carried out by unpaired *t* test or by  $\chi^2$  contingency table analysis followed by Fisher's exact post-hoc test. P values of  $< 0.05$  were considered significant.

## 3. Results

### 3.1. The effect of RIP on *S. aureus* osteomyelitis

One of the diseases caused by *S. aureus* is osteomyelitis [25,26]. While normal bone is highly resistant to infection, a very large organism inoculation, trauma leading to bone damage, or the presence of foreign bodies can lead to osteomyelitis. To test whether RIP can prevent *S. aureus* osteomyelitis, the rabbit osteomyelitis model was used [39]. In this model, injection of *S. aureus* into the tibia initially produces a contiguous focus osteomyelitis that closely mim-

Table 3  
The effect of RIP on *S. aureus* osteomyelitis (30 days post challenge)

Treatment	(n)	Bone destruction				(% animals)
		slight swelling	<10%	10-40%	~100%	
RIP	(8)	2	3	2	1	(12.5)
DMSO	(7)	0	1	1	5	(71.0)

ics acute-subacute osteomyelitis in humans. However, as the infection progresses, the development of macro necrosis is observed, consistent with chronic osteomyelitis in humans. *S. aureus* strain MS ( $10^6$  CFU) that were pre-incubated with  $40\mu\text{g}$  RIP or with 2% DMSO were injected into the left tibia of a rabbit. The infection was allowed to progress and the severity of osteomyelitis was determined radiographically up to thirty days following infection. Radiographic scores (0–4) were assigned by three blinded investigators, based upon the amount of bone architecture disruption (0 = none, 1 = slight swelling, 2 = <10%, 3 = 10–40% and 4 = >40% bone architecture disruption). Radiographs were repeated and compared to the pre-treatment radiographs to evaluate the extent of osteomyelitis. The individual radiographic scores of the rabbits in control and experimental groups 30 days post-infection are given in Table 3. As shown in Table 3, animals injected with bacteria + RIP (in DMSO) had a lower amount of bone destruction as compared to control animals injected with bacteria + DMSO (followed for a period of 30 days). Only one out of eight (12.5%) animals injected with bacteria + RIP showed extensive infection of almost 100% disruption of normal bone architecture. On the other hand, 71% (5/7) of the control animals demonstrated this level of destruction. The difference between these groups was significant ( $P = 0.02$ ). These results suggest that exposure of bacteria to RIP was highly effective in reducing the pathogenic potential of the bacteria and greatly reduced the severity of bone destruction.

### 3.2. The effect of RIP on *S. aureus* sepsis and septic arthritis

*S. aureus* is the most common cause not only of acute osteomyelitis but also of septic arthritis. The rapidly progressive and highly destructive joint disease is difficult to eradicate, with less than 50% of the infected patients recovering without joint damage. Infection introduced at the time of surgery is a significant complication of prosthetic joint implantation [44,45]. To test whether RIP can prevent septic arthritis, a murine septic arthritis model was used [40]. Septic arthritis was induced by injection of the arthritogenic *S. aureus* strain LS-1 into the tail vein of NMRI mice. This strain produces TSST-1 and can cause death if injected in high doses. Injection of  $8 \times 10^6$  bacteria typically results in arthritis and an injection of a higher bacterial load typically

Table 4A  
The effect of RIP on sepsis, using  $1.2 \times 10^7$  *S. aureus* LS-1

Treatment	(n)	Mortality		% mortality
		Day 4	Day 7	
LS-1 + RIP	(11)	0	4	36
LS-1 + DMSO	(10)	2	8	80

Table 4B  
The effect of RIP on septic arthritis, using  $8 \times 10^6$  *S. aureus* LS

Treatment	(n)	no disease (%)	mortality (%)	arthritic (index at 14 days)
LS-1 + DMSO	(10)	1 (10%)	2 (20%)	7 (12)

results in sepsis and death. As shown in Table 4A, RIP treatment of bacteria reduces the frequency and delays the onset of staphylococcal sepsis. While 80% of control animals died (8/10), only 36% of the animals (4/11) died upon the injection of bacteria together with RIP ( $P = 0.04$ ).

As shown in Table 4B, RIP protected animals from septic arthritis. When animals were injected with a lower number of bacteria ( $8 \times 10^6$ ), 70% (7/10) of the RIP-treated ones were free of the disease as compared to 10% (1/10) of control animals ( $P = 0.006$ ). Further more, none of the RIP-injected mice died as compared to 20% of the controls, and the remaining animals had a lower arthritic index than control animals (7 as compared to 12), suggesting that RIP protected the animals both from mortality and from septic arthritis. RIP not only reduced the frequency and severity of disease but also delayed its onset, which in a clinical setting might allow for effective supplemental intervention with other anti-bacterial agents.

### 3.3. The effect of RIP on *S. aureus* keratitis

*S. aureus* is the leading cause of bacterial infection of the human cornea and can result in the loss of visual acuity and blindness [30]. Alpha-toxin has been shown to be the major virulence factor responsible for corneal tissue damage in the rabbit model of keratitis, where bacterial mutants deficient in  $\alpha$ -toxin have been shown to cause significantly less corneal erosion, iritis, and inflammation than their wild type parent strains [6,36,37]. Because RIP inhibits alpha hemolysin production in vitro (Balaban and Booth, unpublished), we tested whether RIP could reduce corneal damage in the rabbit model of keratitis.

For these experiments, RIP, RIP ok, or YSPTNF were used. RIP (YSPWTNF) was synthesized by the Fmoc chemistry and was soluble in DMSO. "RIP ok" has the same sequence (YSPTNF), was synthesized by the Fmoc chemistry and was further purified by reverse phase chromatography. This peptide was soluble both in DMSO and in water. The peptide YSPTNF (missing tryptophan) was also synthesized by the Fmoc chemistry and was used as a control.

As shown in Fig. 2, rabbits injected with bacteria + YSPWTNF (RIP or RIP ok) ( $5\mu\text{g}/100$  bacteria) showed a distinct reduction in pathology at 18 h post infection ( $p = 0.015$ ). Corneas injected with 8325–4 together with same concentration of YSPWTNF or DMSO showed no reduction in pathology at this time. Both RIP and RIP ok prevented keratitis equally well, suggesting that the solubility of the peptide and the way it was prepared did not alter its effectiveness.

### 3.4. The effect of RIP on *S. aureus*-induced mastitis

*Staphylococcus aureus* is a frequent cause of contagious mastitis in dairy cows [29]. Pathologic changes induced by contagious intramammary infections (IMI) can cause necrosis of the mammary stromal and parenchymal tissue, facilitating establishment of foci of chronic infection. Such damage can be permanent, reducing milk-producing potential. A majority of *S. aureus* that cause IMI are resistant to a number of  $\beta$ -lactam antibiotics and to a wide range of other anti-microbial agents [46,47], emphasizing the need for alternative methods of prevention and treatment. To test if RIP can prevent or treat cow mastitis, experiments were carried out both in experimental settings and in rural settings. In the experimental setting, 25–200 CFU *S. aureus* Newbould strain 305 (which produces contagious mastitis if over 300 bacteria are injected) were pre-incubated with RIP or with DMSO, and were injected into separate quarters of the same cow. A total of 5 cows were used, where each cow was injected with bacteria/RIP in one quarter and bacteria/DMSO in another quarter. During the observation period, aseptic duplicate secretion samples were obtained to determine the presence of *S. aureus* in the udder. Milk samples ( $10\ \mu\text{l}$ ) were plated on blood agar plates and CFU counted the following day. As shown in Table 5A, after 5 days, protection rate varied from 70%–100%, where the least protected animal had 187 CFU in the quarter injected with bacteria+DMSO, and 57 CFU in the quarter injected with bacteria+RIP, i.e. 70% reduction in number of bacteria colonizing the udder. On average, while quarters of cows injected with bacteria + DMSO had 283 CFU during the 5 days post challenge, quarters of cows injected with bacteria + RIP had only 23 CFU, suggesting on average, an 81% reduction in number of bacteria colonizing the udder.

Experiments were carried out also with free-stall housed dairy cows to test whether RIP could prevent *S. aureus*-AE-1 strain induced mastitis or cure natural ongoing *S. aureus* infections. Two sources of RIP were used for these experiments. RIP (YSPWTNF) which was synthesized by the Fmoc chemistry and RIP b which has the same sequence but which was further purified by reverse phase chromatography (Genemed Synthesis, Inc., South San Francisco, CA.). *S. aureus* AE-1 cells pre-incubated with RIP or with DMSO were injected via intracisternal inoculation into separate quarters (one quarter per cow, a total of 3 cows), followed by two daily injections of  $150\ \mu\text{g}$  RIP in 15 ml

Table 5  
The effect of RIP on *S. aureus* mastitis

A. Experimental Newbould <i>S. aureus</i> mastitis				
Cow #	CFU in quarters infected with bacteria with			
	DMSO		RIP	
	Day 0	Day 5	Day 0	Day 5
7231	0	188	0	0
8446	0	119	0	2
8463	0	800	0	20
8227	0	122	0	36
5076*	0	187	0	57

B. Experimental AE-1 and environmental <i>S. aureus</i> mastitis:				
Cow #	Treatment	<i>S. aureus</i>	<i>S. aureus</i> infection (CFU)	
			Day 0	Day 23
1	RIP	AE-1	0	0
2	RIPb	AE-1	0	0
3	DMSO	AE-1	0	105
4	RIP	natural	114	0
5	RIP	natural	205	0
6	RIP	natural	1000	0

PBS. Animals were followed for 23 days. As shown in Table 5B, RIP can effectively prevent *S. aureus* AE-1 infection in a rural farm setting. This was demonstrated by fully protecting experimental quarters injected with  $1.5\text{--}10^3$  to  $1.5 \times 10^7$  CFU, a bacteria load that normally results in development (within 48 h) of clinical mastitis. The potential therapeutic values of RIP were next tested in naturally occurring *S. aureus* infections. Naturally infected quarters (one quarter per cow, a total of 3 cows) were injected via intracisternal inoculation with three daily injections of  $150\mu\text{g}$  RIP in 15 ml PBS. As shown in Table 5B, RIP effectively inhibited the occurrence of clinical mastitis in all RIP-treated quarters, and milk samples were free of bacteria for the duration of experiment (as observed after 23 days). Control, naturally infected udders that were not treated with RIP, showed no sign of decreased bacterial load and were treated with antibiotics a day after detection of infection (not shown).

## 4. Discussion

The present study demonstrates that RIP significantly reduced the overall pathology and delayed the onset of disease symptoms in several models of *S. aureus* infection. Such protective effects were observed in animal models of keratitis (tested in rabbits against *S. aureus* 8325–4), osteomyelitis (tested in rabbits against *S. aureus* MS), mastitis (tested in cows against *S. aureus* Newbould 305, AE-1, and environmental infections) and septic arthritis (tested in mice against *S. aureus* LS-1). These findings substantiate that RIP is not strain specific in its inhibitory activity and that RIP is an effective inhibitor of bacterial pathology at multiple body sites following diverse routes and doses of ad-

ministration. These findings strongly evidence the potential value of RIP as a chemotherapeutic agent. Both Gram-negative and Gram-positive bacterial virulence has been shown to be autoinduced by various signaling molecules. Inhibition of bacterial pathogenesis by interfering with the signal transduction of virulence is an exciting new approach to the control of bacterial infections [53].

The rationale for our experiments was as follows: The strain and the number of bacteria used in our studies were the ones commonly used in each infection model. The amount of RIP used in each infection model ranged between 6–600 nmoles, a dose shown to be effective in previous *in vivo* studies [18]. DMSO was used as a carrier buffer because all RIP preparations were soluble in DMSO. The amount of DMSO never exceeded 3%, which is the concentration range shown not to have any effect on *S. aureus in vitro* (Collins et al., unpublished). Bacteria were pre incubated with RIP or with carrier buffer as a control, to mimic the application of RIP to devices (such as catheters or sutures) or during procedures (such as surgical procedures) normally associated with *S. aureus* infections.

*S. aureus* infections, often involving methicillin resistant strains, have been associated with the use of tampons, catheters, prosthetic valves or joints, and with trauma such as surgery [2,49,50,51]. Comprehensive measures of prevention, including the usage of local and systemic antibiotics and operation management, have been implemented successfully in Japan [52], but is limited by antibiotic resistance, suggesting the need for effective preventive measures. As our studies show, when *S. aureus* is pre-incubated in the presence of RIP prior to challenge, the pathogenic potential of the bacteria is greatly reduced. Our results suggest that RIP could be used as a preventive measure, and could for example be used in local application at the site of surgery or for coating devices known to be associated with *S. aureus* infections.

The effectiveness of RIP therapy as observed in the animal models of infection described here was obtained using empirically selected RIP concentrations and dosing schedules. Further experimentation using more ideal doses and dose schedules could produce even more impressive results. Further experiments will also need to be carried out using RIP derivatives shown to inhibit cellulitis [19]. Moreover, drug delivery devices capable of providing higher drug concentrations in tissue and sustaining drug delivery for longer intervals per application could enhance the beneficial effects of RIP therapy. Drug delivery devices have been shown to augment antibiotic therapy in models of *S. aureus* keratitis [48] and osteomyelitis [25] and are currently used in medical practice. In ophthalmology, collagen shields shaped-like contact lenses have been used to deliver antibiotics (e.g. tobramycin or ciprofloxacin) to infected corneas. In orthopedics, implant materials, such as polymethylmethacrylate impregnated with antibiotics, have long been used to manage the dead space created by debridement surgery for patients suffering from osteomyelitis.

The RIP peptide was tested following preparation by two methods and peptide in each form was found to be effective in suppressing RNAPIII synthesis *in vitro* and suppressed *S. aureus in vivo*. Similar positive results were obtained for those RIP samples prepared by the Fmoc system (solubilized in DMSO) and for those that were prepared in a similar fashion then further purified by HPLC (solubilized in water). The material prepared by the Fmoc system and solubilized in DMSO has been demonstrated to be more stable (i.e. longer shelf life) than the more purified peptide which was water-soluble. The water soluble form of RIP could have a greater direct penetration of biologic fluids while the DMSO preparations could release RIP to aqueous fluids in a sustained fashion. Comparisons of the two preparations are needed to determine if the drug's uptake from each formulation differs.

It is not yet known how a temporary exposure to RIP causes the bacteria to become non-pathogenic, as RIP has not been demonstrated to be cytotoxic *in vitro*. Furthermore, RIP causes inhibition of RNAPIII synthesis, and as a consequence an increase in expression of adhesion molecules. In theory, expression of adhesion molecules should increase bacterial colonization and therefore increase rather than decrease pathogenesis. It is known, however, that the pathogenic potential of an *agr* null strain is diminished. This strain is deficient in the production of toxins but can over-express adhesion molecules [7]. We therefore hypothesize that an equilibrium exists between the host and the bacteria. If the bacteria's ability to produce toxins is blocked even temporarily (by RIP), equilibrium is disrupted to benefit the host.

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