

# Screening and Characterization of Surface-Tethered Cationic Peptides for Antimicrobial Activity

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DOI 10.1016/j.chembiol.2008.11.006

## SUMMARY

There is an urgent need to coat the surfaces of medical devices, including implants, with antimicrobial agents to reduce the risk of infection. A peptide array technology was modified to permit the screening of short peptides for antimicrobial activity while tethered to a surface. Cellulose-amino-hydroxypropyl ether (CAPE) linker chemistry was used to synthesize, on a cellulose support, peptides that remained covalently bound during biological assays. Among 122 tested sequences, the best surface-tethered 9-, 12-, and 13-mer peptides were found to be highly antimicrobial against bacteria and fungi, as confirmed using alternative surface materials and coupling strategies as well as coupling through the C and N termini of the peptides. Structure-activity modeling of the structural features determining the activity of tethered peptides indicated that the extent and positioning of positive charges and hydrophobic residues were influential in determining activity.

## INTRODUCTION

The rapid progress of biomedical technology and an aging population places increasing demands on medical implants to treat serious tissue disorders and replace organ function. In the field of orthopedic implant surgery alone, about 2 million fracture-fixation devices and 600,000 joint prostheses are implanted every year in the United States (Darouiche, 2004). The risk of infection after surgical implantation ranges from 1% and 7%, but is associated with considerable morbidity, repeated surgeries, and prolonged therapy (Anderson and Marchant, 2000). Infections associated with the insertion of vascular and urinary catheters are the most common serious complications of surgical implants (Darouiche, 2003). Furthermore, the mortality rate of an infected aortic graft can approach 40%

(Anderson and Marchant, 2000), and infections of knee joint prostheses can, in up to 52% of cases, lead to ankylosis and, in up to 9% of the cases, to amputation (Gollwitzer et al., 2005). Prevention of such infections remains a priority (Darouiche, 2003).

A new strategy for preventing implant-associated infections involves coating the implants with a polymer that contains common antibiotics. Such approaches are currently in clinical trials (Darouiche, 2003; Gollwitzer et al., 2005). However, the rising problem of infections caused by multiply antibiotic-resistant bacteria, so-called superbugs, limits the value of this approach. In addition, the standard procedure for treating implant-associated infections, using high doses of antibiotics over a long period of time, might exacerbate this situation by contributing to selection of antibiotic-resistant bacteria with potential life-threatening complications for patients. The development of an implant coating with broad spectrum antimicrobial activity and one that has no relationship to common antibiotics would be highly advantageous.

Cationic antimicrobial peptides are among the most prominent antimicrobial substances produced by most complex organisms for local protection (e.g., of the skin and mucosal surfaces) against microbial infections (Harder et al., 2001). They have emerged as central components of the innate defenses of animals, insects, and plants, and peptides with activities against Gram-negative and Gram-positive bacteria, fungi, enveloped viruses, and eukaryotic parasites have been identified (Harder et al., 2001). Indeed, the therapeutic potential of cationic antimicrobial peptides as alternatives to conventional antibiotics is currently being explored with synthetic peptides demonstrating efficacy in phase IIIa clinical trials (Hamill et al., 2008). The mechanisms of action of these peptides are complex and different from conventional antibiotics, and it has proven extraordinarily difficult to select resistant mutants to such peptides. The available evidence indicates that cationic antimicrobial peptides interact with bacterial surfaces to either permeabilize them or to translocate across the cytoplasmic membrane to attack cytoplasmic targets. Although the majority of studies of the cationic antimicrobial peptides have focused

on free peptides in solution, it was previously demonstrated that the cationic lipopeptide polymyxin B could inhibit the growth of the Gram-negative bacterium *Escherichia coli* while covalently bound to an agarose bead (LaPorte et al., 1977). Subsequently, Haynie et al. (1995) demonstrated that surface-immobilized (tethered) cationic antimicrobial peptides had broad spectrum activity, including the ability to kill Gram-positive bacteria and yeast.

Thus, short-tethered cationic antimicrobial peptides appear to be excellent candidates for protecting surfaces against microbial growth, such as those of medical implants. Although many investigations of soluble antimicrobial peptides have served to establish the structure-activity relationships that dictate peptide antimicrobial activity and cytotoxicity, this is not the case for tethered antimicrobial peptides. Indeed, as immobilization of peptides to a surface would result in limitations to peptide mobility and thus the ability of peptides to enter into or translocate across membranes, it is imperative that structure-activity relationship investigations among tethered cationic antimicrobial peptides be established. Previously, we developed a high-throughput antimicrobial peptide activity screening assay utilizing *Pseudomonas aeruginosa* with a constitutively expressed luciferase (*luxCDABE*) gene cassette; however, this method was limited to free peptides in solution (Hilpert et al., 2005, 2006). Here we have adapted and made key modifications to this methodology to enable the identification of surface-bound peptides with antimicrobial activity using a high-throughput screening assay format. By creating a large library of peptides, we were able to investigate the influence of charged and hydrophobic residues on the antimicrobial activity of tethered peptides, as well as the influence of their positioning within the peptide sequence relative to the tethering surface. The resultant strategy will assist the development of peptidic antimicrobial surfaces that might exhibit certain advantages over those presently used in the clinic.

## RESULTS

### Methods Development

Peptides were produced by a variation of the SPOT synthesis method involving synthesis of peptides on a membrane support as described in detail previously (Frank, 1992; Hilpert et al., 2007). A variety of linkers were tested, and we eventually decided to use the CAPE linker strategy, designed for high stability by utilizing an ether bond (Kamradt and Volkmer-Engert, 2004). Spontaneous peptide release from the respective tethering surface of the CAPE-linked active peptide Bac2A and negative control peptide Tet000 was monitored by HPLC after 4 hr of incubation at 37°C in 100 mM Tris-HCl buffer (pH 7.5). No spontaneous peptide release was detected by HPLC using the CAPE linker, supporting the use of this linker strategy. By comparison, peptides were almost completely released after overnight treatment with ammonia gas.

Using the SPOT synthesis technology, the peptides Bac2A and variants of Bac2A were synthesized at a peptide density of 50 nmol/spot (Hilpert et al., 2005). The peptide Tet000, an inactive unrelated peptide (Hilpert et al., 2005), was synthesized and included as a negative control. Following cleavage of the side-chain protecting groups and an intense washing procedure,

the peptide spots were punched out of the cellulose sheet and transferred into a standard 96-well microtiter plate suitable for luminescence measurements (one peptide spot per well). A luminescent *Pseudomonas aeruginosa* reporter strain, H1001, containing the luciferase reporter gene *fliC::luxCDABE* was employed. For this strain, bacterial luminescence is dependent on cellular energization and is therefore directly related to bacterial proliferation. Bacteria, glucose, and buffer were added, and luminescence monitored over time after peptide addition. The peptides Tet008 and Tet009 were clearly able to decrease the luminescence of *P. aeruginosa*, with only limited reduction of luminescence upon incubation with the inactive control peptide Tet000 (Table 1). Thus, the decreased luminescence noted for the active peptides was not a result of residual chemicals used during peptide synthesis. As described previously (Hilpert et al., 2005), it could be demonstrated that a nearly perfect correlation existed between bacterial luminescence and the residual colony counts of surviving bacteria after overnight incubation (Figure 1), confirming that the *lux* screening protocol could be used accurately for the assessment of tethered peptide antimicrobial activities.

### Screening for Antimicrobial Activity

Cathelicidins are a family of variable, naturally occurring antimicrobial peptides that are grouped based on their common prepro sequences, even though the mature active peptide fragments tend to be structurally very different (Hancock and Sahl, 2006). To decrease any potential for bias, and permit screening for tethered peptides with enhanced antimicrobial activity, two very different cathelicidin peptides were chosen as starting points for making variant peptides. The 12 aa peptide Bac2A (RLARIV VIRVAR) (Wu and Hancock, 1999a), a linear variant of the naturally occurring cyclic peptide bactenecin (also called bovine dodecapeptide), is one of the smallest naturally occurring cationic antimicrobial peptides (Romeo et al., 1988) and is active in solution against Gram-positive and Gram-negative bacteria. The 13 aa extended peptide indolicidin (ILPWKWPWWPWR) (Rozek et al., 2000), containing the highest proportion of Trp of any natural peptide (Zanetti et al., 1995), was also chosen, as an indolicidin derivative, MX-226, is currently in phase IIIa human clinical trials (Hamill et al., 2008).

The screening procedure was initially performed using a peptide density of 50 nmol/spot and/or 200 nmol/spot. At both peptide densities, the screen was repeated three times for each tethered peptide spot, resulting in three independent rounds of biological assays. Using a peptide concentration of 200 nmol/spot, 23 different sequences were discovered that showed inhibitory values of more than 90% at one or both peptide concentrations (Table 1), representing 17.2% of the total tested library of 122 peptides (other peptides screened appear in Table S1 available online). It was also noted that 9-, 12-, and 13-mer peptides were primarily represented among these highly active peptides. In general, as the inhibitory effect increased, the standard deviation decreased, indicating a higher confidence for higher values of inhibition. Illustrating this point, the peptide Tet009 showed a mean inhibition of *P. aeruginosa* luminescence of  $84.7\% \pm 7.6\%$  as compared with peptide Tet052 ( $44.6\% \pm 8.8\%$  inhibition) and Bac2A ( $16.9\% \pm 15.7\%$  inhibition). An exception to this correlation was found for proline-containing

**Table 1. Assessment of the Growth Inhibitory Potential of a Range of Peptides by Inhibition of Energy-Dependent Luminescent *Pseudomonas aeruginosa* Strain H1001 Containing the Constitutive Luciferase Reporter Gene *fliC::luxCDABE***

Name	Sequence	% Inhibition of Luminescence ( $\pm$ SD)		MIC Soluble Peptide <sup>a</sup>	Tethered Peptide RBC
		50 nmol/spot	200 nmol/spot		Toxicity (200 nmol/spot)
					% HB Release ( $\pm$ SD)
Tet000	GATPEDLNQKLS	0.01 $\pm$ 9	10 $\pm$ 12	>250 (Hilpert et al., 2006)	10 $\pm$ 2
Tet003	RLARIVPIRVAR	40 $\pm$ 4	47 $\pm$ 36	>250 (Hilpert et al., 2006)	9 $\pm$ 2
Tet008	RRWRIVIRVRR	93 $\pm$ 6	79 $\pm$ 7	2 (Hilpert et al., 2006)	12 $\pm$ 1
Tet009	RRWKIVIRWRR	96 $\pm$ 3	85 $\pm$ 8	2 (Hilpert et al., 2006)	11 $\pm$ 1
Tet013	VRFRIRVAVIRA	50 $\pm$ 9	12 $\pm$ 10	6 (Hilpert et al., 2005)	9 $\pm$ 2
Tet021	KKWKIVIKWKK		97 $\pm$ 2	4	9 $\pm$ 2
Tet022	KKWKIVIRWRR		88 $\pm$ 2	2	11 $\pm$ 2
Tet023	RRWKIVIRWKK		96 $\pm$ 1	2	11 $\pm$ 1
Tet024	RRRWIVWRRR		85 $\pm$ 4	2	11 $\pm$ 1
Tet032	RRRRWRRRWR		98 $\pm$ 1	2	11 $\pm$ 1
Tet033	RRRWWRRWRR		98 $\pm$ 1	4	12 $\pm$ 2
Tet037	IVRVAVALRRIR	40 $\pm$ 8	51 $\pm$ 2	73	11 $\pm$ 2
Tet052	RRAAVLVIRRR	39 $\pm$ 15	45 $\pm$ 9	11 (Hilpert et al., 2005)	10 $\pm$ 1
Tet083	ILPWKWPWWPWR		36 $\pm$ 53	38	11 $\pm$ 1
Tet086	ILKWKWPWWPWR		42 $\pm$ 43	10	11 $\pm$ 1
Tet091	FLPKKFRWWKYRK		98 $\pm$ 1	16	11 $\pm$ 1
Tet092	FIKWKFRWWKWRK	90 $\pm$ 5	97 $\pm$ 1	2	14 $\pm$ 1
Tet099	FIRWRFRWWRR		74 $\pm$ 25	6	11 $\pm$ 2
Tet110	RWRWWRRR		68 $\pm$ 19	62	9 $\pm$ 2
Tet112	KWKWWKWK		86 $\pm$ 2	125	12 $\pm$ 2
Tet124	KLWWMIRRW	13 $\pm$ 2	33 $\pm$ 4	8	10 $\pm$ 2
Tet127	KRWKWWRR	92 $\pm$ 8	94 $\pm$ 3	0.7	11 $\pm$ 1

Luminescence was measured after 4 hr of incubation with a range of peptides that were C-terminally tethered to cellulose, at a peptide density of 50 nmol/spot or 200 nmol/spot. The percentage of inhibition was determined by comparing the residual luminescence signal of treated bacteria with that of nontreated ones. The assay was performed three times and the SD determined. For some peptides, the minimal inhibitory concentration (MIC) of the soluble peptide had been previously determined (see parenthetical reference citations); the remainder was calculated here. Toxicity was measured by a hemolysis assay (monitoring release of hemoglobin from red blood cells).

<sup>a</sup> MIC of the corresponding soluble peptide against *Pseudomonas aeruginosa* (three biological repeats).

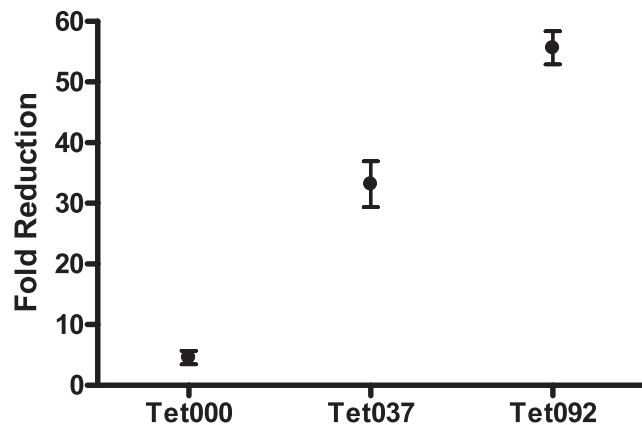
peptides, which generally showed a remarkably high standard deviation in three independent repeat assays.

The cytotoxicity of selected tethered antimicrobial peptides was assessed through human red blood cell (RBC) hemolysis (Table 1). None of these peptides demonstrated substantial hemolytic activities when tethered. For example, the soluble peptides Tet052 and Tet083 demonstrated the highest hemolytic activities at peptide concentrations of 200  $\mu$ g/ml (23%  $\pm$  3% and 64%  $\pm$  2%, respectively); however, upon tethering, the hemolytic activities of both peptides were reduced (11%  $\pm$  1% and 11%  $\pm$  1% hemolysis, respectively). Thus, tethering of antimicrobial peptides reduced the hemolytic activities associated with the respective soluble peptide analogs.

No correlation could be demonstrated between the measured antimicrobial activity (minimal inhibitory concentration; MIC) in solution of the soluble untethered peptides and the luminescence inhibition of the tethered peptides (Figure 2A); however, although no overall pattern was observed, the 10 most active peptides (with MICs between 0.8 and 4  $\mu$ g/ml) were also highly active when tethered (>90% inhibition of luminescence; Figure 2B). For example, the soluble version of peptide Tet013

had an MIC value of 6  $\mu$ g/ml, but at 200 nmol/spot the tethered peptide inhibited luminescence by only 12%  $\pm$  10%. Conversely, the soluble version of peptide Tet091 exhibited an MIC of 31  $\mu$ g/ml, but when tethered demonstrated 98%  $\pm$  1% inhibition. Further, peptide Tet003 demonstrated intermediate activity as a tethered peptide (40%–47% inhibition), whereas the soluble analog was completely inactive.

Among the Bac2A variants, there were many highly antimicrobial peptides. Interestingly, the reversed sequence of Bac2A (RAVRIVIRALR) showed a 4-fold stronger activity than Bac2A (RLARIVIRVAR). For further refinement, peptide Tet009 (RRWKIVIRWRR) was selected, and new variants were synthesized and tested for the effects of Arg and Lys substitution within the tethered peptides, indicating that these residues were essentially interchangeable (cf. Tet009 versus Tet023, Tet021, and Tet022). Rearranging the molecule such that all positively charged amino acids were localized furthest from the cellulose surface (Tet024) or in the middle of the peptide (Tet025) resulted in reduced antimicrobial activities. Conversely, localization of all charged amino acids at the tethered C terminus increased antimicrobial activity (Tet026), indicating that the hydrophobic



**Figure 1. Correlation between Bacterial Luminescence Inhibition and Antimicrobial Activities of Selected Tethered Antimicrobial Peptides**

*P. aeruginosa* H1001 was incubated with the described tethered antimicrobial peptides or cellulose alone for 4 hr at 37°C. The assays were carried out in 0.1M Tris-buffer (pH 7.3) supplemented with 20 mM glucose at a starting concentration of between  $1 \times 10^5$  and  $10^6$  colony forming units (CFU) per ml. The values indicated represent fold-change in CFU of the cultures treated with tethered peptides as compared with the control group incubated with cellulose.

residues in these tethered peptides should be optimally exposed to form direct contacts with bacteria.

As there appeared to be a relationship between peptide antimicrobial activity, charge, and hydrophobicity distribution, structure-activity relationship studies were performed. To determine the effect of charge and hydrophobic properties of the peptides on the peptides' inhibitory activity we generated simple linear regression models. There was a clear relationship between both charge and hydrophobicity distribution in the peptides when plotted against the tethered peptides antibacterial activity (Figure S1). There is a somewhat Gaussian distribution for both chemical properties across the peptide population, whereas the antibacterial activity levels are fairly evenly distributed throughout the peptide set with a slightly higher number of superactive peptides than completely inactive peptides (Figure S1).

As neither charge nor hydrophobicity alone could explain the peptide activity, we attempted combining several easily calculated peptide properties, identifying a relationship between them and the peptide inhibitory activity. For this purpose we calculated a multiple linear regression model considering a total of eight peptide properties, described previously. After successive removal of properties that did not significantly contribute to the model, a total of four properties remained in the final model shown in the following equation: charge, hydrophobic fraction, polar fraction, and hydrophobic moment along the length of the peptide.

$$I_{pred} = aQ + bH_f + cP_f + dH_m$$

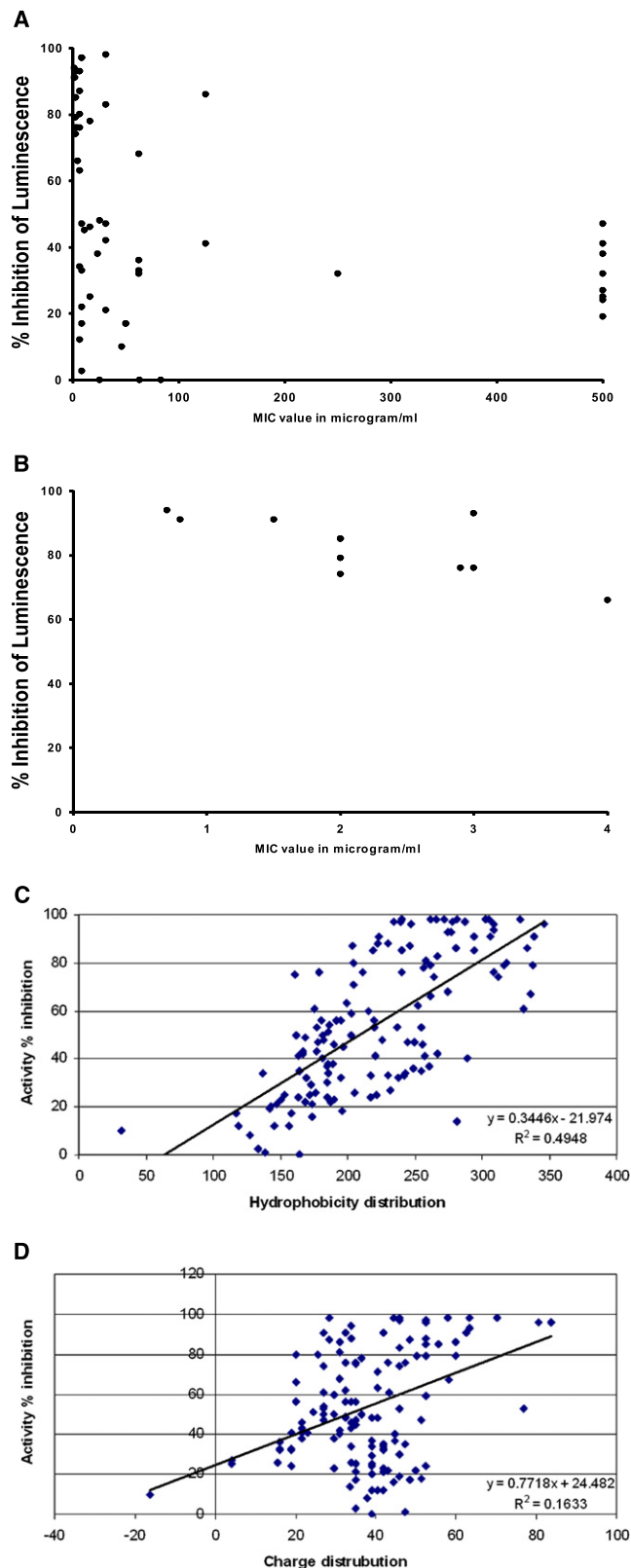
The equation models the predicted inhibitory activity ( $I_{pred}$ ) based on physical properties charge ( $Q$ ), hydrophobic fraction ( $H_f$ ), polar fraction ( $P_f$ ), and hydrophobic moment ( $H_m$ ). The coefficients for the contribution of each physical property is given by the coefficients for each  $a$ ,  $b$ ,  $c$ , and  $d$ .

A comparison of predicted inhibitory activity (using Equation 1) to measured inhibitory activity is shown in Figure S2. With this simple model, we found a reasonable quality of fit for all data with  $R^2 = 0.58$  ( $R$  is the Pearson correlation coefficient). A 10-fold cross-validation yielded a similar  $R^2$  of 0.54. The values of the predicted activities tend to cluster around the 40% predicted inhibitory activity value for a wide range of measured values (around 0%–60% measured activity). Importantly, only a limited number of peptides predicted to have good activity were found to be inactive in vitro, lending credence to the use of this model for computational screening of large numbers of “virtual” peptides prior to laboratory evaluation. Table 2 shows the expected model performance using thresholds of 80%, 90%, and 95% predicted inhibition. This demonstrates that the model is expected to be powerful in identifying additional peptides with high activity. For example, based on this, virtually all peptides predicted to have an activity above 95% inhibition are expected to demonstrate inhibition of bacterial growth by at least 95% (positive predictive value = 1.0). Because only 11% (15 of 136 peptides) have this level of activity, this gives an impressive enhancement ratio (ER) of 9.1 (1.0/0.11).

A direct relationship between the hydrophobic characteristics of the peptide and antibacterial activity was demonstrated such that insertion of hydrophobic amino acids away from the linker site results in tethered peptides with higher antibacterial activity (Figure 2C). Similar trends were observed for charge properties, although this appears to be limited by the necessity for presentation of the charged residues in a specific pattern. Thus, the concentration of charged residues away from the tethering surface did not result in increased antibacterial activity (Figure 2D). There appeared to be a complex relationship between antibacterial activity and overall cationicity and hydrophobicity, requiring the use of more-sophisticated quantitative structure-activity relationship approaches. As a result we were able to identify a reasonably meaningful relationship between these two peptide characteristics and the antibacterial activity.

To further explore structure-activity relationships, 48 sequence-scrambled peptides of Bac2A were examined for activity when tethered (Table S1). All analogs were composed of the same amino acids and consequently had conserved physicochemical properties (length, net charge, and proportion of hydrophilic and hydrophobic amino acids); however, the activities of these scrambled tethered peptides varied substantially. This clearly indicated that activity was not solely dependent on the specific composition of amino acids or the overall charge or hydrophobicity, but rather required particular sequence patterns, consistent with the structure-activity analyses described herein. Previously, the soluble versions of these randomly scrambled peptide variants had been tested for antimicrobial activity (Hilpert et al., 2006) and analogous conclusions were made; however, in comparing these data sets there was no specific relationship observed between peptide activity in the soluble or tethered forms. Thus it is clear that tethered peptides cannot be optimized using data from the soluble versions of the peptides.

The tethered analog of full-length bovine indolicidin Tet083 demonstrated weak-to-medium activity, whereas two indolicidin variants, Tet091 (FLPKKFRWWKYRK) and Tet092 (FIKWKFRWWKWRK), had very strong antimicrobial activities. Based on its potent antimicrobial activity, Tet092 was selected to design new peptide



**Figure 2. Correlation between Measured Antimicrobial Activity in Solution of the Soluble Peptides (MIC) and Luminescence Inhibition of the Tethered Peptides and Peptide Charge and Hydrophobicity**

**Table 2. Estimated Coefficients for the Model Describing the Inhibitory Activity of Tethered Peptides**

Coefficient (Property)	Estimated Value $\pm$ SE	p Value
a (charge)	$10.6 \pm 1.3$	$1.93 \times 10^{13}$
b (hydrophobic fraction)	$56.9 \pm 10.6$	$3.80 \times 10^7$
c (polar fraction)	$34.4 \pm 7.4$	$8.28 \times 10^6$
d (hydrophobic moment)	$0.38 \pm 0.10$	$5.76 \times 10^4$

Coefficients for a linear model of inhibitory activity were determined by multiple regression using Equation 1 as described in the text, namely  $I_{pred} = aQ + bH_f + cP_f + dH_m$ , where  $I_{pred}$  is the predicted inhibitory activity, and the variables are charge (Q), hydrophobic fraction (proportion of hydrophobic residues,  $H_f$ ), polar fraction as an assessment of charge distribution ( $P_f$ ), and hydrophobic moment ( $H_m$ ) as an assessment of amphipathicity along the length of the peptide. The contributions of each of these physical properties was given by the coefficients for each factor a, b, c, and d, and the final estimates provided here. The p value indicates the statistical significance of the estimate (i.e., probability coefficient is nonzero using Student's t test).

variants. The substitution of both Phe residues with other hydrophobic residues, Val or Trp, did not alter activity, nor did the substitution of both basic Arg residues with the equivalently basic residue Lys. In contrast, substituting all Lys residues with Arg (creating Tet099) resulted in decreased antimicrobial activity. Substituting one Arg of peptide Tet099 with Trp along with rearrangement of the sequence such that a hydrophobic patch appeared at the exposed N terminus led to the weakest activity in this series.

In addition to peptide variants of the same length as indolicidin (13-mer), shorter (7-, 9-, and 11-mer) peptides were tested for antimicrobial activity. The most active variants were found to be Tet110–Tet113, with Tet112 (KWKWWKWK) exhibiting the strongest activity.

Selected semirandom peptides exhibiting potent antimicrobial activity in solution (Cherkasov et al., 2008) were selected here to be synthesized as tethered peptides (Tet122–Tet134; Table S1) and tested for their ability to kill H1001. Most of these peptides demonstrate substantial inhibition of luminescence, with the only exception being Tet124 (KLWWMIRRW). The strongest antimicrobial activity at both peptide densities was demonstrated by Tet127 (KRWWKWWRR), which is consistent with the general conclusion that these peptides act electrostatically at the surface of bacteria rather than penetrating into the bacterial cell.

#### Confirmation of Antimicrobial Activity of Tethered Peptides on Other Surfaces

To enable examination of the peptide's antimicrobial activity in a regular MIC assay, a selected few of the peptides were tethered

(A) All assessed data pairs. The MIC values were taken as indicated from the references in Table 1 or measured here. The MIC values were used for the x axis values, whereas the measured values of inhibition of luminescence (at 200 nmol/spot) were used as y axis values. MIC values greater than 250  $\mu\text{g/ml}$  were set to 500  $\mu\text{g/ml}$ .

(B) Data pairs in the MIC range between 0.8 and 4  $\mu\text{g/ml}$ .

(C and D) The relationship between distribution of hydrophobic properties (C) and charged residues (D) along the peptide from the tethering site and out toward N-terminal end, with respect to the peptides antimicrobial activity. The units on the scales are relative values, calculated as described in Methods Development. In both cases, the p value was found to be  $<0.0001$ .

to the surface of a microtiter plate via biotin-streptavidin interaction. No peptide release could be demonstrated using HPLC when employing either strategy under the antimicrobial assay conditions (4 hr, 37°C, and 0.1 M Tris buffer [pH 7.5]). For these studies, a model peptide with intermediate activity, Tet052 (RRAAVLVIRRR), and the inactive control peptide Tet000 were selected.

Both peptides were coupled to biotin through the side chain amine group of a C-terminally added lysine. These purified and soluble peptides were incubated overnight in a microtiter plate coated with streptavidin, resulting in complete binding as the biotin/streptavidin interaction is extremely strong ( $K_d \sim 10^{-15}$  M). Washing was able to remove all nonspecifically associated peptide molecules (as confirmed using nonbiotinylated soluble Tet052). After an intensive washing procedure the *P. aeruginosa* strain H1001, buffer, and glucose were added to the wells of the streptavidin-coated microtiter plate. In the case of the biotin-labeled active peptide Tet052, the measured luminescence decreased to baseline over 4 hr. In addition, antimicrobial activity was also investigated by counting the surviving colonies at various times after addition of microbes to the wells of the microtiter tray, using *P. aeruginosa* strain H1001 (Gram-negative), *Staphylococcus aureus* (Gram-positive), and the yeast *Candida albicans*. Complete killing of each of these microbes was observed within 6 hr (Figure 3B).

Using the streptavidin/biotin-bound peptides, it was possible to freely change the density of the tethered peptides on the surface by utilizing different concentrations of biotinylated peptide solutions to bind to the streptavidin-coated plates. As expected, antimicrobial activity was clearly concentration dependent, decreasing sigmoidally as a function of decreasing peptide concentration. The minimum concentration of biotin-labeled Tet052 for a strong decrease in luminescence was determined to be 375  $\mu\text{g/ml}$ . A control experiment in which biotinylated Tet052 was added to a normal microtiter plate (not streptavidin coated and omitting the incubation and washing steps) resulted in a minimal inhibitory concentration of 4.6  $\mu\text{g/ml}$  for eliciting a strong reduction in luminescence. This indicated a requirement for a high surface density of peptide for killing.

### Characterization of the Mode of Action of Tethered Peptides

Scanning electron microscopy (SEM) was used to visualize any potential membrane damage of *P. aeruginosa* strain H1001 after 4 hr of contact with the tethered peptides. Four individual spots of the CAPE membrane (200 nmol/spot) were punched out; two positive controls (Tet009 and Tet052), as well as two negative controls (Tet000 and a spot containing no peptide). Contact with the active tethered peptides led to a dramatic effect on the morphology of the bacterial surface. Bacteria that were in contact with the control cellulose membranes with no attached peptides had cell surfaces with smooth appearance (Figure 4A). In contrast, the surfaces of bacteria that were in contact with the tethered peptides were characterized by a dense distribution of small protuberant structures, or blebs (Figure 4B). These observations indicated that the tethered peptides can destabilize the bacterial envelope.

Cellular membranes are normally impermeable to ATP. Because cytoplasmic membrane destabilization could lead to

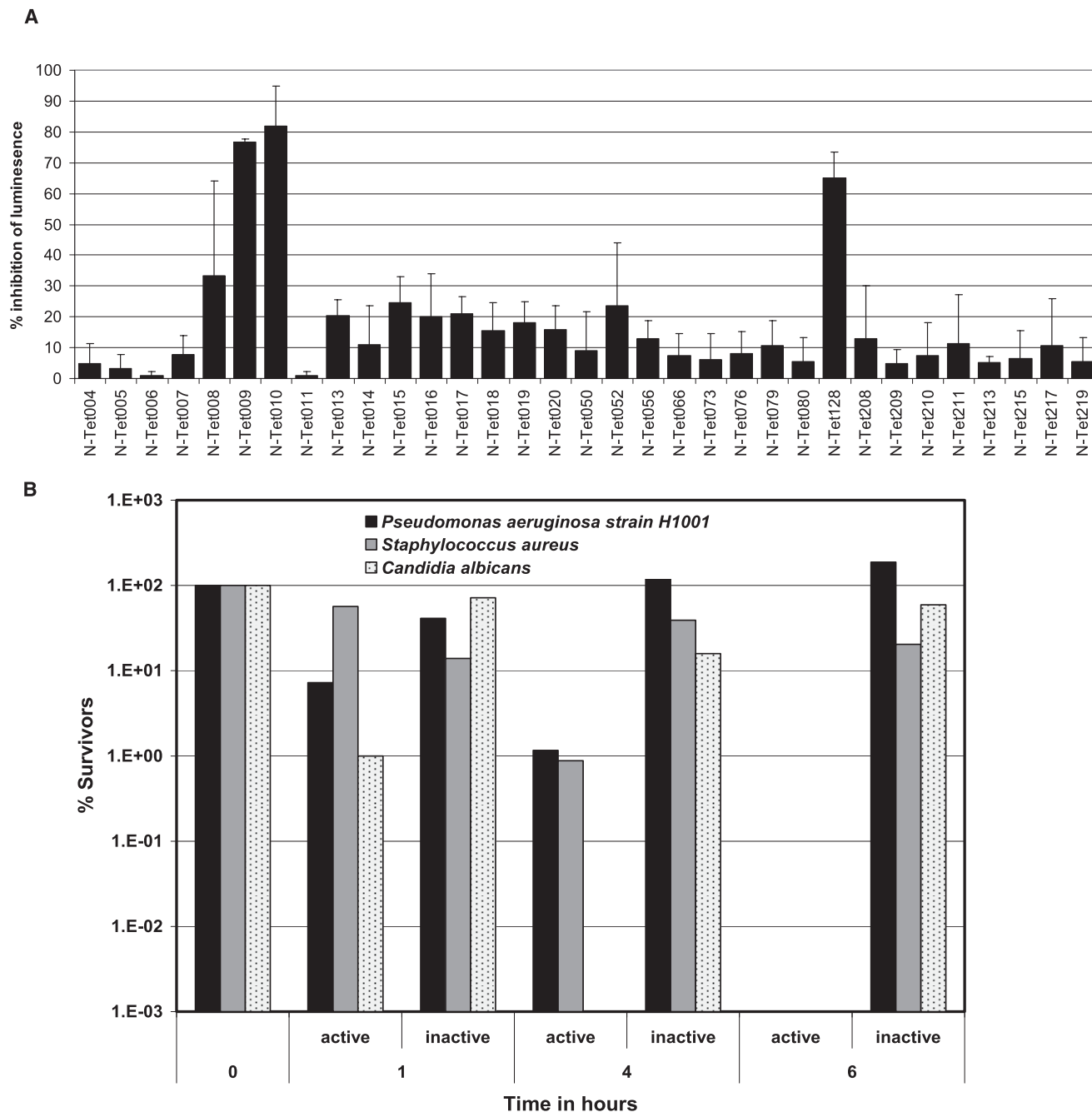
the leakage of normally impermeable substances, the level of ATP in the supernatant following contact of the bacteria with tethered peptides was determined (Table 3). After 30 min of contact with Tet052, a substantial leakage of ATP into the supernatant was demonstrated for all three tested pathogens, with greater leakage from the Gram-positive organism *S. aureus* compared with the yeast *C. albicans* and the Gram-negative bacterium *P. aeruginosa*. Treatment with the nonactive tethered peptide Tet000 led only to background levels of ATP in the supernatant. The different amounts of leakage of the three pathogens may relate to their individual susceptibilities and/or the greater cell envelope complexity of the latter two organisms.

The resin-bound tethered active peptide Tet052, the negative control peptide Tet000, Bac2A, the Bac2A parent peptide bactericidin, and the strongly depolarizing peptide gramicidin S were examined for the effects of the peptides on the membrane potential of the Gram-positive bacterium *S. aureus* (Figure 5A). As these cellulose-linked peptides did not allow for adjustments to peptide concentration, the peptides were instead tethered to resin utilizing a noncleavable linker. All peptides, with the exception of the negative control peptide Tet000, led to a measurable depolarization of membrane potential as assessed with the membrane potential-sensitive fluorescent dye diSC<sub>3</sub>5. The soluble analog of the active peptide Tet052 caused some depolarization at 3  $\mu\text{g/ml}$ , which corresponded to about 1/4 of the MIC (Hilpert et al., 2006), and full depolarization at 30  $\mu\text{g/ml}$ . Depolarization by the tethered analog of Tet052 occurred in a concentration-dependent manner between 0.3 and 1.2 mg/ml of resin-coupled peptide (assuming that each coupling step of the peptide synthesis was 100% successful, where 1.2 mg/ml resin represents 415  $\mu\text{g/ml}$  of surface-available peptide). Tethered Tet052 demonstrated sigmoidal depolarization kinetics as opposed to the hyperbolic kinetics observed with the soluble peptides. The lag phases (which ranged from 5 to 20 min), slopes, and the final heights of the sigmoidal curves obtained from the resin-tethered peptides were concentration dependent.

To investigate whether the observed lag phase reflected a bacterial event that had to occur after contact with the tethered peptides, the same depolarization experiment was performed following a 30 min pretreatment of bacteria with 50  $\mu\text{g/ml}$  rifampicin, an inhibitor of DNA-dependent RNA polymerase. The resulting inhibition of transcription would block any new protein synthesis that might occur as a result of bacterial contact with the tethered peptides (Figure 5B). The data showed that the depolarization caused by the tethered active peptide was independent of induced signaling pathways involving the production of new mRNA.

### Proposed Working Model

The active peptides Tet052 and Tet009 carry net positive charges of +4 and +6, respectively. Microbes are known to have negatively charged surfaces. The polycationic charges on the peptides, present at high density on the tethering surface, will therefore strongly attract the polyanionic microbial surface. Initially, an electrostatic interaction would occur between the negatively charged outer layer of the bacterium and the positively charged tethered peptides. This attraction would draw the microbes closer to the tethered peptide surface. The high local concentration of the tethered peptides would lead to the



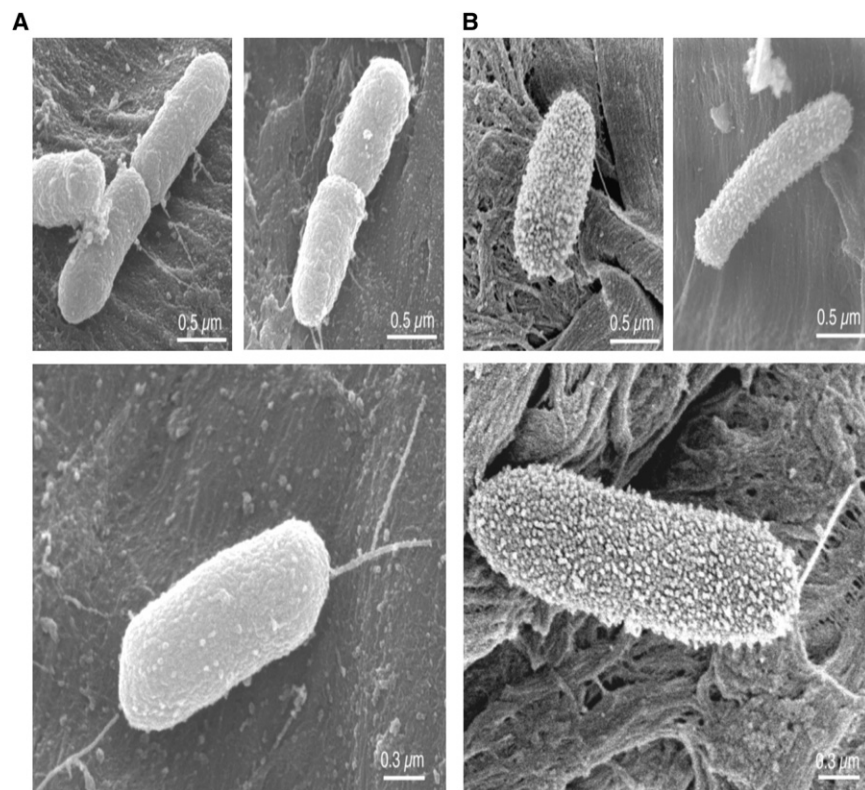
**Figure 3. Antimicrobial Activity of Surface-Bound Peptides Utilizing Biotin-Streptavidin Tethering**

(A) The constitutive luciferase-producing *P. aeruginosa* strain H1001 was exposed to the streptavidin/N-terminal biotin-coupled peptides. The luminescence was measured after 4 hr of incubation. Data shown represents the mean  $\pm$  SD of two independent data sets. The biotinylation of the peptides was performed at the N terminus. These peptides have identical sequences as peptides in Table 1, and have for comparative reasons been given the same numbers.

(B) *P. aeruginosa* H1001, *S. aureus*, and *C. albicans* were exposed to streptavidin/N-terminal biotin-coupled Tet052, and surviving colonies were counted at various time points following addition of microbes to the surface-bound peptides.

displacement of positively charged counterions attached to the outer surface layers, and could thus induce a dramatic change in bacterial surface electrostatics. This process may introduce an electrostatic imbalance—e.g., an alteration of the Donnan potential across the cytoplasmic membrane—which could trigger a lethal event such as activation of autolytic enzymes or

disruption of the ionic balance of more-internal layers. To examine this possibility, we investigated the ability of ethylenediamine tetraacetate (EDTA), a divalent cation chelator and known permeabilizer of Gram-negative bacterial outer membranes, to depolarize the cytoplasmic membrane of the Gram-positive bacterium *S. aureus* (Figure 5C). At elevated concentrations



**Figure 4. Bacterial Membrane Damage Induced by Tethered Peptides**

Scanning electron micrographs of *P. aeruginosa* cells that were in contact with (A) membrane without peptide (bottom large picture and small picture to the right), an inactive peptide (small picture to the left), and (B) membrane with the active tethered peptide Tet009 (bottom large picture and small picture to the left right) and the active tethered peptide Tet052 (small picture to the left). The bacteria were incubated in contact with membranes for 4 hr at 37°C before fixation and preparation for SEM.

lose was chosen for this work because it is a time- and cost-efficient method for primary screening (Hilpert et al., 2007; Frank, 2002). Central to this unique methodology is the combination of cellulose-bound peptide arrays with a CAPE linker strategy and a rapid screening procedure that employs the Gram-negative bioluminescent bacterium *P. aeruginosa* (H1001). The genetically introduced bioluminescence of this bacterium is strictly dependent on a supply of energy in the form of flavin mononucleotide, and killing of bacteria by soluble peptides

>5 mM (compared with the 1.5 mM, which permeabilizes Gram-negative bacteria), EDTA caused the depolarization of *Staphylococcus*, demonstrating both a concentration-dependent lag phase and slope reminiscent of that observed for the tethered active peptides.

## DISCUSSION

Because it has previously been demonstrated that peptides can kill bacteria while tethered to surfaces (LaPorte et al., 1977; Haynie et al., 1995), we focused here on the development of a screening technology for peptide variants that retain antimicrobial activity when bound to a surface. This was clearly appropriate, as we have also demonstrated that there are independent determinants of activity for tethered peptides as compared with their free soluble analogs. Peptide spot synthesis on cellulose

results in a loss of luminescence, demonstrating rapid kinetics that parallel the loss of viability of the bacteria (Hilpert et al., 2005). Using this screen, most peptides showed a consistent inhibition rate throughout the three rounds of biological assays.

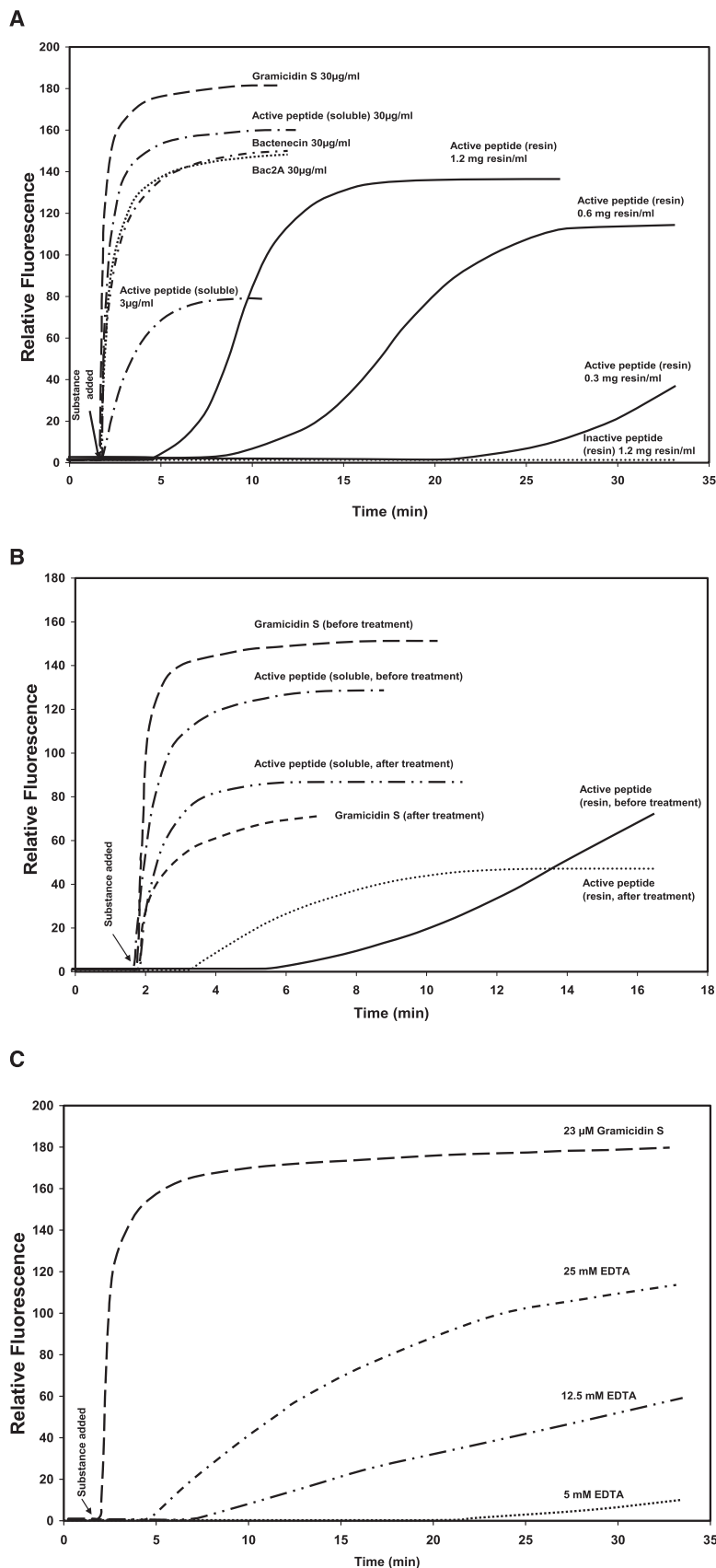
The observed antimicrobial activity of the tethered peptides is influenced by the coupling method, the linker, the spacer, and the context of the sequence. Through structure-activity relationship studies we have demonstrated that the context of hydrophobic and cationic residues within a tethered peptide dictates the direct antimicrobial activity. This would be anticipated as these two physicochemical properties have previously been demonstrated to be critical contributors to the activity of antimicrobial peptides in numerous investigations (Jenssen et al., 2006; Yeaman and Yount, 2003). Indeed, the placement of cationic residues close to the linker site correlated with increased antimicrobial activity as compared with peptides with cationic residues localized to the N terminus or within the middle portion of the peptide. Peptides with cationic residues at both termini had comparable activities to those with N-terminally localized basic residues and would suggest that although cationic residues are not a necessity for tethered peptide antimicrobial activity, they must occupy the residue sites closest to the linker. Equally as important to activity is the positioning of hydrophobic residues within the tethered peptides; the positioning of hydrophobic residues proximal to the N terminus was critical for activity. This is perhaps unsurprising because an overall hydrophobic region close to the N terminus would allow for optimal interaction with the bacterial membrane and may therefore act as a tethering point between the microbe and the surface. This would also extend to N-terminal basic residues,

**Table 3. ATP Release by Tethered Peptides**

Peptide	ATP Released into Supernatant (fM)		
	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>	<i>Candida albicans</i>
Bac020 <sup>T</sup> (active)	154 ± 14	552 ± 98	224 ± 9
Neg <sup>T</sup> (inactive)	22 ± 4	18 ± 1	25 ± 6
Ratio	7	29	9

Bacteria or yeast ( $1 \times 10^6$  cfu/ml) were exposed for 30 min at 37°C to tethered (via biotin/streptavidin) peptides and then centrifuged to obtain extracellular supernatants. Released ATP was measured using a luminescence kit. The data are mean values of at least three independent measurements performed in duplicate.





**Figure 5. Membrane Depolarization of *S. aureus* Cells Using the Membrane Potential Sensitive Dye diSC<sub>35</sub>**

(A) Measurements of membrane depolarization of nontethered compounds gramicidin S, Bac2A (RLARIVVIRVAR-NH<sub>2</sub>), active peptide Tet052 (RRAAVLVIRR-NH<sub>2</sub>), and bactenecin (RLCRIVVIRVCR-NH<sub>2</sub>), as well as the tethered compounds active peptide Tet052 (RRAAVLVIRR) and inactive peptide (GATPEDLNQKLS) bound to resin. The concentrations used in the assay are stated on the graph. For this and other parts of this figure, the fluorescence values were measured every second. Smoothed lines were used to describe the data points. The graphs represent results from one experiment of at least three that demonstrated similar trends.

(B) Measurements of membrane depolarization were performed before and after treatment with rifampicin. In this assay we used nontethered gramicidin S before treatment and after treatment, active peptide Tet052 before treatment and after treatment, and the tethered compounds: Tet052 before treatment bound to resin and after treatment. The concentrations for the soluble peptides were 30  $\mu$ g/ml and for the tethered one 0.6 mg resin/ml.

(C) Membrane depolarization of *S. aureus* assessed with different concentrations of EDTA and gramicidin S as a positive control. The concentrations used in the assay are stated on the graph.

but the limitation in this context may be the absolute concentration of cationic residues in this region. Indeed, the cationic residues of antimicrobial peptides can interact electrostatically with negatively charged microbial lipopolysaccharide or lipoteichoic acid; however, it has been postulated that increased cationicity past a specific threshold may in fact reduce overall peptide activity due to the immobilization of the peptide at the microbial surface. Thus, the hydrophobic core may act to optimally expose charges to permit more-effective formation of direct contacts with bacteria, especially as these peptides are far too short to penetrate far into the bacterial membrane.

The N-terminal biotinylation and streptavidin-tethering of peptides (synthesized in a high-throughput array on cellulose, followed by cleavage) provides a tool for screening variants with a free C terminus. In the initial experiments, all peptides were directly tethered via covalent amino acid linkers at the C terminus. In subsequent screens, three peptides also showed strong antimicrobial activity when N-terminally biotinylated and tethered, and two of these also showed strong activity when tethered via C-terminal biotinylation. Nevertheless, the present data show that cellulose-bound peptides with various linker chemistries, streptavidin/biotin-bound peptides, and resin-bound peptides are all able to kill bacteria. This observation strengthens our hypothesis that the killing of the bacteria by tethered peptides is in principle independent of the support to which they are coupled and does not require the actual penetration of the peptide into the bacteria. Indeed the observation that very short peptides as small as 9 aa had good activity when tethered is consistent with this view as it requires 14 aa in a beta conformation to stretch across the cytoplasmic membrane and Gram-negative bacteria like *P. aeruginosa* have an additional outer membrane protecting the cells.

Bac2A is only 12 aa long, and the maximum length of the stretched molecule coupled to cellulose via, for example, a glycine linker, would be about 5.5 nm. The cell envelopes of Gram-negative bacteria (comprising an outer membrane and narrow peptidoglycan layer) and Gram-positive bacteria (comprising a thicker peptidoglycan layer) are between 20 and 50 nm thick. The data gathered to this point clearly indicate that tethered peptides can kill Gram-positive bacteria, Gram-negative bacteria, and yeast, indicating that they must act in some analogous manner on all three. Results of SEM, ATP release, and depolarization assays indicate strongly that the peptides destabilize the cell envelope of the pathogens. Thus we conclude that it is likely that the disturbance of surface electrostatics must trigger an autolytic and/or cell death mechanism.

The methods described here can be used to screen large numbers of tethered peptides for antimicrobial activity against any chosen pathogen very rapidly at low cost. This may open up a new field of application for host defense peptides and may lead to new ways of protecting medical devices and other surfaces against the growth of pathogens.

## SIGNIFICANCE

**Due to the increasing incidence of infections associated with medical devices, we have proposed a methodology for coating the surfaces of medical devices with antimicrobial peptides. Importantly, this procedure utilizes a linker**

**strategy that provides a covalent, noncleavable linkage between the peptide and the surface. A library of 122 tethered antimicrobial peptides were screened utilizing a high-throughput antimicrobial peptide screening assay that measures luciferase production from a *Pseudomonas aeruginosa* strain with a constitutively expressed luciferase (*luxCDABE*) gene cassette. This study has identified that the activities of tethered antimicrobial peptides do not directly mimic those of their soluble analogs. Through structure-activity relationship studies of the tethered peptide library it has been demonstrated that antimicrobial activity of tethered peptides is influenced by the linker and the context of the sequence. Indeed, the distribution of hydrophobic and cationic residues within a tethered peptide dictates the direct antimicrobial activity; cationic residues located proximal to the linker site correlated with increased antimicrobial activity; and the positioning of hydrophobic residues close to the N terminus were critical for activity.**

## EXPERIMENTAL PROCEDURES

### Strains

For the screening assay, a mini-*Tn5-lux fliC::luxCDABE* mutant strain H1001 of *P. aeruginosa* PAO1 was used. This strain contained a transcriptional fusion, resulting in constitutive expression of luciferase (Lewenza et al., 2005). Additional strains used for determining survivors after contact with tethered peptides included *S. aureus* ATCC25923 and a lab isolate of *C. albicans* obtained from Dr. B. Dill (Department of Microbiology and Immunology, University of British Columbia).

### Peptide Synthesis

Peptide syntheses on cellulose were performed using a pipetting robot (Intavis; Köln, Germany) and Whatman 50 cellulose membranes (Whatman; Maidstone, UK) (Hilpert et al., 2007). The CAPE linker chemistries were applied as described previously (Frank, 1992). For further characterization, peptides were synthesized by Fmoc chemistry in our laboratories (Humboldt-Universität, Berlin). For the synthesis of peptides tethered to resin, a bifunctional resin, TGS (1-6-dichloro-1- $\beta$ -dideoxy- $\beta$ -fructofuranosyl-m-chloro-4-deoxy-galactopyranoside) NH<sub>2</sub>/RAM (p-[(R,S)-a-[1-(9H-Fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phen-oxyacetic acid) (Rapp Polymere; Tübingen, Germany) was used. The ratio of NH<sub>2</sub> and RAM was 0.243 mmol/g to 0.017 mmol/g. The peptide concentration was estimated from the weighed sample.

### Determination of Linker Stability

The peptides were synthesized on cellulose using three different linker strategies (Frank, 1992). After side-chain deprotection by trifluoroacetic acid (TFA) treatment, all peptide spots were punched out with a standard paper puncher and transferred to individual wells of a 96-well plate. All cellulose spots containing the bound peptides were washed five times with 98% ethanol for 5 min, and then equilibrated with 100 mM Tris buffer (pH 7.5) five times for 5 min. Subsequently, 100  $\mu$ l of 0.1 M Tris buffer (pH 7.5) was added and all spots were incubated for 4 hr at 37°C. The supernatant (80  $\mu$ l) of each well was transferred into vials and HPLC of the supernatant was performed. Forty microliters of the sample solution was injected into a LC-20 System (Shimadzu; Kyoto, Japan). As eluents, water (+0.1% TFA; eluent A) and acetonitrile (+0.1% TFA; eluent B) were used. The analytical HPLC was performed using a C-18 RP-HPLC column (Grace Vydac; Hesperia, CA) with a linear gradient (0% B to 50% B in 20 min), followed by a 5 min wash with 95% B and final equilibration with 100% A for another 5 min at a flow of 1 ml/min.

### Screening Assay for the Peptides on Cellulose Support

The peptide spots were punched out and transferred to a 96-well microtiter plate. The spots were washed five times with 98% ethanol for 5 min and then equilibrated with 100 mM Tris-HCl buffer (pH 7.5) five times for 5 min. An overnight culture of *P. aeruginosa* strain H1001 was diluted 1:50 in

Mueller-Hinton (MH) medium and incubated at 37°C until an OD<sub>600</sub> of 0.35 was observed. This bacterial culture was diluted 1:25 in 100 mM Tris-HCl buffer (pH 7.5) containing 20 mM glucose. Fifty microliters of this culture was added to the wells containing the peptide spots and incubated at 37°C for 4 hr. The luminescence produced by the FMN-dependent luciferase system was detected in a time-dependent manner using a Tecan Spectrafluor Plus (Tecan U.S., Inc.; Durham, NC). At the end of the experiment, the cellulose spots were washed once with 98% ethanol for 5 min. After removing the ethanol, the membrane was air-dried overnight. Before performing an additional biological repeat, the spots were washed five times with 98% ethanol for 5 min and then equilibrated with 100 mM Tris-HCl buffer (pH 7.5) 5 times for 5 min. After that, the new diluted overnight culture was added as described.

#### Killing Assay for Peptides Bound via Biotin/Streptavidin Complex

(A) For peptides synthesized on cellulose, biotinylated peptides were punched out and transferred into a standard noncoated microtiter plate. Into each well that contained a peptide spot, 70  $\mu$ l of 100 mM Tris-HCl buffer (pH 7.5) was added. The microtiter plate was agitated for 4 hr at room temperature. Fifty microliters of each solution was then transferred into independent wells of a high-density coated streptavidin plate (Perkin Elmer; Vaudreuil, Quebec, Canada) and incubated overnight at 4°C. (B) For peptide solutions with known concentration, the desired peptide concentrations were prepared and transferred into high-density coated streptavidin plates (Perkin Elmer) and incubated overnight at 4°C. For (A) and (B) the plate was washed 50 times with distilled water and 10 times with 100 mM Tris buffer (pH 7.5). An overnight culture of *P. aeruginosa* strain H1001 was diluted 1:50 in MH medium and incubated at 37°C until an OD<sub>600</sub> of 0.35 was observed. This bacterial culture was diluted 1:50 in 100 mM Tris buffer (pH 7.5) containing 20 mM glucose. Fifty microliters of this culture was added to all wells containing peptides bound to the streptavidin plate and incubated at 37°C. The luminescence produced by the FMN-dependent luciferase system was detected in a time-dependent manner using a Tecan Spectrafluor Plus (Tecan U.S., Inc.).

#### Scanning Electron Microscopy

CAPE membranes with or without tethered peptides were incubated with *P. aeruginosa* (1:25 dilution of cells suspended at an OD<sub>600</sub> of 0.35). Samples were then fixed with 1% glutaraldehyde for 1 hr, postfixed with 1% osmium tetroxide for 30 min, stained with 2% uranyl acetate for 30 min, and dehydrated in an ethanol series (30%, 50%, 70%, 85%, 95%, and 100%; 5 min each). Samples were dried in a Tousimis Critical Point Dryer (Tousimis; Rockville, MD), sputter coated with Pt/Pd (2 nm), and observed on a Hitachi S4700 scanning electron microscope (Hitachi High Technologies America, Inc.; Pleasanton, CA) operated at 5 KV with a working distance of 8 mm.

#### ATP Determination

The ATP concentration was determined using the ATP Bioluminescent Assay Kit (Sigma Aldrich; Oakville, Ontario, Canada), according to the manufacturer's instructions. After incubating the microbes for 30 min with biotin-tethered peptides, 10  $\mu$ l of sample was taken and mixed with 60  $\mu$ l 100 mM Tris-HCl buffer (pH 7.5) containing 10  $\mu$ l assay kit solution. By using an ATP standard curve, which was measured for each experimental set, the amount of ATP in the samples was calculated. The integration time for the luminescence was set to 1500 ms, and the measurement was performed at 37°C.

#### Membrane Depolarization

The resin was weighed and transferred into a 1.5 ml tube, and 1500  $\mu$ l of 20 mM carbonate-bicarbonate buffer (pH 10) was added and incubated on a shaker for 30 min at room temperature. The resin was centrifuged at 10,000 rpm and washed five times using 1500  $\mu$ l of 20 mM carbonate-bicarbonate buffer (pH 10); equilibrated with 100 mM Tris buffer (pH 7.5) by washing five times with this buffer; and then washed three times with distilled water and resolved in distilled water. The assay was performed as reported previously (Wu and Hancock, 1999b). Briefly, depolarization was monitored over time by a membrane potential-sensitive fluorescence dye, diSC<sub>3</sub>5, in a Luminescence Spectrometer LS50B (Perkin Elmer; Woodbridge, Ontario, Canada) at room temperature. The depolarization of the cytoplasmic membrane was determined with the Gram-positive bacterium *S. aureus* ATCC 25923. Exponential-phase bacteria were washed and resuspended in 5 mM HEPES 20 mM

glucose buffer (pH 7.2) to an optical density of 0.05. This cell suspension was incubated with 100 mM KCl, and after 10 min 0.4  $\mu$ M DiSC<sub>3</sub>5 were added. After 30 min, or until there was a stable (~90%) reduction in fluorescence, a 2 ml aliquot of cell suspension was placed in a cuvette, and the desired concentration of peptide was added. Fluorescence was monitored at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

#### Minimal Inhibitory Concentration (MIC) Determination

The MIC of the peptides was measured using a modified broth microdilution method (Wiegand et al., 2008) in MH medium. After incubation at 37°C for 12–15 hr, the MIC was taken as the concentration at which no growth was observed.

#### Structure-Activity Relationship Studies

To illustrate the relationship between the molecular distribution of charge and hydrophobicity along the peptide sequence, charge and hydrophobicity properties were calculated for each of the peptides in Table 1 and Table S1. The respective amino acids were given a numerical value of 1.349 Å, corresponding to the length of the peptide bond multiplied by its numerical position in the peptide from the C-terminal end (tethering position). Cationic residues were given the numerical value of +1 multiplied by the numerical value for the respective amino acid, resulting in a higher weighting of charge when present further away from the cellulose. Anionic residues were given a numerical value of -1, and neutral amino acids were set to zero. The hydrophobic potential of the different amino acids was determined using the scale of Black and Mould (1991).

To determine the relationship between the inhibitory activity and physical properties of the peptides, we used a simple multiple linear regression using generalized linear model fitting (from the glm package of the R project, <http://www.r-project.org/>). We considered eight physical properties: total charge at pH 7.0, charge distribution along the peptide, hydrophobic moment along the length of the peptide (both as described in the previous section), plus five values reflecting the composition of the amino acids independent of position. These five were the composition of aliphatic, aromatic, hydrophilic, hydrophobic, and polar residues. To identify the most significant properties to explain inhibitory activity, all eight properties were initially used in a multiple regression model. The property with the least significant coefficient (largest p value) was dropped from consideration and the model was recalculated. Successive elimination of properties was repeated until all coefficients were found to be significantly different from zero using t test (p value < 0.05). Four properties were used in the final model: charge, polar fraction, hydrophobic fraction, and hydrophobic moment, shown in Table 2. A 20-fold cross-validation was performed as usual by randomly assigning each peptide to one of ten groups, training on nine of the ten and predicting the group not used for training, reiterated a total of ten times. Therefore, for the cross-validation the peptide data used for prediction was not used for training the models used for prediction.

Classification analysis was performed considering the peptides correctly or incorrectly classified above the threshold in predicted inhibitory activity (true positive, TP; false positive, FP) or correctly or incorrectly classified below the threshold in predicted activity (true negative, TN; false negative, FN). Sensitivity [TP/(TP + FN)], specificity [TN/(FP + TN)], positivity predictive value [TP/(TP + FP)] and enhancement ratio [(TP/TP+FP)/((TP+FN)/(TP+FN+TN+FP))] were calculated.

#### SUPPLEMENTAL DATA

Supplemental Data include two figures, one table, and Supplemental References and can be found with this article online at [http://www.cell.com/chemistry-biology/supplemental/S1074-5521\(08\)00453-5](http://www.cell.com/chemistry-biology/supplemental/S1074-5521(08)00453-5).

#### ACKNOWLEDGMENTS

We are grateful to Derrick Horne at the University of British Columbia Bioimaging Facility for his assistance with SEM. We acknowledge the financial assistance of the Advanced Foods and Materials Network, and the Natural Sciences and Engineering Council of Canada through their Collaborative Health Research Projects program. R.E.W.H. was supported by a Canada

Research Chair award. K.H. was supported by a fellowship from the Canadian Institutes of Health Research (CIHR). C.D.F. received a Doctoral Research Award from CIHR. J.K. was supported by a fellowship from the Canadian Cystic Fibrosis Foundation. The authors declare competing financial interest as they have submitted a patent application, assigned to the University of British Columbia, on some of these peptides.

Received: July 9, 2008

Revised: November 14, 2008

Accepted: November 24, 2008

Published: January 29, 2009

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