

Research article

# Binding of protegrin-1 to *Pseudomonas aeruginosa* and *Burkholderia cepacia*

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## Abstract

**Background:** *Pseudomonas aeruginosa* and *Burkholderia cepacia* infections of cystic fibrosis patients' lungs are often resistant to conventional antibiotic therapy. Protegrins are antimicrobial peptides with potent activity against many bacteria, including *P. aeruginosa*. The present study evaluates the correlation between protegrin-1 (PG-1) sensitivity/resistance and protegrin binding in *P. aeruginosa* and *B. cepacia*.

**Methods:** The PG-1 sensitivity/resistance and PG-1 binding properties of *P. aeruginosa* and *B. cepacia* were assessed using radial diffusion assays, radiiodinated PG-1, and surface plasmon resonance (BiaCore).

**Results:** The six *P. aeruginosa* strains examined were very sensitive to PG-1, exhibiting minimal active concentrations from 0.0625–0.5 µg/ml in radial diffusion assays. In contrast, all five *B. cepacia* strains examined were greater than 10-fold to 100-fold more resistant, with minimal active concentrations ranging from 6–10 µg/ml. When incubated with a radiiodinated variant of PG-1, a sensitive *P. aeruginosa* strain bound considerably more protegrin molecules per cell than a resistant *B. cepacia* strain. Binding/diffusion and surface plasmon resonance assays revealed that isolated lipopolysaccharide (LPS) and lipid A from the sensitive *P. aeruginosa* strains bound PG-1 more effectively than LPS and lipid A from resistant *B. cepacia* strains.

**Conclusion:** These findings support the hypothesis that the relative resistance of *B. cepacia* to protegrin is due to a reduced number of PG-1 binding sites on the lipid A moiety of its LPS.

**Keywords:** *Burkholderia cepacia*, lipid A, lipopolysaccharide, protegrin, *Pseudomonas aeruginosa*

## Introduction

Cystic fibrosis (CF) is a generalized exocrine disease resulting from the defective regulation of epithelial chloride ion transport within various organ systems, most importantly the lungs [1–4]. This dysfunction can arise from a variety of mutations within the cystic fibrosis transmembrane conductance regulator (CFTR) gene on chromosome 7, which encodes the transmembrane pump responsible for regulating transepithelial ion levels [1,2,4]. This defect is associated with an increased viscosity of the airway surface fluid, and according to some studies, an altered salt concentra-

tion [5,6]. These and other pleiotropic effects of the CF gene abnormality increase the susceptibility of CF patients to bronchopulmonary bacterial infection by opportunistic pathogens.

*Pseudomonas aeruginosa* is the most common pathogen recovered from the lungs of CF patients [2,3]. This predisposition to *P. aeruginosa* infection has been attributed to several factors. For example, an increase in asialo-GM<sub>1</sub> glycoproteins on the apical surface of CF airway epithelial cells provides greater opportunity for adhesion by the type

IV pili of *P. aeruginosa*[3,7,8]. Furthermore, the first extracellular domain of wild type CFTR has been found to act as a *P. aeruginosa*-specific receptor, which is involved in the clearance of *P. aeruginosa* from the airways via epithelial cell desquamation [9–11], a process greatly impaired in the CF lung. Other conditions that abet colonization of the CF lung include impairment of the "mucociliary escalator" and macrophage-mediated phagocytosis by the viscous airway secretions. If elevated salt concentrations exist within these secretions [5,6], they would inhibit  $\alpha$  and  $\beta$ -defensins, endogenous antibacterial peptides produced by neutrophils and airway epithelia, respectively [2,3,12–15]. This last point is still under review as recent evidence suggests that CF airway surface fluid may be isotonic not hypertonic [16].

In recent years *Burkholderia cepacia* has become the second leading cause of death in patients with chronic granulomatous disease (CGD). The phagocytes of such patients are deficient in their capacity to kill organisms by oxidative means because of a defective NADPH oxidase [17,18]. *B. cepacia* also poses a threat to patients with CF, either co-colonizing those already infected with *P. aeruginosa* or as the sole infecting agent [2,18]. Although rates of colonization can vary widely (between 5 and 70%), *B. cepacia* is isolated from sputum in only 10%–20% of patients with CF [2,3]. *B. cepacia*, a very diverse species, has been divided into several "classes" of bacteria, i.e., genomovars, collectively referred to as the *B. cepacia* complex. Genomovar III is the most common *B. cepacia* genomovar isolated from CF patients, accounting for approximately 80% of *B. cepacia* isolates in CF patients. Genomovars II and V have also been recovered from CF patients [18]. Of critical concern are *B. cepacia*'s transmissibility from one patient to another and its propensity to give rise to the *B. cepacia* syndrome, which results in a rapid decline in pulmonary function [2,18]. The ability of *B. cepacia* as well as *P. aeruginosa* to cause chronic bronchopulmonary infections in CF patients is exacerbated by their intrinsic or acquired resistance to many conventional antibiotics. Peptide antibiotics, including protegrins, are currently under consideration as novel agents for treating pulmonary infections in CF patients.

Antimicrobial peptides are synthesized either nonribosomally or ribosomally [19]. Protegrin-1 (PG-1), a 2 kDa cationic octadecapeptide (RGGRLCYCRRRFCVVCVGR-amide) was originally isolated from porcine leukocytes [20] and falls into the latter category. We selected it for this study because it is rapidly bactericidal, functions well at elevated physiological salt concentrations such as those that may occur in the CF lung, and has a broad spectrum of activity [21–23]. PG-1's antiparallel  $\beta$ -hairpin structure and antimicrobial activity in physiological and elevated salt concentrations are maintained by its two intramolecular cystine disulfide bonds [21,24–28]. The antimicrobial spectrum of

PG-1 includes *Chlamydia trachomatis*, *Candida albicans*, *Escherichia coli*, *Fusobacterium nucleatum*, *Haemophilus ducreyi*, *Listeria monocytogenes*, *Neisseria gonorrhoeae*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *P. aeruginosa*, and *Staphylococcus aureus*[20,22,25,29–32].

This study tested the hypothesis that the relative sensitivity or resistance of *P. aeruginosa* and *B. cepacia* strains to PG-1 correlates to the extent of peptide binding. A previously described two-stage radial diffusion technique designed for peptide antibiotics was used to determine susceptibility. Binding of protegrins to intact *P. aeruginosa* and *B. cepacia* cells and to purified lipopolysaccharide (LPS) and lipid A prepared from these organisms was measured by three different techniques.

## Materials and methods

### Bacterial strains and growth media

Two sets of genetically related *P. aeruginosa* strains were studied. Strain 144M, a serum-sensitive mucoid isolate from a CF patient, contains short O-side chain LPS, while its serum-resistant derivative, 144M(SR), which is also mucoid, has long O-side chain LPS [33]. Strain FRD-1 is a mucoid CF isolate [34] and FRD-2 is its spontaneous nonmucoid derivative [35]. *P. aeruginosa* ATCC strains 10145 and 9027 are nonmucoid strains with long O-side chain LPSs [N L Schiller, unpublished results]. We also studied *B. cepacia* ATCC strains 25416 and 25609, as well as three clinical strains isolated from patients with CF in Vancouver, Canada, kindly provided by David P Speert, University of British Columbia. These strains included: C4813, genomovar IIIa, *B. cepacia* epidemic strain marker (BCESM) positive, cable pilin subunit gene A (cable A) negative; C4878, genomovar IIIa, BCESM positive, cable A negative; and C6159, genomovar IIIb, BCESM and cable A negative. BCESM and cable A are putative transmissibility markers [36]. Log-phase cultures were grown at 37°C with shaking in trypticase soy broth (TSB) (Becton-Dickinson Microbiology, Cockeysville, MD).

### Protegrin

PG-1 was prepared as previously described [25] and was approximately 96.5% pure. PG-1 was dissolved in and diluted with filter-sterilized acidified water (0.01% acetic acid) supplemented with 0.1% human serum albumin (Sigma, St. Louis, MO). The albumin in the PG-1 vehicle helped reduce nonspecific adsorption of protegrins when the stock solutions were serially diluted [22,37]. These PG-1 stock solutions were filter-sterilized prior to storage at either -20°C for short term (<3 months) or -80°C for long-term storage.

### Radial diffusion assay

The two-stage radial diffusion assay protocol as described by Steinberg and Lehrer [37] and Lehrer et al. [38] was al-

tered by supplementing the underlay agar with 150 mM NaCl to ascertain PG-1's ability to kill *P. aeruginosa* and *B. cepacia* at an elevated salt concentration. These assays were interpreted as described below. The sample wells received 5  $\mu$ l of PG-1 (various concentrations) or its vehicle. We analyzed the radial diffusion assay results in two ways. In one, we calculated the lethal concentration ( $C_L$ ) as described by Hultmark *et al.* [39,40] from wells surrounded by net clear zones of 1 to 2.5 mm. In the other, we defined the minimal active concentration (MAC) as the lowest concentration of PG-1 that produced a measurable, totally clear, inhibition zone around the well.

#### **Whole cell binding assay**

Binding of PG-1 to intact cells was measured with a monoiodinated variant of PG-1. This protegrin variant differed from PG-1 in two ways: Phe7 replaced Tyr7 in the protegrin domain, and an N-terminal glycine-rich hexapeptide extension (GGGYGG) with a single tyrosine residue was present. In this report the modified peptide and its monoiodinated variant will be called "GGPG" and "I-GGPG", respectively. The full sequence of GGPG is GGGYGGRG-GRLCFCRRRFCVVCVGR-CONH<sub>2</sub>. In preliminary experiments, we determined that PG-1, GGPG, and I-GGPG exhibited virtually identical antimicrobial potency and kinetics (data not shown).

GGPG was iodinated with NaI or Na<sup>125</sup>I (Pierce, Rockford, IL) by the iodobead method [41,42]. Unincorporated NaI or Na<sup>125</sup>I was removed by solid phase extraction on a Sep-Pak C-18 Plus Cartridge. I-GGPG was purified from uniodinated and di-iodinated GGPG by reverse phase-HPLC (Vydac, Hesperia, CA). Binding assays were done with stationary phase *P. aeruginosa* 9027 or *B. cepacia* 25416, both typically at  $2.5 \times 10^7$  CFU/ml. The bacteria were incubated with <sup>125</sup>I-GGPG at 0° C for 60 min at pH 7.4 in a 10 mM Tris-acetate buffer containing 100 mM NaCl, 1% TSB, and 1% BSA. Samples were layered over 0.3 ml of a cushion composed of 5 parts of dibutyl phthalate and 3 parts of di-isodecyl phthalate (density<sub>20</sub> = 1.01) and centrifuged at approximately  $14,000 \times g$ . After removing the supernatant and phthalate oil, the base of the tube was severed with a razor blade and the stub, which contained the pellet, was recovered for radioactivity counting.

#### **LPS and lipid A extraction and analysis**

Bacterial LPS was isolated according to the protocol of Darveau and Hancock [43] with some modifications, as described below. Strains were grown overnight in TSB, collected via centrifugation ( $10,000 \times g$  for 20 min at 0°C), and suspended in a pH 8.0 buffer (5.0 g of wet weight bacteria/30 ml of buffer) that contained 10 mM Tris-hydrochloride, 2 mM MgCl<sub>2</sub>, 100  $\mu$ g/ml DNase I, and 25  $\mu$ g/ml RNase I. The slurry was passed twice through a French Press set at 20,000 lbs/in<sup>2</sup>. DNase and RNase were again

added, and the mixture was incubated at 37°C for 2 hours. Then, to each 15 ml of suspension, the following were added: 5.0 ml of 0.5 M tetrasodium EDTA in 10 mM Tris buffer, 2.5 ml of 20% SDS in 10 mM Tris buffer, and 2.5 ml of 10 mM Tris buffer. The preparation was mixed and centrifuged at  $50,000 \times g$  for 30 min at 20°C to remove the peptidoglycan. Protease (Sigma P5147) at 200  $\mu$ g/ml was added to the supernatant and incubated at 37°C overnight with agitation. On the next day, 0.375 M MgCl<sub>2</sub> in 95% ethanol was added and the suspension was cooled to 0°C before centrifugation at  $12,000 \times g$  for 15 min at 0°C. The resulting pellet was resuspended in 2% SDS, 0.1 M tetrasodium EDTA in 10 mM Tris buffer and sonicated. This solution was incubated at 85°C for 30 min, and cooled to room temperature before protease (25  $\mu$ g/ml) was added and incubated overnight at 37°C. After re-precipitation the following day with ethanol, the pellet was resuspended in 10 mM Tris buffer and centrifuged at  $200,000 \times g$  for 2 hours at 20°C in the presence of 25 mM MgCl<sub>2</sub>. The final LPS extract was suspended in 10 ml dH<sub>2</sub>O and dialyzed overnight against dH<sub>2</sub>O to remove any residual salt and SDS prior to being lyophilized. Contamination of the LPS samples by protein represented no more than  $3.6\% \pm 0.21\%$ , as estimated by measuring the dry weight and protein concentration with the Bio-Rad Protein Assay Kit. Nucleic acid contamination was considered to be minimal due to the repeated DNase and RNase treatments. LPS preparations were diluted using the PG-1 vehicle prior to assaying with PG-1.

Lipid A was extracted from isolated LPS preparations via acid hydrolysis [44,45]. Isolated LPS (25–30 mg) was suspended in 4 ml of a 1% acetic acid solution and heated in a water bath to 100°C under refluxing conditions for 3 hours. Once the lipid A was hydrolyzed, it precipitated out of solution and was extracted with five sequential 4 ml aliquots of chloroform. Lipid A extracts were collected and washed three times with 10 ml aliquots of dH<sub>2</sub>O before being lyophilized. Lipid A was dissolved in 100% dimethyl sulfoxide (DMSO), which was diluted to 2.5% with the PG-1 vehicle before application in the radial diffusion assay.

In order to measure the ability of LPS and lipid A to bind PG-1, we modified the two stage radial diffusion assay so that we could ascertain the amount of LPS or lipid A (in ng), that reduced the antimicrobial activity of 50 ng of PG-1 by 50 percent (called the effective concentration 50% or EC<sub>50</sub>). Briefly, 2.5  $\mu$ l of a 20  $\mu$ g/ml PG-1 solution and 2.5  $\mu$ l of LPS or lipid A (at various concentrations based on their dry weight) were combined, and added to the radial diffusion wells. After a 3-hour incubation, a nutrient rich overlay gel was poured and the plates were incubated overnight. The following day, zone diameters (including the wells) were measured in mm. Since the height (h) of the underlay gel was always 1.2 mm, the zone diameters were converted to volumes cleared of viable bacteria with the for-

mula for the volume of a cylinder:  $V = \pi r^2 h$ . Since the 3 mm diameter sample well was devoid of both bacteria and peptide, its volume (8.5  $\mu\text{l}$ ) was subtracted from the gross clear zone volume ( $\pi r^2 h$ ) to derive the net volume rendered free of bacteria. As will be shown below, this net volume was a linear function of the amount of PG-1 placed in the well, for amounts of protegrin between 0–50 ng/ well.

### Surface plasmon resonance

Binding of LPS or lipid A to PG-1 was assessed with a Biacore 2000 system (Biacore AB, Piscataway, NJ); a very similar approach was used to study LPS binding by peptide domains of horseshoe crab factor C [46]. This innovative technology uses surface plasmon resonance to study biomolecular interactions in real time. Briefly, the binding takes place on a small chip coated with a thin layer of metal. It is monitored by monochromatic, p-polarized light whose electric vector component is parallel to the plane of incidence. Under these conditions, the intensity of the reflected light is markedly reduced at a specific incident angle, producing a sharp "shadow". This phenomenon is called "surface plasmon resonance" (SPR) and the incident light angle at which the shadow is observed is the "SPR angle". Detection of the SPR angle depends on changes in the refractive index (RI) of the medium on the nonilluminated side of the chip. Since this RI depends on the mass concentration of macromolecules at this surface, monitoring the SPR angle provides a real-time measure of changes in the surface concentration. The SPR spectrum is a plot of the SPR angle against time, and displays the interaction's progress at the sensor surface. The SPR signal is expressed in resonance units (RU), and 1000 RU is equivalent to a change in surface protein concentration of about 1 ng/mm<sup>2</sup> or of about 6 mg/ml in the bulk protein concentration.

In our study, LPS and lipid A samples from *P. aeruginosa* 9027 and *B. cepacia* 25416 were immobilized on a hydrophobic HPA sensor chip (BR-1000–30, Biacore AB) that was found, in our preliminary experiments, to provide an optimal matrix for LPS and lipid A. The surface of HPA sensor chips is composed of long-chain alkanethiol molecules that form a flat, quasi-crystalline hydrophobic layer that facilitates hydrophobic adsorption of liposomes or micelles on a user-defined polar lipid monolayer. The chip was pre-cleaned and conditioned by washing twice with 40 mM n-octyl glucoside (Sigma Chemical Co.). To immobilize the LPS, 60  $\mu\text{l}$  of a 1 mg/ml LPS preparation was dispersed in 100 mM NaCl containing 10 mM HEPES buffer (pH 7.4), and passed across the chip's surface for 30 min, at flow rate of 2  $\mu\text{l}/\text{min}$ . Lipid A was dissolved and immobilized similarly, except that its concentration was reduced to 0.2 mg/ml. Residual unbound LPS and lipid A were washed away with 0.1 M HCl and 0.1 M NaOH. BSA (0.1 mg/ml) was used to detect nonspecific binding on the surfaces coated by LPS or lipid A. PG-1 binding was measured by observ-

ing the change in the SPR angle of the sample bound to the chip as 30  $\mu\text{l}$  of PG-1 (20  $\mu\text{M}$ , 10  $\mu\text{M}$ , 6  $\mu\text{M}$ , 3  $\mu\text{M}$ , 2  $\mu\text{M}$ , and 1  $\mu\text{M}$ ) flowed over the sample for 3 min at a rate of 10  $\mu\text{l}/\text{min}$ . These PG-1 solutions were also prepared in 100 mM NaCl containing 10 mM HEPES buffer (pH 7.4). Each binding study was done in triplicate and the chip was regenerated between trials by washing it with 0.1 M HCl and 0.1 M NaOH.

## Results

### Activity of PG-1 on *P. aeruginosa* and *B. cepacia* strains

The  $C_L$  and MAC for PG-1 on *P. aeruginosa* and *B. cepacia* were determined in two-stage radial diffusion assays (Table 1). The mean  $C_L$  of PG-1 for the six *P. aeruginosa* strains was 0.30  $\mu\text{g}/\text{ml}$ , and the mean  $C_L$  for the five *B. cepacia* strains was 5.02  $\mu\text{g}/\text{ml}$ , a 16.7-fold difference. The mean MACs for *P. aeruginosa* and *B. cepacia* were 0.17  $\mu\text{g}/\text{ml}$  and 8.40  $\mu\text{g}/\text{ml}$ , respectively. The  $C_L$  and MAC values for *P. aeruginosa* and *B. cepacia* differed significantly ( $P$  exact = 0.004) when the data were analyzed by the Mann-Whitney test.

We also compared two sets of genetically related *P. aeruginosa* strains: 144M/144M(SR) and FRD-1/FRD-2. Whereas the LPS of strain 144M has a very short O-antigen side chain, LPS from strain 144M(SR) has a very long O-antigen side chain. However, despite these differences both strains had comparable  $C_L$  and MAC values. Similarly, comparison of the alginate producer FRD-1 with FRD-2, its spontaneous nonmucoid derivative, revealed that both strains had very similar  $C_L$  and MAC values.

### Intact/whole cell binding

Incubation of <sup>125</sup>I-GGPG with intact *P. aeruginosa* 9027 (PG-1 sensitive) and *B. cepacia* 25416 (PG-1 resistant) cells demonstrated a marked difference in protegrin binding between the two strains (Fig. 1). When the bacteria were exposed to 12.5  $\mu\text{g}$  <sup>125</sup>I-GGPG /ml, approximately sixfold more protegrin molecules bound to *P. aeruginosa* than to *B. cepacia*, and at an <sup>125</sup>I-GGPG concentration of 25  $\mu\text{g}/\text{ml}$ , 12 times as many protegrin molecules bound to *P. aeruginosa*. Another factor also merits mention in this regard. By light microscopy, the *B. cepacia* cells appeared larger than the *P. aeruginosa* cells. By electron microscopy, we found that the mean cross sectional diameters and lengths of *B. cepacia* 25416 cells were 0.755  $\mu\text{m} \times 2.55 \mu\text{m}$ . The corresponding values for *P. aeruginosa* 9027 cells were 0.615  $\mu\text{m} \times 2.35 \mu\text{m}$ . Assuming a cylindrical structure for both, we calculated that the surface area of the *B. cepacia* cells (6.00  $\mu\text{m}^2$ ) was approximately 33% larger on average than that of *P. aeruginosa* (4.54  $\mu\text{m}^2$ ).

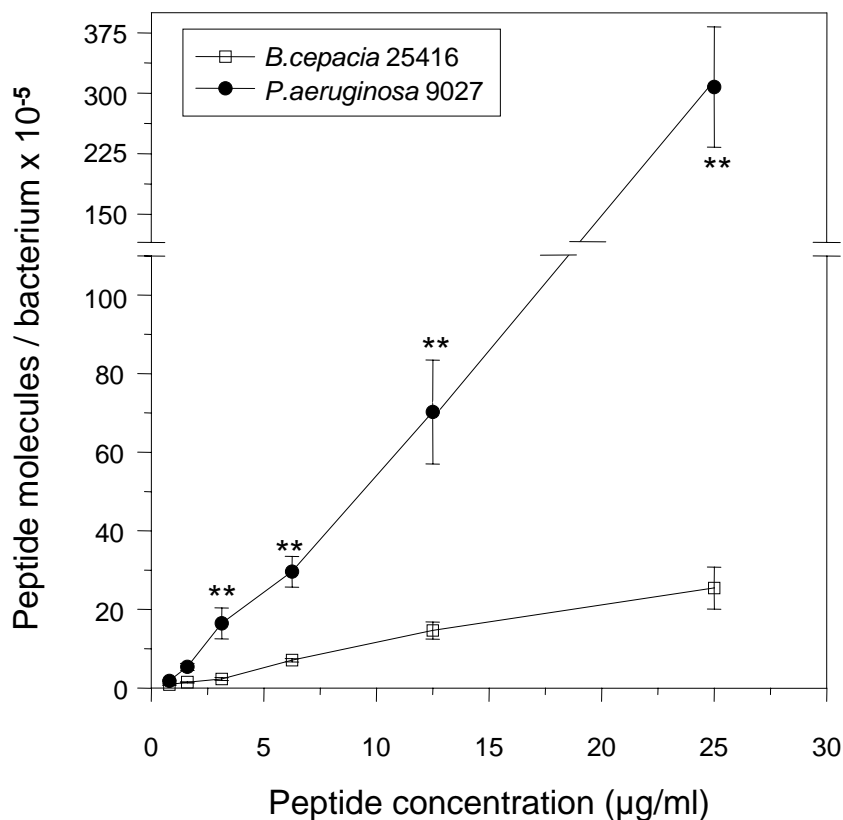
### Binding of PG-1 to LPS and lipid A

To compare binding of PG-1 to LPS and lipid A from *P. aeruginosa* 9027 and *B. cepacia* 25416, we devised a simple

**Table 1** **$C_L$  and MAC values for various *P. aeruginosa* and *B. cepacia* strains determined using the radial diffusion assay.**

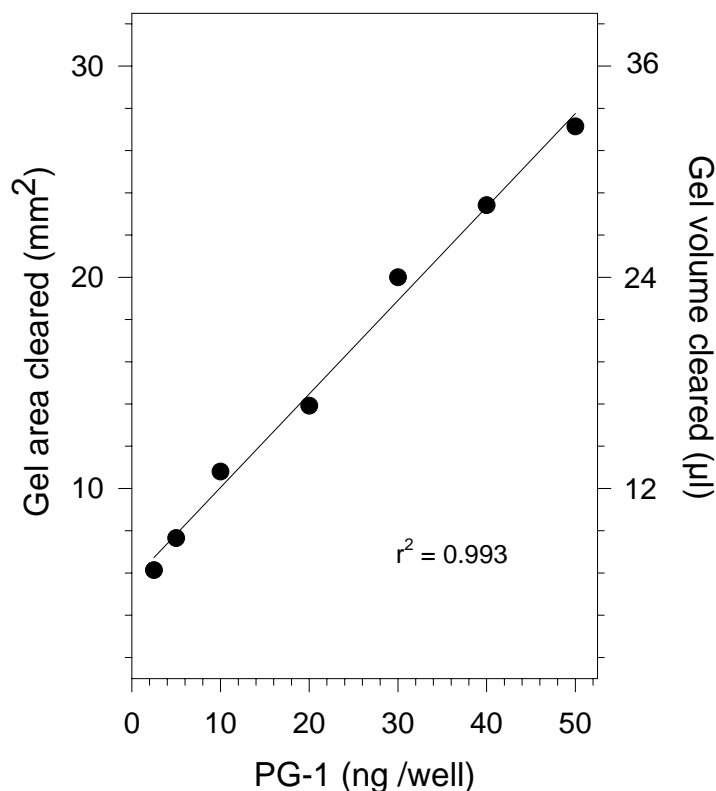
Organism	Strain	Comments	$C_L^*$	MAC*
<i>P. aeruginosa</i>	144M	Serum sensitive, mucoid, short chain LPS	0.20	0.0625
<i>P. aeruginosa</i>	144M (SR)	Serum resistant, mucoid, long chain LPS	0.18	0.0625
<i>P. aeruginosa</i>	FRD-1	Mucoid strain	0.15	0.125
<i>P. aeruginosa</i>	FRD-2	Nonmucoid strain	0.19	0.125
<i>P. aeruginosa</i>	10145	ATCC strain	0.25	0.125
<i>P. aeruginosa</i>	9027	ATCC strain	0.81	0.50
Mean $\pm$ SEM	(all 6 strains)		$0.30 \pm 0.10^{**}$	$0.17 \pm 0.07^{**}$
<i>B. cepacia</i>	C6159	Clinical isolate	1.35	6
<i>B. cepacia</i>	C4813	Clinical isolate	5.70	8
<i>B. cepacia</i>	C4878	Clinical isolate	1.53	8
<i>B. cepacia</i>	25416	ATCC strain	7.57	10
<i>B. cepacia</i>	25609	ATCC strain	8.95	10
Mean $\pm$ SEM	(all 5 strains)		$5.02 \pm 1.55^{**}$	$8.40 \pm 0.75^{**}$

\*Each value represents the mean of three replicate assays, shown in  $\mu\text{g/ml}$ . \*\*Mann-Whitney test, *P. aeruginosa* versus *B. cepacia*,  $P$  exact for  $C_L = 0.004$ , MAC = 0.004.

**Figure 1**

Binding of  $^{125}\text{I}$ -GGPG. Stationary phase bacteria,  $2.5 \times 10^7$  CFU/ml, were incubated with the indicated concentrations of  $^{125}\text{I}$ -GGPG for 60 min at  $0^\circ\text{C}$ , and centrifuged through a phthalate ester "cushion" to separate free and bound peptides. The data points are mean  $\pm$  SEM values from 5 to 9 replicates obtained in three separate experiments. Using the Mann-Whitney test,  $P$  was  $< 0.05$  at values indicated by \*\*.

Figure 2



Quantitative aspects of PG-1 mediated killing measured by two-stage radial diffusion assays. The left ordinate scale shows the net area of underlay gel totally cleared of viable bacteria using the equation  $(\pi d^2 - 9) \div 4$ , where  $d$  is the diameter of the cleared zone including the well diameter and 9 is subtracted to account for the area of the well, which is devoid of bacteria. The right ordinate scale shows the volume cleared (area cleared times the height of the underlay gel, 1.2 mm). All data points are means ( $n = 3$ ). Note that  $Y$  is a linear function of  $X$  for these peptide concentrations. Area ( $A$ ) and volume ( $V$ ) are related by the equation:  $A \text{ (mm}^2\text{)} = V \text{ (}\mu\text{l)}/1.2 \text{ mm}$ .

bioassay based on the radial diffusion assay. As shown in Fig. 2, for protegrin amounts between 0 and 50 ng, the volume of underlay gel that was rendered completely free of viable bacteria was linearly related to the amount of peptide introduced into the well. By mixing a constant amount of protegrin (within this linear range) with graded amounts of purified LPS and lipid A, we could readily determine how much of these ligands were needed to reduce the activity of 50 ng of PG-1 by 50% (the  $EC_{50}$  values). The results of our assays with LPS and lipid A purified from the strains we used in the above  $^{125}\text{I}$ -GGPG binding studies are shown in Fig. 3A. The  $EC_{50}$  values for LPS isolated from *P. aeruginosa* and *B. cepacia* differed by 4.6-fold (100 ng versus 460 ng) while the  $EC_{50}$  values for lipid A isolated from the same strains differed by approximately 16.7-fold (60 ng versus 1000 ng).

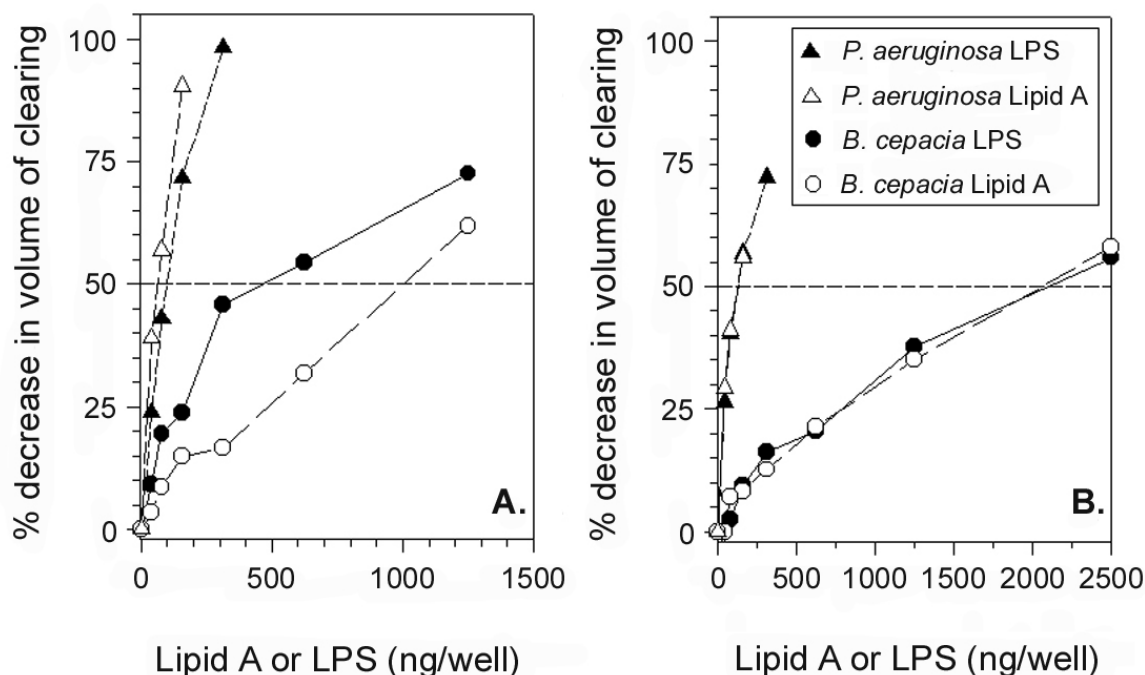
To ascertain if these findings were representative, we prepared LPS and lipid A from another pair of bacteria (*P. aer-*

*uginosa* FRD-2 and *B. cepacia* C4813) and repeated the studies. The  $EC_{50}$  values for both LPS and lipid A obtained from *P. aeruginosa* FRD-2 were each 120 ng. The values for *B. cepacia* LPS and lipid A were estimated as approximately 2050 ng for both (Fig. 3B), a difference of 17.1-fold when compared to *P. aeruginosa* FRD-2 LPS and lipid A.

#### Surface plasmon resonance studies

We also used SPR spectroscopy to compare the binding of PG-1 to LPS and lipid A purified from *P. aeruginosa* 9027 and *B. cepacia* 25416. Injection of LPS or lipid A from both strains was stopped when the increase in RU of both reached 1000. Loosely bound molecules were washed away by increasing the flow rate to 100  $\mu\text{l}/\text{min}$ , followed by two, one-minute short pulse injections of 100 mM NaOH. Injections of 0.1 mg/ml BSA prepared in the eluent buffer were passed across the LPS or lipid A coated surfaces for a five-minute period and showed an increase of around 100 RU, whereas the same amount of BSA passed

Figure 3



Binding of PG-1 to LPS and lipid A. Binding/radial diffusion assays were conducted by mixing various amounts of LPS or lipid A with 50 ng of PG-1 in a final volume of 5  $\mu$ l. In Panel A, the LPS and lipid A preparations were isolated from *P. aeruginosa* strain 9027 and *B. cepacia* strain 25416. In Panel B, the LPS and lipid A were isolated from *P. aeruginosa* FRD-2 and *B. cepacia* C4813. The mixtures of PG-1 with LPS or lipid A were introduced into the wells of radial diffusion assay plates seeded with *P. aeruginosa* FRD-1, a highly susceptible test organism. All data points are means ( $n = 3$ ), and  $EC_{50}$  values were extrapolated from these graphs. When these binding isotherms were subjected to linear regression analysis, the 95% confidence limits of the LPS and lipid A binding isotherms for *P. aeruginosa* and *B. cepacia* were non-overlapping, and the isotherms for the LPS preparations in Panel A overlapped only at the lowest ligand concentrations (data not shown).

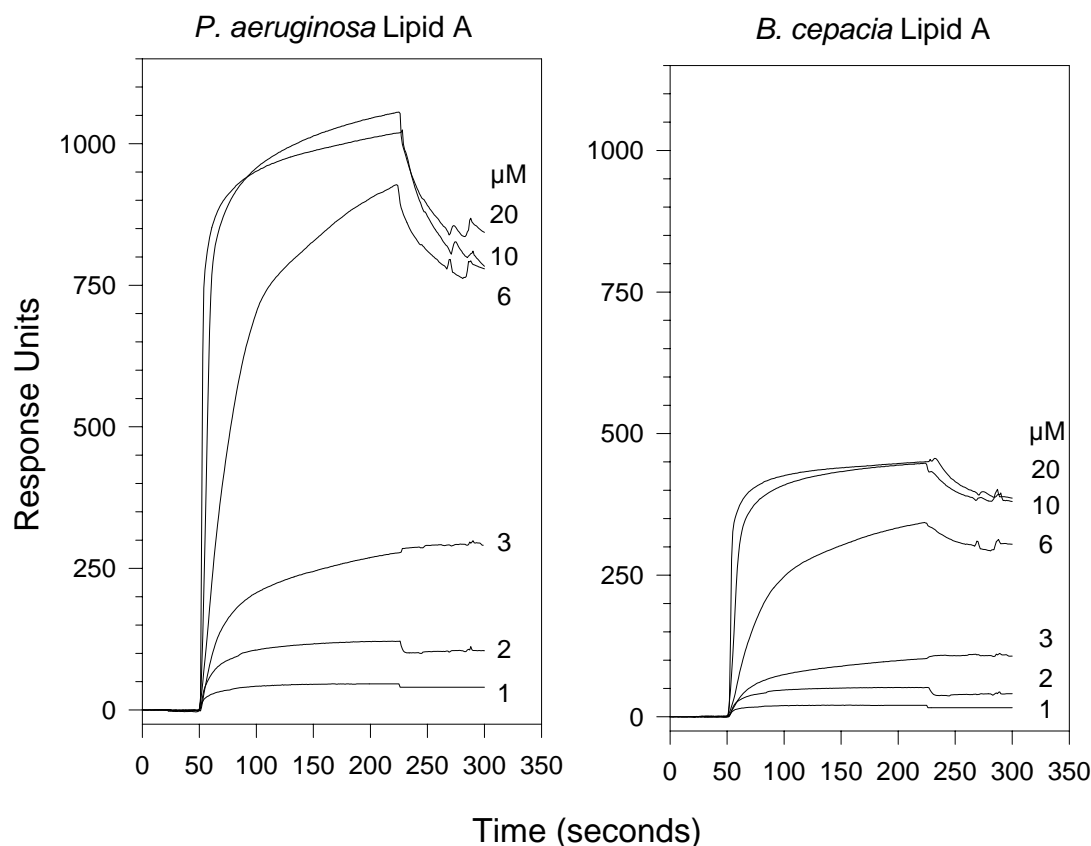
across the uncoated, n-octyl glucoside washed surface showed an increase of roughly 1000 RU. This demonstrated that the surface of the chip was fully covered by LPS or lipid A. Since similar amounts of these ligands had bound to the chip, the response unit differences seen at concentrations between 1 and 20  $\mu$ M indicate that approximately twice as much PG-1 bound *P. aeruginosa* lipid A as bound lipid A from the *B. cepacia* strain (Fig. 4). Binding differences were less marked when we studied LPS from these organisms (Fig. 5). Although up to one third more protegrin molecules bound to *P. aeruginosa* LPS than to *B. cepacia* LPS at protegrin concentrations of 1–6  $\mu$ M, these differences disappeared when we tested higher PG-1 concentrations. At 10  $\mu$ M PG-1, LPS from *P. aeruginosa* and *B. cepacia* bound PG-1 equally ( $\approx 750$  response units). Equal binding was also seen at 20  $\mu$ M PG-1 ( $\approx 1000$  response units).

## Discussion

In this study we compared the sensitivity of *P. aeruginosa* and *B. cepacia* strains to protegrin PG-1 with the ability of

the intact bacteria and key components of their outer membranes (LPS and lipid A) to bind this peptide. The indices of susceptibility ( $C_L$  and MAC values) obtained from our radial diffusion assays established that *P. aeruginosa* was considerably more sensitive to PG-1 than *B. cepacia*. We also found that at any given peptide concentration, many fewer protegrin molecules bound to intact *B. cepacia* and its lipid A than to the corresponding *P. aeruginosa* preparations. In model systems, the ability of protegrins to permeabilize membranes shows pronounced "concentration-gating", an indication that it is influenced by the density of bound protegrin molecules per unit area of membrane [47]. As prior quantitative studies of peptide binding are few in number, some readers may be surprised to note that Fig. 1 shows many millions of protegrin molecules are bound to an average bacterial cell, even at concentrations around the minimal active concentration. To acquire an equivalent density of surface-bound PG-1 molecules, *B. cepacia* needed exposure to much higher concentrations of PG-1 than *P. aeruginosa*. Thus, it is a reasonable inference that the relative resistance of *B. cepacia* to PG-1 is a consequence of

Figure 4



Surface plasmon resonance (SPR) studies of isolated lipid A. PG-1 binding was measured by observing the change in the SPR angle of the sample bound to the chip (*P. aeruginosa* 9027 lipid A in the left panel, *B. cepacia* 25416 lipid A in the right panel) as 30  $\mu$ l of PG-1 at the indicated concentrations (1 to 20  $\mu$ M) flowed over the sample. All data points are means ( $n = 3$ ).

the smaller number of protegrin molecules bound per unit area of bacterial surface.

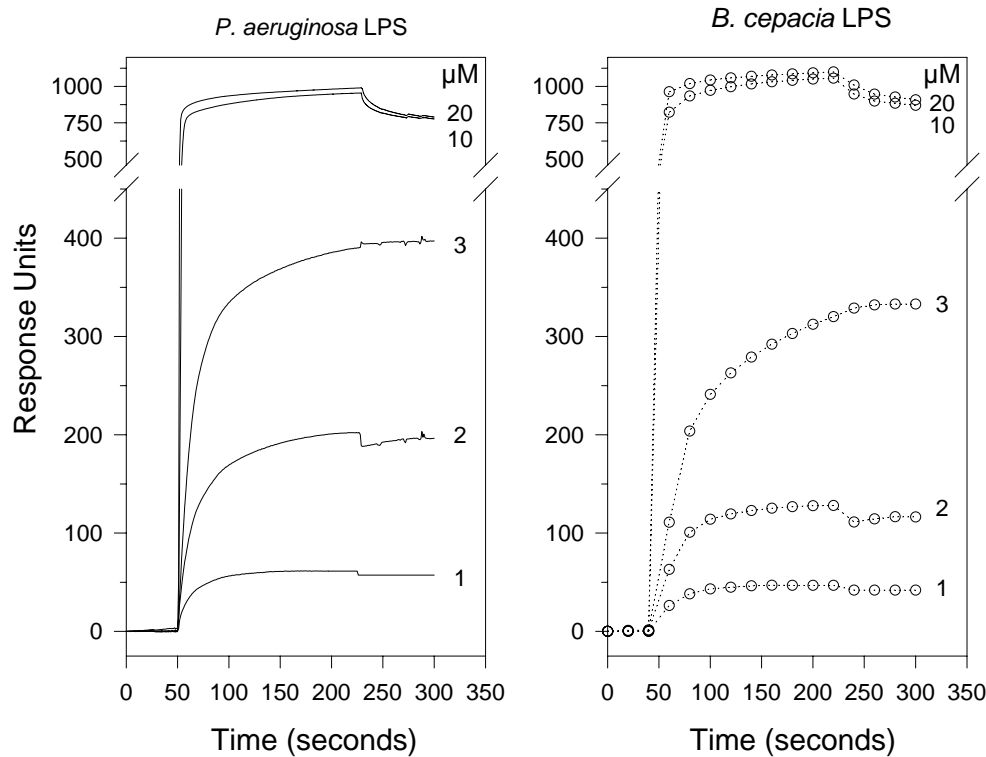
Earlier studies have shown that certain structural differences in the LPS of Gram-negative bacteria correlate with their susceptibility or resistance to antimicrobial (lipo)peptides [19,23,48–51]. Particular attention has been given to alterations of the outer membrane that could modify binding to the cell surface. For example, sensitivity to the antibacterial lipopeptide polymyxin B is affected by the PmrA-PmrB regulon, which mediates substitution of 4-aminoarabinose on the 4' phosphate of lipid A [52,53]. By reducing the negative charge of lipid A, this modification decreases its electrostatic interaction with cationic peptides. Resistance to polymyxin B is also imparted by the absence or masking of critical binding sites required for polycation-mediated permeabilization of the outer membrane [54]. Thus, attachment of polymyxin B to *Yersinia enterocolitica* is reduced by steric hindrance generated by the size and/or branched shape of the outer core region of its LPS macromolecule

[55]. Additionally, resistance to cationic peptides can be due to cytoplasmic clearance of the antimicrobial peptide by an efflux pump; e.g., *Yersinia* resistance to polymyxin B caused by the RosA/RosB efflux pump/potassium antiporter [56] and *Neisseria gonorrhoeae* resistance to protegrin PG-1 via an energy-dependent efflux system termed mtr [57].

Given the notorious resistance of *B. cepacia* to conventional antibiotics [2], its relative resistance to PG-1 was not unexpected. What bacterial properties influenced sensitivity to protegrins? Certainly, the striking correlation with the extent of protegrin binding must be one of these. Our studies with genetically related *P. aeruginosa* strains did not implicate the length of the O-antigen side chain as a key factor. Furthermore, genetically related *P. aeruginosa* strains that differed in alginate production were equally sensitive to PG-1. Both results were somewhat surprising, since long O-side chain and alginate molecules should both bind PG-1. Perhaps limited alginate production under our experi-



Figure 5



Surface plasmon resonance (SPR) binding studies of isolated LPS. PG-1 binding was measured by observing the change in the SPR angle of the sample bound to the chip (*P. aeruginosa* 9027 LPS in the left panel, *B. cepacia* 25416 LPS in the right panel) as 30  $\mu$ l of PG-1 at the indicated concentrations (1 to 20  $\mu$ M) flowed over the sample. All data points are means ( $n = 3$ ).

mental conditions explains its failure to influence susceptibility to PG-1. Additional studies focusing on the influence of alginate on PG-1 activity in a biofilm model are in progress.

Why did fewer protegrin molecules bind to *B. cepacia* than to *P. aeruginosa*? Our radial diffusion/binding and SPR studies strongly implicated lipid A as a prime suspect in the case. The acyl chains of lipid A form an integral part of the outer membrane bilayer, and electrostatic or hydrophobic binding of protegrin to lipid A could easily disturb the organization and acyl-chain packing of the outer membrane, both vital in maintaining its integrity and barrier function. Accepting a direct proportionality between measured plasmon resonance (RU) and the extent of peptide bound to the sensor surface, the responses shown in Fig. 4 indicate that at saturation (20  $\mu$ M), lipid A from *B. cepacia* bound almost exactly one-half as many protegrin molecules as lipid A from *P. aeruginosa*. How might this be rationalized?

Lipid A typically contains two phosphate groups, one on each glucosamine residue. In the lipid A of *B. cepacia*, one

of these phosphates is modified by a phosphodiester-linked 4-amino-4-deoxyarabinose residue [58,59]. It is noteworthy that a previous study reported that the phosphate content of *B. cepacia* LPS was only one-third of that of *P. aeruginosa*[60]. If most of the *P. aeruginosa* lipid A phosphates are unmodified, and if these phosphates are principal protegrin binding sites, then one would expect exactly the results shown in Fig. 4. This is also consistent with the suggestion, made in a recent report showing that cystic fibrosis isolates of *P. aeruginosa* had lipid A isoforms derivatized with aminoarabinose (or palmitate), that these structural modifications could enhance resistance to cationic antimicrobial peptides [61]. Although the acyl chains of lipid A might also provide alternative binding sites for protegrin, the orientation of lipid A on the HPA sensor chip ("butter-side down") probably removes them from consideration, at least under our study conditions.

The core and outer polysaccharide regions of LPS should provide additional binding sites, including the carboxylate moieties of KDO or KO (D-glycero-D-talo-oct-2-ulopyranosylonic acid [62]. This could explain why *B. cepacia* LPS

binds about as much PG-1 as *P. aeruginosa* LPS (Fig. 5) especially at peptide concentrations of 10–20  $\mu\text{M}$ , which allow binding to lower affinity sites. This interpretation helps explain why Fig. 3 suggests that *B. cepacia* LPS binds protegrin less effectively than the corresponding *P. aeruginosa* preparation, while the SPR results in Fig. 5 show that *B. cepacia* and *P. aeruginosa* LPS bound equivalent amounts of PG-1 at high (10–20  $\mu\text{M}$ ) protegrin concentrations. To resolve these seemingly discrepant results, it is important to recall that in SPR assays, the sensor chips were constantly bathed with a fixed protegrin concentration ranging from 1–20  $\mu\text{M}$  and that in radial diffusion assays, the wells received a fixed initial amount of protegrin (50 ng). In the latter assays, the peptide was dispersed in a total volume of 10  $\mu\text{l}$ , making its maximal initial concentration approximately 2.3  $\mu\text{M}$  – well below the 10–20  $\mu\text{M}$  concentrations needed to saturate LPS. Moreover, in addition to binding the LPS or lipid A molecules we added to the well, some of the initially added protegrin could diffuse radially into the underlay gel. Both initial binding and radial diffusion will decrease the concentration of free (i.e., unbound) protegrin that remains in the well, and further skew its binding towards higher affinity binding sites on lipid A.

Although our data clearly establish the presence of a reduced number of binding sites for protegrin in lipid A from *B. cepacia*, as compared to lipid A from *P. aeruginosa*, they do not identify the cause of this phenomenon. These alterations could result from 4-aminoarabinose substitutions on the lipid A, from other changes that reduce the number of ion-binding sites, or from changes that affect hydrophobic interactions or membrane insertion. Additional studies that characterize *B. cepacia* LPS and lipid A can help pinpoint the exact structural differences that relate to the relative resistance of *B. cepacia* to protegrins and to endogenous antimicrobial peptides of humans.

## Conclusion

Our studies demonstrate a correlation between protegrin sensitivity/resistance and protegrin binding in *P. aeruginosa* and *B. cepacia*, and support a hypothesis that the relative resistance of *B. cepacia* to protegrin is due principally to a reduced number of protegrin binding sites on the lipid A moiety of its LPS.

## Abbreviations

BSA = bovine serum albumin; CF = cystic fibrosis; CFTR = cystic fibrosis transmembrane conductance regulator;  $C_L$  = lethal concentration; DMSO = dimethyl sulfoxide;  $EC_{50}$  = effective concentration 50%; EDTA = ethylenediamine tetraacetic acid; GGPG = modified PG-1 with a glycine-rich hexapeptide extension (GGGYGG) containing a single tyrosine residue; HEPES = N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; HPLC = high performance liquid chromatography; I-GGPG = monoiodinated variant of GGPG; LPS = lipopolysaccharide; MAC =

minimal active concentration; PG-1 = protegrin-1; RU = resonance units; SDS = sodium dodecyl sulfate; SPR = surface plasmon resonance; TSB = trypticase soy broth.

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