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RNAIII inhibiting peptide (RIP), a global inhibitor of *Staphylococcus aureus* pathogenesis: structure and function analysis

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Abstract

Staphylococcus aureus are gram-positive bacteria that can cause serious diseases in humans and animals. *S. aureus* infections can be prevented by the heptapeptide RNAIII inhibiting peptide (RIP). RIP was originally isolated from culture supernatants of coagulase negative staphylococci presumed to be *S. xylosus*. The sequence of RIP was identified as YSPXTNF. Native RIP and its synthetic analogue YSPWTNF have been shown to be effective inhibitors of diseases caused by various strains of *S. aureus*, including, cellulitis, keratitis, septic arthritis, osteomylitis and mastitis. RIP is therefore considered to be a global inhibitor of *S. aureus*. We show here that: 1) the amide form of RIP (YSPWTNF-NH2) is highly stable and is therefore the one recommended for use. 2) RIP inhibits *S. aureus* pathogenesis by inhibiting the synthesis of both *agr* transcripts RNAII and RNAIII. 3) Although RIP inhibits *agr*, it also reduces bacterial adherence to mammalian cells and to plastic (tested on HEp2 cells and on polystyrene by fluorescence and atomic force microscopy), suggesting that RIP can be used safely as a therapeutic molecule. 4) RIP derivatives were designed and tested for their ability to inhibit RNAIII *in vitro* and cellulitis *in vivo*. Not all peptides that inhibited RNAIII also inhibited an infection *in vivo*, indicating that studies must be carried out *in vivo* before considering a peptide to be of therapeutic potential. 5) The RIP derivative containing Lysine and Isoleucine at positions 2 and 4, respectively, inhibited *S. aureus* infections *in vivo* (tested on cellulitis), suggesting that both RIP YSPWTNF and its derivative YKPITNF are effective inhibitors of infections caused by *S. aureus*.

Keywords: *Staphylococcus aureus*; Bacterial virulence; Quorum sensing; Synthetic peptide inhibitors; Atomic force microscopy; Adhesion; RIP; RAP; *agr*

1. Introduction

1.1. Regulation of S. aureus *virulence*

S. aureus is one of the major pathogenic bacteria in humans and animals, and is one of the most common causes of hospital-acquired lethal infection [17]. During its proliferation, the bacteria produce a repertoire of different toxins that can cause life-threatening diseases [17]. Moreover, this bacterium can proliferate in foodstuff and secrete the same toxins, thus causing food poisoning [7]. Regulation of toxin production is a key feature in *S. aureus* pathogenesis.

S. aureus express surface molecules such as fibronectin binding-proteins, fibrinogen binding-protein and protein A, in the early exponential phase of growth, when the bacteria are in lower density [17]. Expression of adhesion molecules allows the bacteria to adhere to and colonize host cells. When in higher densities, like in the postexponential phase of growth, the bacteria no longer produce adhesion molecules and instead, they produce toxic exomolecules such as Toxic Shock Syndrome Toxin-1 (TSST-1), enterotoxins, proteases and hemolysins, that allow the bacteria to survive, disseminate and establish the infection [17]. Mutant bacteria that do not produce toxins do not cause disease and are considered to be non-pathogenic.

The ability of the bacteria to express adhesion molecules when in lower densities and instead, to express toxic exomolecules when in higher densities, is due to a complex regulatory process, which involves quorum sensing (QS) mechanisms and activation of genetic loci such as *agr*

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[1,5,19,21,23,24], *sar* [9–11,25,27], *sae* [12] and *trap* [3]. These processes act in parallel or in concert to regulate virulence. Our studies are aimed at interfering with *trap* and *agr* function using the peptide RNAIII Inhibiting Peptide (RIP) [2,4,6,8].

1.2. Regulation of S. aureus *virulence by quorum sensing mechanisms*

As the cells multiply, signaling molecules (proteins and peptides) are secreted and when a critical level of these molecules is obtained, a signaling pathway is activated, leading to the induction of genes encoding toxins [14]. Staphylococcal Quorum Sensing mechanisms will be referred herein as SQS. To date, two quorum sensing systems have been described in staphylococci [3], which will be referred herein as SQS 1 and SQS 2.

SQS 1 consists of the autoinducer RNAIII-activating protein (RAP) and its target molecule TRAP [3]. RAP is a ~38 kDa protein containing the NH2 terminal sequence YKPITN [8] that activates toxin production via its sensor TRAP, a membrane-associated ~21 kDa protein. As the cells multiply and the colony grows, the cells secrete RAP. When RAP reaches a threshold concentration, it induces the histidine phosphorylation of its target molecule TRAP. In the absence of an intact *trap* gene, *agr* is not activated by RAP [3]. TRAP does not have a transmembrane domain, but immuno-electron microscopy studies indicate that it is membrane-associated, suggesting that TRAP is bound to the membrane via another molecule (Balaban, unpublished). The *trap* gene is constitutively transcribed, and the transcript is monocistronic. Its transcription is controlled by a sigma A-dependent promoter and a putative rho-independent terminator (Ilya Borokov, personal communication). TRAP seems to be unique to staphylococci and is highly conserved among staphylococcal strains and species.

The phosphorylation of TRAP leads, in a yet unknown mechanism, to the activation of the gene regulatory system *agr*, which encodes the second quorum sensing system, SQS 2. Activation of SQS 2 leads to the production of toxins (see below).

SQS 2 is composed of the products of the *agr* system. *agr* is a chromosomal locus that is active from the midexponential phase of growth and encodes two divergently transcribed transcripts, RNAII and RNAIII [23,24]. RNAII is a polycistronic transcript that encodes AgrA, AgrC, AgrD and AgrB, where AgrD is a pro-peptide that yields an octapeptide pheromone (AIP) that is hypothesized to be processed and secreted with the aid of AgrB [19]. AIP contains a thiolactone structure that is proposed to be necessary for its activity [19], although other laboratories have produced AIPs that are active also as linear peptides (Hume, personal communication). AgrC and AgrA are part of a bacterial two-component system, AgrC being the sensor of AIP [16], and AgrA a regulator [20]. Once *agr* is activated in the midexponential phase of growth and AIP is secreted, it induces the phosphorylation of AgrC [16], leading to the production of RNAIII [20]. RNAIII has two main functions. RNAIII is a polycistronic transcript that has four potential open reading frames, one of which encodes δ hemolysin [5]. RNAIII is also a regulatory RNA molecule that upregulates the production of numerous secreted toxins, including enterotoxins B, C and D (SEB, SEC and SED), α and β hemolysins, and TSST-1, while repressing the transcription of genes encoding cell surface proteins, including genes that encode protein A and fibronectin binding proteins [1,24].

Members of SQS 1 and SQS 2 interact with one another and may therefore be part of a more complex phosphorelay system [13], where once the *agr* system is activated and the AIP is produced, AIP indirectly down regulates the phosphorylation of TRAP [3]. TRAP is hypothesized, then, to be available for interacting with another RAP molecule, leading again to the activation of *agr* and to the production of toxins (Fig. 1A).

1.3. Inhibition of S. aureus *virulence by RIP*

S. aureus virulence can be inhibited by the heptapeptide RNAIII-inhibiting peptide (RIP). RIP inhibits *S. aureus* from producing toxins by interfering with the known function of SQS 1. RIP competes with RAP on inducing TRAP phosphorylation, thus leading to inhibition of the phosphorylation of TRAP [3]. This leads to inhibition of RNAIII synthesis and to suppression of the virulence phenotype (Fig. 1B). The peptide RIP was first isolated from culture supernatants of coagulase negative staphylococci that were identified with 99% certainty to be *S. xylosus* [8]. The sequence of RIP was identified as YSPXTNF, where X can be a Cys, a Trp, or a modified amino acid [4]. Synthetic RIP analogues were designed as YSPWTNF and shown to be extremely effective in inhibiting RNAIII *in vitro* and in suppressing *S. aureus* infections *in vivo*, including: cellulitis (tested in mice against *S. aureus* Smith Diffuse), septic arthritis (tested in mice against *S. aureus* LS-1), keratitis (tested in rabbits against *S. aureus* 8325-4), osteomyelitis (tested in rabbits against *S. aureus* MS), and mastitis (tested in cows against *S. aureus* Newbould 305, AE-1, and environmental infections) [2]. These findings indicate that RIP YSPWTNF can suppress virulence of any strain of *S. aureus* so far tested, which is not surprising, considering the fact that the signaling molecule it interacts with (TRAP) is highly conserved among strains. Worth noting, this is in contrast to the situation with AIPs that activate RNAIII of self but inhibits RNAIII of other strains because of hypervariability in both peptide and receptor [19].

RIP shows sequence similarity to the NH2-terminal sequence of RAP (YSPXTNF versus YKPITN respectively), suggesting that both molecules may bind to the same receptor, one as an agonist (RAP) the other as an antagonist (RIP). In support of this hypothesis is the fact that both

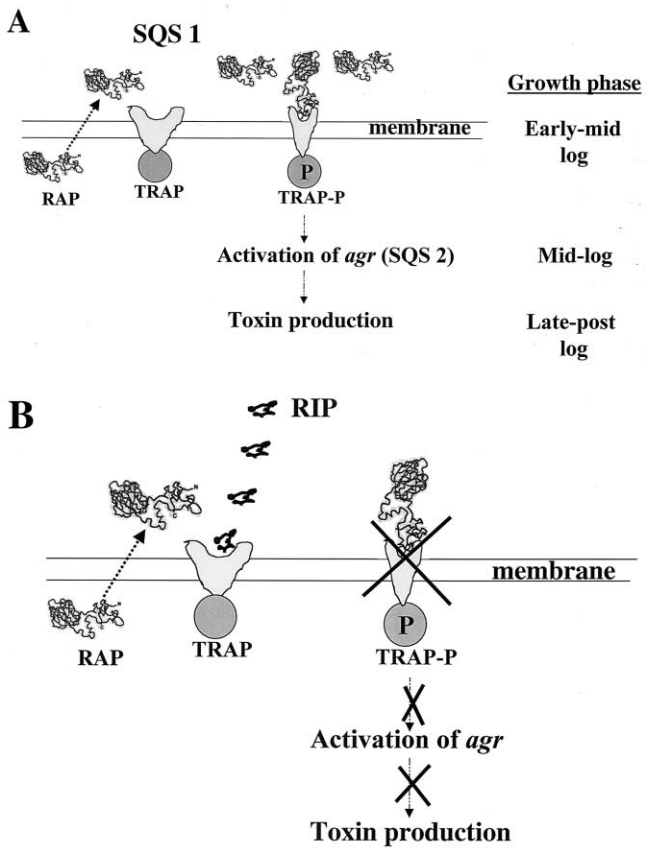

Fig. 1. Activation (A) and inhibition (B) of toxin production: proposed mechanism: As the cells multiply, RAP accumulates in the supernatant, binds to its receptor, and induces the phosphorylation of TRAP (all components of SQS 1). *agr* is then activated (at the mid exponential phase of growth) and RNAII is produced and the components of SQS 2 are made (AgrA-D). This leads to the production of RNAIII, which upregulates the production of toxins at the postexponential phase of growth (A). In the presence of RIP, TRAP is not phosphorylated, *agr* is not activated and toxins are not produced (B).

regulate TRAP phosphorylation and by the fact that RIP derivatives designed according to the NH2 terminal sequence of RAP, are most efficient inhibitors of *S. aureus* infections [8].

To further study the structure and function of RIP, peptide derivatives were designed and tested for their ability to repress *agr* function. We show here that while various RIP derivatives inhibit RNAIII *in vitro*, not all inhibited infections *in vivo*. The peptides that were most effective in suppressing *S. aureus* also *in vivo* are RIP peptides that contain Lys and Ile at positions 2 and 4, respectively. We also show here that RIP inhibits the production of both *agr* transcripts RNAII and RNAIII. Interestingly, in addition to inhibiting *agr* and there for toxin production, RIP reduces the adhesion of *S. aureus* both to mammalian cells and to plastic surfaces. These results further confirm the potential value of RIP and its derivatives as therapeutic agents for the suppression of diseases caused by *S. aureus*. Because RIP inhibited all strains of *S. aureus* so far tested [2,4], RIP is suggested to be a global inhibitor of *S. aureus* pathogenesis.

2. Methods

2.1. Cells

Bacteria: Wild type *S. aureus* strain RN6390B (ATCC 55620) and *S. aureus* strain Smith Diffuse [4]. *S. aureus agr*-null mutant strain RN6911 [24]. RIP-producing, coagulase negative staphylococcus strain RN833 (ATCC 55619) [4,6,8]. Bacteria were grown at 37°C with shaking in CY broth supplemented with β-glycerophosphate (1% case amino acid, 1% yeast extract, 100 mM NaCl, 100 mM sodium glycophosphate, and 0.5% glucose, pH 7.4) [22].

Mammalian cells: Human epithelial HEp2 cells were grown at 37°C in a 5% CO2 humidified incubator in bicarbonate-buffered Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal calf serum (FCS).

2.2. Synthesis of RIP and derivatives

RIP peptides and derivatives (YSPWTNF, YKPITNF, YSPITNF, YKPWTNF, PWTNF, PITNF and YKPITN) were synthesized commercially, essentially as described [2] by the Fmoc chemistry using Knorr resin, resulting in an amide form of the peptides (UC Davis core facilities, CA, and Neosystem, Strasbourg). Alternatively, peptides (YSPWTNF, YSPCTNF and YSPCTNFF) were synthesized by the Fmoc chemistry using FMOC-amino acyl Wang resin, resulting in carboxyl form of the peptides (LSUMC, New Orleans, LA). Routinely, proper molar ratios of the amino acids in the peptides were confirmed by amino acid analysis and by mass spectrometry. Purity of all peptides was confirmed by HPLC trace. Single letter abbreviations for the amino acid residues are: C, Cys; F, Phe; I, Ile; K, Lys; N, Asn; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr.

2.3. Preparation of native RIP and RAP

Preparation of RIP and RAP and testing for activity was carried out essentially as described [3]. Briefly, wild type *S. aureus* RN6390B cells producing RAP, coagulase negative staphylococcus RN833 producing RIP, and *agr*-null strain RN6911 (that were used to prepare control media), were grown to the postexponential phase of growth. Growth culture was centrifuged at 6,000 $\times$ *g* for 10 minutes at 4°C. Supernatants were collected, filtered through a 0.22 μm filter, lyophilized and resuspended in water to a tenth of the original volume (10$\times$ total). To partially purify RAP, 15 ml total 10$\times$ of RN6390B was applied to a 10 kDa cutoff membrane (Centriprep 10 (Amicon, Inc. Beverly, MA)). Resulting 1 ml concentrated material greater than 10 kDa was washed twice in PBS by resuspending it each time in 15 ml PBS and re-concentrating it on the Centriprep 10, and the material greater than 10 kDa collected (>10). 100 μl of >10 was applied to 900 μl early exponential wild type *S. aureus* RN6390B cells (containing about 1 $\times$ 10^9 cells) to test for activation of RNAII and RNAIII synthesis (see below).

To partially purify native RIP from supernatants of RN833, 15 ml of 10× postexponential supernatants prepared as described above (10× total) were applied to a 3 kDa cutoff membrane (Centriprep 3 (Amicon, Inc. Beverly, MA)) and the flow through containing RIP was collected. Control material <3 kDa was similarly collected from postexponential supernatants of RN6911, an *agr* null strain. Control material does not contain any RAP, AIP or RIP and was used as a control for the delivery buffer. 100 μl of the flow through was applied to 900 μl early exponential wild type *S. aureus* RN6390B cells (containing about 1×10^9 cells) to test for activation of RNAII and RNAIII synthesis (see below).

2.4. Regulation of RNAII and RNAIII synthesis

Early exponential wild type RN6390B cells (900 μl, containing about 1×10^9 cells) were incubated with 100 μl of PBS, native RAP, native RIP, or control medium (all prepared as described above) for 40 min (with PBS or RAP) or for 2.5 hrs (with RIP or control medium), with constant shaking at 37°C. Cells were collected by centrifugation (2 min 12,000 × *g*) and total RNA purified and northern blotted as described [3]. Synthetic peptides were used at 10 μg peptide/10^7 cells. To detect RNAII and RNAIII, radiolabeled DNA probes were prepared by PCR using RN6390B DNA as a template. For RNAIII, primers identical to the 5′ and 3′ sequences of the gene encoding for RNAIII were used as described [24]. For RNAII, an internal 624-bp fragment of *agrC* was prepared, using the primers 5′-GTTTGATAGCGCGTCCTTAAT-3′ (*agr* nt 2669-2690) and 5′-GAAATAATCACGTAGGCCAGG-3′ (*agr* nt 3293-3272).

Regulation of RNAIII synthesis was also tested using a reporter gene as described [19]. Briefly, *S. aureus* containing *agr* P3-*blaZ* (β lactamase) fusion plasmid was grown to the early exponential phase. 30 μl (containing about 10^6 cells) were applied to wells of microtiter plates together with 5 μl RIP (0–10 μg) in water. Cells were incubated at 37°C with shaking for 2.5 hrs, and β lactamase activity was determined using the nitrocefin method as described [19] and absorbance determined at 490/650 nm in a microtiter plate reader. All assays were performed in triplicate.

2.5. Bacterial adherence (fluorescence)

2.5.1. Labeling bacterial cells with Fluorescein Isothiocyanate (FITC)

Bacterial cells (20 ml) were grown to early or late exponential phase of growth. Cells were collected by centrifugation, and resuspended in 1.5 ml PBS and 10.5 ml sodium bicarbonate 0.5 M pH 9.5 containing 10 mg FITC (Sigma Chemical Co. St. Louis, MO) were added. Cells were stirred gently overnight at 4°C. Cells were washed by centrifugation ×3 with sodium bicarbonate and colony-forming units (CFU) determined.

2.5.2. Adherence (fluorescence experiments)

FITC-labeled bacterial cells were diluted 1:100 in PBS. 10^6 cells/well (in 90 μl PBS) were applied to Costar 96 well cell culture polystyrene plates (Corning Inc. Corning, NY) together with 5 μl FCS (to make a final FCS of 5%) or with 5 μl PBS as a control, and with 5 μl 1 mg/ml RIP in water (to make a final RIP concentration of 5 μg), or with 5 μl water as a control. To test the adherence of bacterial cells to mammalian cells, 10^4 HEp2 cells were applied to the wells a day before the addition of bacterial cells, so that Hep2 cells reached confluency when exposed to the bacteria.

Microtiter plates containing bacteria ± HEp2 cells were incubated for 30 min at 37°C, washed in PBS and fluorescence determined at 485/530 nm in a Microplate Fluorescence Reader (FL 600, Bio-Tek, Vermont, USA) using KC4 software. Microtiter plates were also observed under the fluorescent microscope (Olympus 1 × 50) at 400×. Each condition was tested in 10 wells.

2.6. Bacterial adherence (Atomic Force Microscopy)

Native RIP affects cell adhesion like the synthetic molecule. Therefore, for some of the AFM studies that required higher amounts of material, the native RIP was used instead of the synthetic one. *S. aureus* cells were grown overnight at 37°C on TSA agar plates. Cells (approximately 10^{10}) were suspended in PBS with 1× native RIP (supernatant <3 kDa from RN833, prepared as described above) or with 1× supernatant of RN6911 <3 kDa as a control. Mixtures were applied to cell culture plates (60 mm polystyrene plates, Corning Inc. Corning, NY). Plates were placed at 37°C for 30 min and then placed at 4°C overnight. Unbound cells were removed by washing the plates × 5 with PBS, and cells that adhered to the plastic were fixed with 2.5% glutaraldehyde for 15 min at room temperature and rinsed with PBS. Bacteria were recorded in PBS using Atomic Force Microscopy (AFM, BioScope, Digital Instruments, Santa Barbara, CA) in tapping mode. V-shaped silicon nitride cantilevers with a nominal spring constant of 0.01 N/m (Park Scientific Instruments, Sunnyvale, CA) were used in all experiments for bacterial imaging. Typical resonance frequencies were chosen between 6 and 9 kHz. Images of bacteria were obtained by a serial window narrowing, from the typical 30 μm scale in the first scan to several hundred nanometers scale in the final scan. Both the initial large scan and following smaller scans were performed in the scan rate range between 0.1–1.0 Hz to provide tip velocity of about 2–4 μm/s. All images were carried out at light tapping conditions and a typical set point ratio of about 0.9.

2.7. Suppression of S. aureus cellulitis by RIP

Suppression of *S. aureus* using the peptide RIP was carried out essentially as described [2,4,8]. Briefly, *S. aureus* strain Smith diffuse was grown overnight at 37°C on TSA agar plates [22]. Bacteria was suspended in PBS at 3.5×10^9 cells/ml. 100 μl (containing 3.5×10^8 cells) were incubated for 30 min at room temperature in the presence of 200 μg synthetic RIP peptides (solubilized in 75% DMSO) or with DMSO only as a

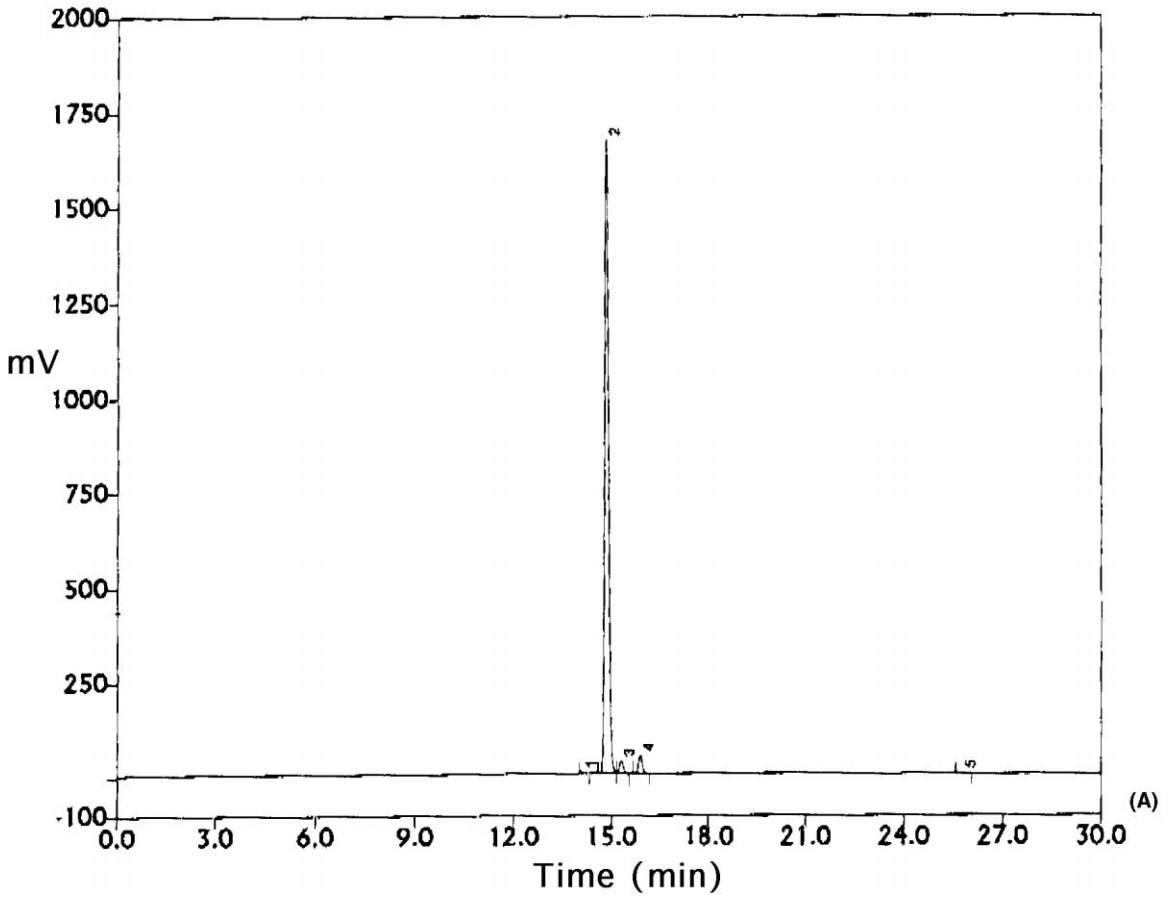

Fig. 2. (A) HPLC trace of YSPWTNF-NH2 (Neosystem, Strasbourg): Synthetic peptide was applied to a C18 HPLC reverse phase column and eluted by a 10–40% acetonitrile in 0.1% TFA gradient at 1 ml/min. Eluted peptide was detected at 210 nm. (B) Synthetic RIP inhibits RNAIII synthesis: β lactamase assay: Early exponential *S. aureus* containing an *agr* P3-blaZ (β lactamase) fusion plasmid (about 10^6 cells) were grown together with 0–10 μg synthetic RIP for 2.5 hrs, and β lactamase activity detected using the nitrocefin method, and absorbance (A, $\times 1000$) was determined at 490/650 nm.

control, both at a final DMSO concentration of 3%. The bacteria ± RIP was injected subcutaneously together with 1 mg cytodex beads into 6-week-old male Balb/C mice to induce a local infection. Animals were observed daily for mortality and development of lesion. The size of the lesion was measured after a week (area = 0.5 [π (length) (width)]).

2.8. Statistical analysis

Statistical analysis was performed using Student's *t*-Test by Microsoft Excel (Microsoft, WA). Values of $p < 0.05$ were considered significant.

3. Results

3.1. Synthesis of RIP

RIP YSPWTNF has been synthesized commercially by various methods, resulting in peptides of different efficacy and solubilization properties. Having compared the products of different methods, we now routinely synthesize the peptide in an amide form, followed by HPLC purification (Fig. 2A). Resulting peptide YSPWTNF-NH2 was determined by mass spectrometry to have a molecular weight of 912.7 dalton. This peptide is soluble in water, can inhibit RNAIII synthesis at 5 μg RIP/10^6 *S. aureus* cells (Fig. 2B), and is stable at higher temperatures (was kept up to a week at 0–42°C with no loss of activity (not shown)). Of note is that since both native and synthetic RIP were equally active [4], some of the experiments that required higher amounts of RIP were done using the native molecule instead of the synthetic one.

3.2. RAP activates whereas RIP inhibits agr activity (synthesis of RNAII and RNAIII)

The *agr* locus encodes two divergently transcribed transcripts, RNAII and RNAIII [23,24]. RNAII encodes for proteins involved in upregulation of the synthesis of RNAIII [23], and in the absence of RNAII, RNAIII is not produced [23,24]. RAP has been shown to activate, and RIP has been shown to inhibit the synthesis of RNAIII [3,4,6]. Because the synthesis of RNAIII depends on the synthesis of RNAII, it seemed reasonable to assume that RAP and RIP regulate the synthesis of RNAIII by regulating RNAII synthesis. To

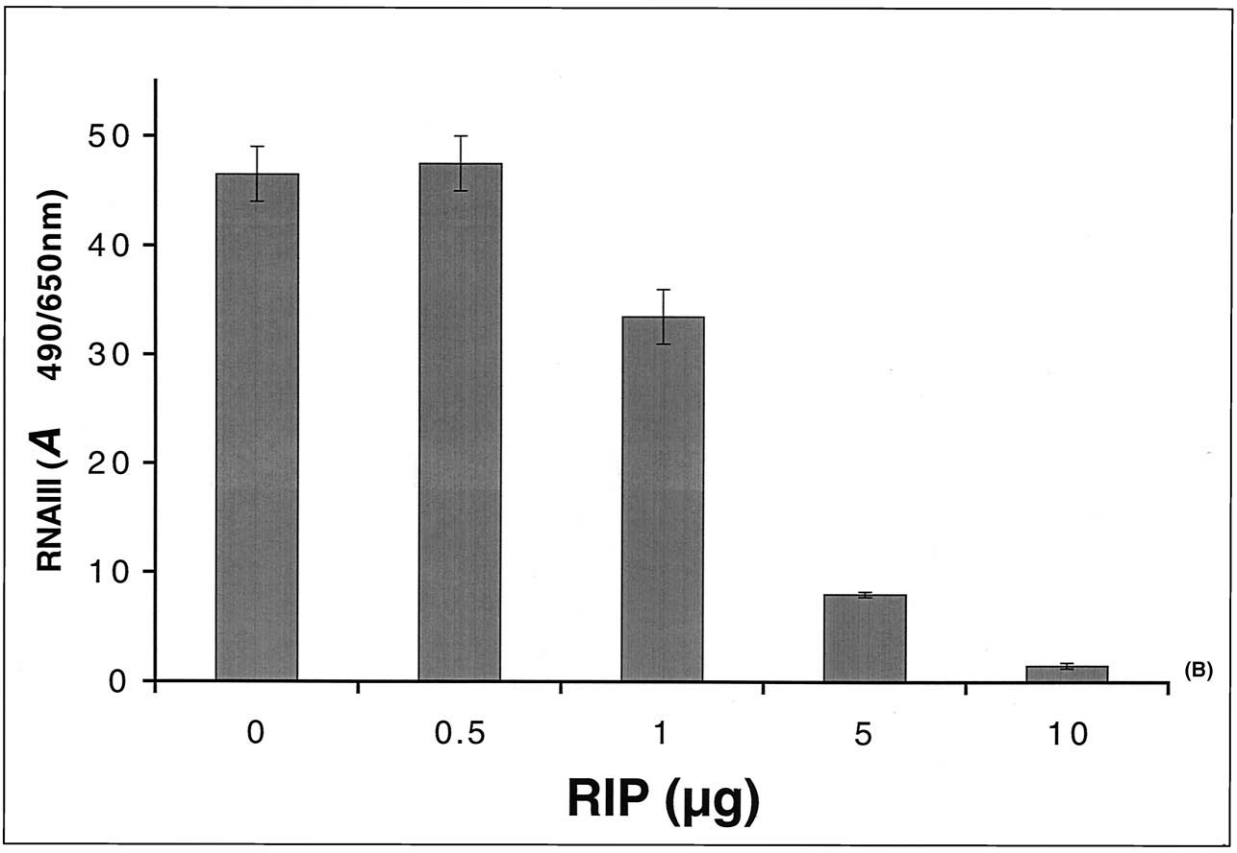

Fig. 2. *(continued)*

test for this hypothesis, wild type *S. aureus* cells were incubated with RAP or PBS for 40 min or with native RIP or control buffer for 2.5 hrs. Native molecules and control buffer were prepared as described in the methods section. Cells were collected, RNA purified and northern blotted. RNAII was detected by hybridizing the membrane with RNAII-specific radiolabeled DNA followed by hybridization with RNAIII-specific radiolabeled DNA, and the membrane autoradiographed. As shown in Fig. 3, RAP activates (A) whereas RIP inhibits (B) the synthesis of both RNAII and RNAIII.

3.3. RIP reduces cell adhesion

The ability of staphylococci to adhere is thought to be crucial for the early colonization of host tissues or implanted biomaterials [29]. RNAIII up-regulates the production of toxins and downregulates the production of adhesion molecules, suggesting that RIP, which down regulates RNAIII synthesis, might increase cell adhesion and therefore cell colonization. Although RIP has already been shown to be a successful inhibitor of *S. aureus* infections *in vivo*, the knowledge that theoretically it could increase cell colonization would hinder on the use of RIP as a therapeutic [28]. It was therefore important to investigate whether RIP in fact increases bacterial adhesion to mammalian cells and to plastic, both known to be colonized by staphylococci. Bacterial adhesion was tested both by fluorescence and by atomic force microscopy. To determine experimental conditions, increasing numbers of FITC-labeled bacteria (10^5–10^8) were placed in polystyrene plates for increasing lengths of time (30 min–2 hrs) and optimal adhesion conditions were chosen to be 10^6 bacteria/well for 30 min. For fluorescence microscopy, FITC-labeled cells were placed in microtiter polystyrene plates for 30 min at 37°C, with or without RIP (5 μg/10^6 cells) to test for bacterial adhesion to plastic (Fig. 4, 5). Early exponential FITC-labeled cells were also placed in microtiter plates that contained confluent HEp2 cells, to test for bacterial adhesion to eukaryotic cells (Fig. 6). As shown in Fig. 4 and Fig. 5A, B, RIP significantly reduced cell adhesion to polystyrene ($p < 0.004$) in the absence of serum. In the presence of serum (Fig. 5C, D), no significant adherence to polystyrene was observed, with or without RIP. As shown in Fig. 6, in the absence of serum, RIP significantly reduced bacterial cell adhesion to HEp2 cells ($p < 0.006$). In the presence of serum there was a slight overall reduction in bacterial adhesion to HEp2 cells, both with or without RIP. RIP reduced bacterial adhesion to plastic and to HEp2 cells also when bacterial cells were in their late exponential phase of growth (not shown), although the relative number of bacteria that adhered was lower, as expected from genetic data. Of note

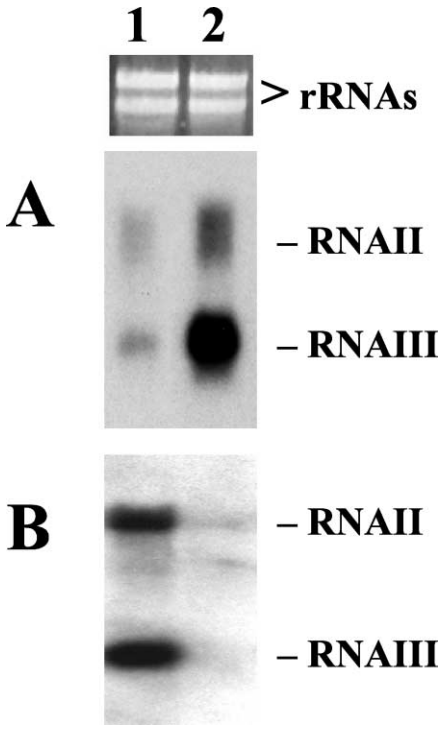

Fig. 3. RAP activates whereas RIP inhibits the production of RNAII and RNAIII: Early exponential wild type *S. aureus* cells were incubated with RAP or PBS for 40 min (A) or with RIP or control buffer for 2.5 hrs (B). Cells were collected, and total RNA of equal number of cells was northern blotted. RNAII and RNAIII were detected by hybridizing the membrane with RNAII-specific radiolabeled DNA followed by hybridization with RNAIII-specific radiolabeled DNA, and the membrane was autoradiographed. Top panel: RNA was applied to an agarose gel and ribosomal RNA (rRNA) visualized by staining the gel with ethidium bromide, indicating that equal amounts of RNA were applied to the gel (shown here as an example for A). A. Cells incubated with PBS (lane 1) or with RAP (lane 2). B. Cells were incubated with control media (<3 kDa of supernatants of RN6911) (lane 1) or with native RIP (<3 kDa of supernatants of RN833) (lane 2).

is that adhesion experiments with *S. aureus* were done using either native or synthetic RIP and results were equivalent (not shown).

Local adhesion properties of single bacteria were examined by AFM. *S. aureus* cells were placed in polystyrene plates in PBS with or without RIP for 30 min, non adherent cells washed and adherent bacteria were recorded in tapping mode by AFM (BioScope, Digital Instruments, Santa Barbara, CA). Height images were collected and cell profiles analyzed. The color code of height images corresponds to changes from 1 μm (light) to 0 μm (dark). The higher areas (shown here in lighter color) of the cell were considered as "caps." As shown in Fig. 7, in the absence of RIP (top panel), the bacteria exhibited layer spread around the whole bacteria, the cell had some protrusions, with a small cap ($\sim$0.0975 μm^2) and a large base ($\sim$0.79 μm^2). In contrast to that, in the presence of RIP (Fig. 7 bottom panel), the bacteria had no visible protrusions, with a base ($\sim$0.623 μm^2) and a cap ($\sim$0.488 μm^2) that were almost similar in

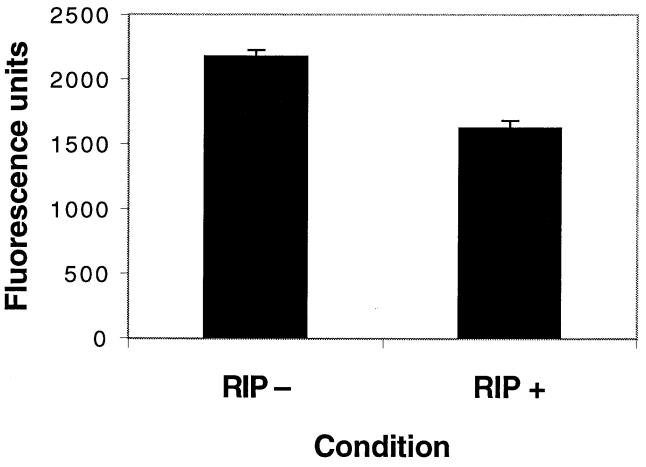

Fig. 4. RIP reduces adhesion of bacteria to polystyrene: FITC-labeled bacterial cells (10^6 CFU) were applied to polystyrene plates with water (RIP−) or with 5 μg RIP in water (RIP+) for 30 min at 37°C, unbound cells removed and fluorescence determined at 485/530 nm.

size. The approximate cap/base ratio of the bacteria was 0.12 as compared to 0.78 of the bacteria in the presence of RIP. These results suggest that in the presence of RIP, the contact area the bacteria made with the plastic surface was much smaller, indicating reduced adhesion level in the presence of RIP.

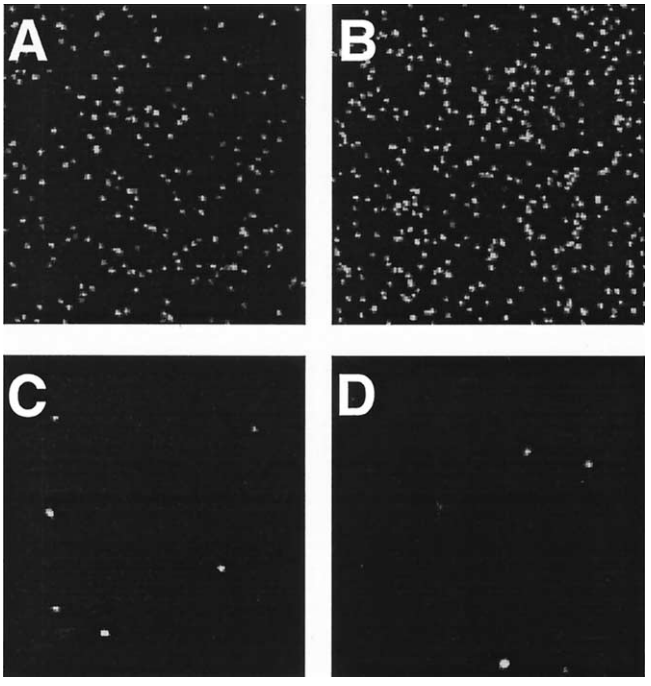

Fig. 5. RIP reduces adhesion of bacteria to polystyrene: FITC-labeled bacterial cells (10^6 CFU) were applied to polystyrene plates with 5 μg RIP in water (A), with water as a control (B), with RIP and 5% FCS (C), or with water and FCS as a control (D). Cells were incubated for 30 min at 37°C, unbound cells removed and adherent cells observed under the fluorescent microscope (Olympus 1 × 50) at 400×.

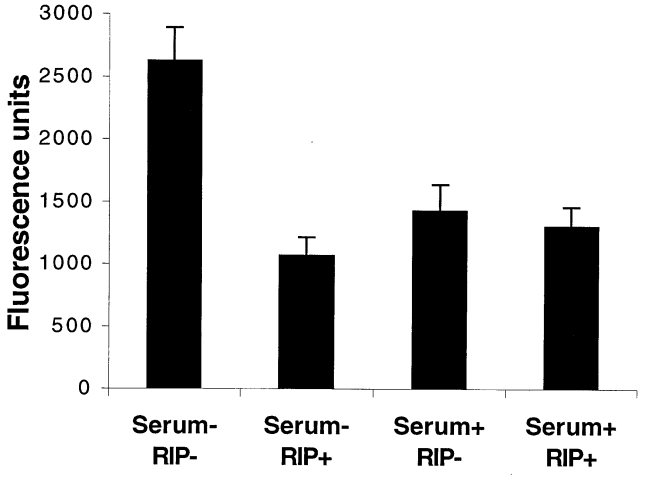

Fig. 6. RIP reduces adhesion of bacteria to HEp2 cells: FITC-labeled bacterial cells (10^6 CFU) were applied to microtiter plates containing confluent 10^4 HEp2 cells, in the presence or absence of 5% FCS (Serum ±) and 5 μg RIP (RIP ±), incubated for 30 min at 37°C, washed in PBS and fluorescence determined at 485/530 nm.

3.4. RIP derivative YKPITNF is an effective inhibitor of cellulitis

RIP inhibits and RAP induces the phosphorylation of TRAP. The sequence similarity between RIP and the NH2 terminal sequence of *S. aureus* RAP (YSPXTNF versus YKPITN) suggests that both may compete on binding to the same site on the receptor [3,8]. If this hypothesis is to hold, increasing the sequence similarity of RIP to the amino terminal of RAP should enhance its inhibitory activity. Thus RIP derivatives were designed to mimic the sequence of the amino terminal sequence of RAP Peptides containing a Cys in the 4th position were also designed because the sequence of native RIP is YSPXTNF, where X could be a Trp or a Cys. In these experiments, the peptides YSPWTNF, YSPCTNF and YSPCTNFF were synthesized in their carboxyl form on a Wang resin, while all other peptides were synthesized in their amide form, on a Knorr resin. All peptides were tested for their ability to inhibit RNAIII synthesis *in vitro* (at a concentration of 10 μg/10^7 cells) and to inhibit *S. aureus*-induced cellulitis *in vivo* (tested at 200 μg RIP per 3.5×10^8 cells).

As shown in Fig. 8A, several of the RIP derivatives inhibited the synthesis of RNAIII to varying degrees, including YSPWTNF (by 80%), PWTNF (by 100%), YSPCTNF (by 88%), YSPCTNFF (by 100%), YSPITNF (by 70%), PITNF (by 68%), YKPWTNF (by 100%), YKPITN (by 100%) and YKPITNF (by 100%).

The peptides that inhibited synthesis of RNAIII *in vitro* were also tested for their ability to prevent cellulitis *in vivo*. Some of the infected animals died of sepsis before a lesion was formed, and they were given an arbitrary lesion size greater than 3000 mm^2. As shown in Fig. 8B, of the 8 control animals injected with bacteria/control buffer (3% DMSO), 6 of them died (75%) and the other two had a

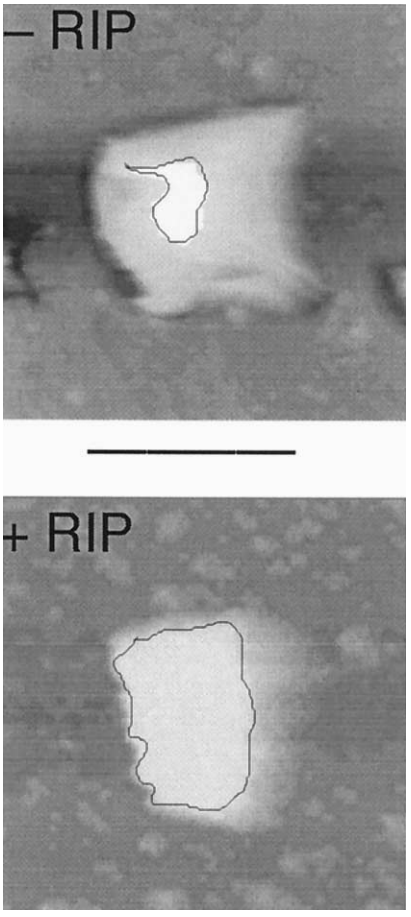

Fig. 7. RIP reduces bacterial adhesion to plastic: AFM. Bacteria ± RIP were applied to polystyrene cell culture plates and cells observed using AFM in tapping mode. Height images were collected and cell profiles analyzed. The color code of height images corresponds to changes from 1 μm (light) to 0 μm (dark). The higher areas (shown here in lighter color) of the cell were considered as "caps." Caps were highlighted by a black line. Bar, 1 μm.

lesion. Of the 4 animals injected with bacteria/YSPWTNF, all had a lesion, but none died (0%). In comparison, of the 8 animals injected with PWTNF, 3 of them died (37.5%), and the rest had a lesion. Of the 8 animals injected with YSPCTNF, 4 died (50%) and the rest had a lesion, while none of the 4 animals injected with bacteria/YSPCTNFF died (0%) but all had a lesion. Of the 8 animals injected with bacteria/YSPITNF, 4 died (50%) and the rest had a lesion, while only 1 animal died of the 8 animals injected with bacteria/PITNF (12.5%) but all the rest had a lesion. Of the 8 animals injected with bacteria/YKPWTNF, none died but all had a relatively smaller lesion. Of the 8 animals injected with bacteria/YKPITN, only 1 animal died but all the rest had no lesion at all and were completely protected, and of the 8 animals injected with bacteria/YKPITNF, all animals were entirely protected; that is, none died (0%) and none had a lesion. Taken together, the results of the *in vivo* and *in vitro* experiments, indicate the importance of Lys and Ile residues at position 2 and 4 of RIP, respectively, with

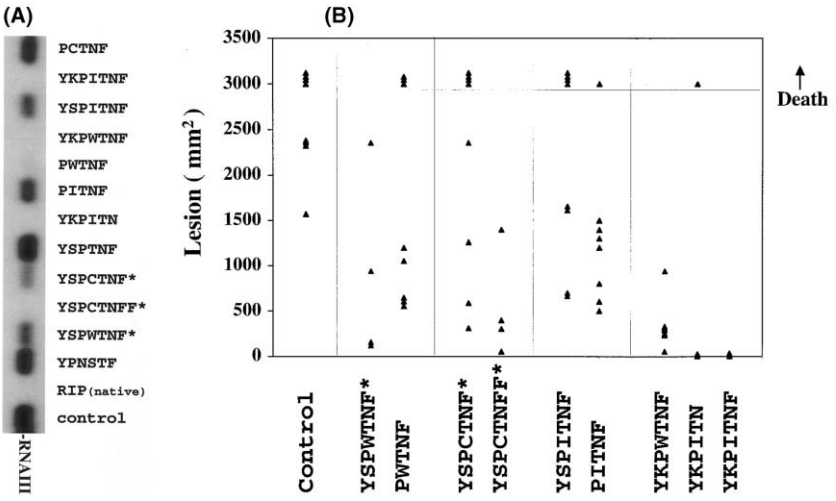

Fig. 8. (A) Inhibition of RNAIII synthesis by synthetic RIP and derivatives: Early exponential wild type *S. aureus* RN6390B (1×10^7 cells) were incubated for 2.5 hrs at 37°C with native RIP, with synthetic RIP peptides and derivatives (10 μg) or with carrier buffer (3% DMSO, final concentration). Cells were collected and assayed for RNAIII by northern blotting, using radiolabeled RNAIII-specific DNA as a probe and membrane autoradiographed. All the peptides were synthesized in their amide form but the ones indicated by a star (*). (B) The effect of RIP derivatives on *S. aureus* sepsis and cellulitis: 3.5×10^8 cells *S. aureus* strain Smith diffuse were incubated with 200 μg synthetic peptides, 1× native RIP or carrier buffer (3% DMSO) for 30 min at room temperature and the mixture was injected subcutaneously into Balb/C mice together with cytodex beads. Mice were followed for mortality and development of lesion. All the peptides were synthesized in their amide form but the ones indicated by a star (*).

YKPITNF exhibiting the highest inhibitory activity *in vivo*, under the experimental conditions tested.

3.5. The predicted structure of RIP YSPWTNF and its derivative YKPITNF

To hypothetically compare the potential structures of YSPWTNF and its derivative YKPITNF, artificial structures of the peptides were made according to known structures in the Protein Data Bank that contain much of the desired sequences. Each structure was then rotated about single bonds by Chem 3D (CambridgeSoft) to generate the conformations shown in Fig. 9. During rotation, conformations that resulted in internal collisions were avoided. Fig. 9 represents 2-d projections of some of the attained conformations. These predictions are merely hypothetical since protein folding involves a balance not only between local interactions but also between non-local interactions, which can lead to structural diversity even of identical heptapeptides [26]. However, one cannot disregard the observation

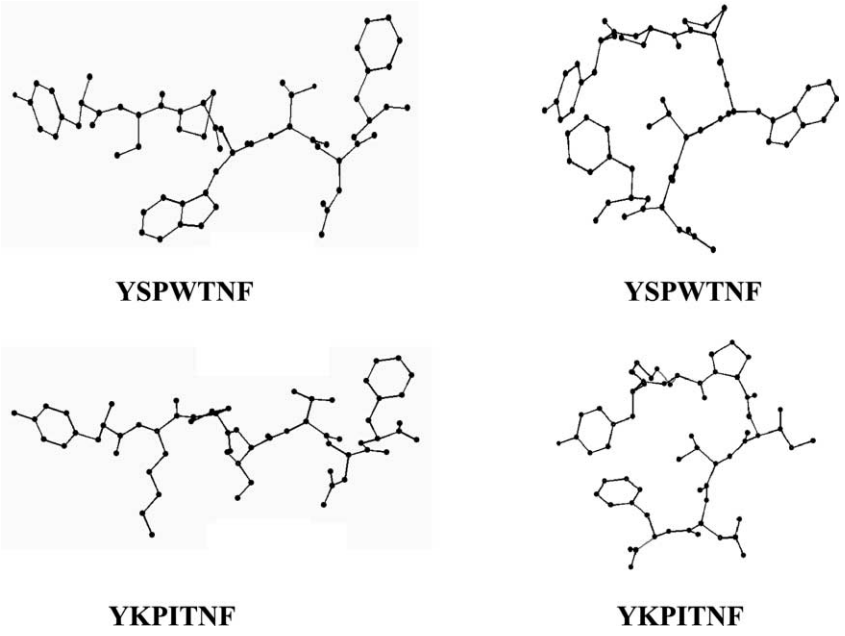

Fig. 9. Secondary structure predictions of YSPWTNF and YKPITNF by Chem 3D (CambridgeSoft) to produce the conformations shown.

that the predicted conformations for both peptide structures are similar, suggesting that the enhanced inhibitory activity of peptide YKPITNF may not be due to its secondary structure but rather to the local interactions it makes with its receptor(s).

4. Discussion

4.1. YSPWTNF-NH2

The synthetic peptide RIP YSPWTNF, produced through different peptide synthesis methodologies, has been shown by various laboratories to be an effective inhibitor of *S. aureus* pathogenesis. However, its stability and solubility varied, resulting in possible lack of activity, which may have lead to the claim that linear peptides do not inhibit RNAIII synthesis [see e.g. 19]. We recently discovered that a difference in peptide synthesis methodology can lead to the variable results. We now recommend to use RIP in its amide form (synthesized for example on a Knorr resin). This peptide can be used effectively at concentrations as low as 5 μg/10^6 cells. If it is HPLC purified, it can be solubilized in water. The peptide was tested by many investigators throughout the world and shown to be a very effective inhibitor of *S. aureus* infections [see e.g. 2].

4.2. Structure and function analysis of RIP

Structure and function analyses of RIP derivatives indicate that the peptides most effective in preventing *S. aureus* infection are the peptides that contain residues Lys and Ile at the second and fourth positions, respectively, making the peptide YKPITNF an effective inhibitor. Because this particular sequence is found in the NH2 terminal sequence predicted for RAP, we suggest that RIP inhibits *S. aureus* pathogenesis by competing with RAP for binding to its receptor. An amide form of RIP would be more similar to the NH2 terminal sequence of RAP, the natural ligand of the receptor. Perhaps this is the reason why an amide form of the synthetic RIP peptide is a better inhibitor than the carboxyl form of RIP. The predicted theoretical 2-d conformations for peptides YSPWTNF and YKPITNF are similar. It may therefore be reasonable to suggest that the enhanced inhibitory activity of peptide YKPITNF may be due to the local interactions the peptide makes with its receptor.

4.3. The effect of YSPCTNF and YSPCTNFF on RNAIII synthesis and cellulitis

The effect of the synthetic linear peptides YSPCTNF and YSPCTNFF on RNAIII synthesis and *S. aureus* infections was also tested. The reason we chose to test these peptides is two-fold: 1) The native sequence of RIP is YSPXTNF, suggesting that it may contain a Cys in the fourth position. 2) It has been suggested that *agr*-encoded AIP of *S. warnerii* origin contains the sequence YSPCTN or YSPCTNFF. It was determined, however, that these peptides must contain a thiolactone structure and that linear peptides were not active [19]. To the contrary, our studies indicate the following: 1) Both YSPCTNF and YSPCTNFF linear peptides, which were synthesized (in their carboxyl form) independently by the Louisiana State University Medical Center Core Facilities by routine methods of peptide synthesis, inhibited the production of RNAIII. Other laboratories (Hume, personal communication), confirmed this observation. Our results clearly demonstrate that these linear peptides can in fact be active. 2) Of the two peptides, only YSPCTNFF had some inhibitory activity *in vivo*, but its inhibitory activity was not as high as the rationally designed RIP-derivative YKPITNF.

4.4. Effectiveness of a peptide in vitro *does not predict with certainty its effectiveness* in vivo

Some of the synthetic peptides that inhibited RNAIII synthesis *in vitro* did not inhibit *S. aureus* infections *in vivo*. The nature of the differences observed in the inhibitory effect(s) in the *in-vivo* experiments as compared with those *in vitro*, are unclear. Barring plausible intrinsic peptide structural properties, the observed differences may be due to differences either in the bacterial strains used (RN6390B for *in vitro* studies and Smith diffuse for the *in vivo* studies), or in the relative stability of the peptide in the host. Overall, our data indicate that effectiveness of a peptide *in vitro* does not predict with certainty its effectiveness *in vivo* (see below, section 4.6).

4.5. RIP reduces bacterial adhesion

RIP inhibits the synthesis of both *agr* transcripts, RNAII and RNAIII. Because transcription from P3 promoter (leading to the production of RNAIII) depends on translational products of RNAII, we propose that RIP inhibits RNAIII synthesis by inhibiting the production of RNAII. Inhibition of *agr* or genetic disruption of *agr* results in the overexpression of adhesion molecules [24]. Phenotypically, this would then lead to enhanced bacterial colonization [24,28, 29], which expectedly would enhance bacterial pathogenesis and disease. Nevertheless, native and synthetic RIP peptides have been repeatedly shown by many investigators throughout the world to suppress infections [see for example 2]. We could therefore argue that inhibition of toxin production by RIP would be sufficient for the suppression of disease, even if the bacteria were to be considered better colonizers as a result of transcriptional downregulation of RNAII and RNAIII by treatment with RIP. To our surprise, however, not only that RIP did not enhance bacterial adhesion to eukaryotic cells (tested on HEp2 cells) or to plastic (tested on polystyrene), but it actually decreased bacterial adhesion. We therefore conclude that RIP effects other factors involved in cell adhesion in addition to *agr*. Of note

is the fact that RIP also reduced adhesion of *S. epidermidis* to HEp2 cells and to polystyrene (not shown). Since cell adhesion is a major factor in the virulence of *S. epidermidis*, it may be possible that RIP is also an effective inhibitor of infections caused by *S. epidermidis*.

The inhibitory effect of RIP on cell adhesion was also examined by AFM, which enabled us to reveal differences in adhesion properties at a single bacterium level [see e.g. 15]. We showed not only that the number of adhered bacteria was lower in the presence of RIP, but also that the contact area each bacterium made with the plastic surface was lower in the presence of RIP. The bacteria were shaped differently, where in the absence of RIP it had protrusions that spread laterally. This can be considered as a result of the action of the adhesion forces of different strengths. In the case of the wild type, these forces are stronger and cause larger spread of the bacterium. But in the presence of RIP, the adhesion forces drop so strongly that the forces almost do not produce any bacterial spread. This feature is confirmed by the fact that atomic force imaging itself was very difficult in the presence of RIP. Only the set of large initial scans allowed finding few bacteria for imaging, which yielded only qualitative information on the strength of the adhesion forces. In the future, we hope to acquire quantitative measurements of the adhesion forces using AFM, in force measurement mode. This can be done once better attachment conditions are found also in the presence of RIP. Thus currently, the AFM imaging can be considered as the first but significant step for the study of bacteria interaction with the substrate.

4.6. Currently, there are three schools of thought concerning the nature and effectiveness of agr-*inhibiting peptides*

1. One school of thought argues that the only peptides that regulate the *agr* are the AIPs [19]. Their primary sequences vary among staphylococcal strains and species, but all are 8–9-aa long. These peptides must contain a thiolactone structure in which the alpha-carboxyl group of the C-terminal amino acid is linked to the sulfhydryl group of a cysteine, which is always the fifth amino acid from the C terminus of the peptide. These peptides have been shown to activate *agr* of self but inhibit *agr* of some of the other *S. aureus* strains.

   While AIPs have been shown to be effective *in vitro*, the results of only a single limited *in vivo* study was so far reported. In that study, one of these peptides (AIPII) was tested in a cellulitis model against only one *S. aureus* strain, in two experimental groups with only 3 mice per group [19]. Recently a truncated version of one of the naturally occurring thiolactone peptides was designed that inhibited *agr in vitro* and was suggested to be a global inhibitor of virulence of *S. aureus* [18]. *In vivo* studies using this peptide, however, have not yet been reported.
2. Our school of thought argues that RIP and its derivative are effective inhibitors of infections caused by *S. aureus*. RIP and its derivative were synthesized as linear peptides, and have been shown to inhibit *agr in vitro* and to inhibit all types of *S. aureus* infections so far tested. *In vivo* studies were carried out throughout the world on a large number of animals (>120), on several strains of *S. aureus* (Smith diffuse, LS-1, 8325-4, MS, Newbould, AE-1), and on different types of animals (mice, rabbits, cows). RIP was shown to be effective in preventing cellulitis, mastitis, sepsis, septic arthritis, keratitis, osteomyelitis and mastitis [2,4,8]. These findings clearly indicate that RIP is a very effective inhibitor of *S. aureus* infections.
3. A third school of thought is that inhibition of *agr* might ultimately cause an increase in chronic infections as a result of upregulation of expression of adhesion molecules, thus resulting in increased colonization. Downregulation of *agr* by AIPs did in fact increase cell adhesion *in vitro*, shedding some doubt on the use of *agr* inhibitors as successful therapeutics [28].

   On the other hand, as we have shown above, RIP inhibits *agr* but also decreases cell adhesion. Our results indicate that RIP inhibits other factors in addition to *agr* and that RIP can be used safely as a therapeutic.

To summarize, the ability of RIP to reduce toxin synthesis and cell adhesion *in vitro* and be an effective inhibitor of all strains of *S. aureus* infections so far tested *in vivo*, indicate once more the potential of RIP as a therapeutic agent to staphylococcal infections.

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