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Susceptibilities of *Bordetella pertussis* Strains to Antimicrobial Peptides

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We examined the susceptibilities of *Bordetella pertussis* strains to several antimicrobial peptides by determining the concentration required to inhibit or kill 50% of the bacterial population. The peptides are ranked in decreasing potency as follows: cecropin B > cecropin A >> melittin > cecropin P1 > (ala^{8,13,18})-magainin II amide > mastoparan = defensin HNP1 > protamine ≥ magainin II = magainin I. By using a radial diffusion assay to compare susceptibilities between strains, wild-type *B. pertussis* BP338 was more resistant than the avirulent *bvg* mutant strain BP347 and the *brk* mutant strain BPM2041 to killing by cecropin P1. In contrast, compared with the wild type, the avirulent BP347 strain was highly resistant to killing by protamine and defensin HNP1.

Antimicrobial peptides are, in general, about 14 to 40 amino acids in size and are predominantly cationic in terms of their net charge (for reviews, see references 1, 8, 17, and 21). They exist either as alpha helices (e.g., cecropins) or as antiparallel beta sheets (e.g., defensins) which are amphipathic in that they have polar as well as hydrophobic faces. In gram-negative bacteria, these cationic peptides interact with the negatively charged lipopolysaccharide-lipid A to perturb the outer membrane. This, in itself, is not sufficient for cell death. The lethal target of the antimicrobial peptides is the cytoplasmic membrane, where the antimicrobial peptides can form channels, resulting in the disruption of the membrane potential, which leads to cell death.

It is being increasingly recognized that antimicrobial peptides such as tracheal antimicrobial peptide (3, 4), defensins (8), cryptdins (5, 13), lingual antimicrobial peptide (18), and cecropin P1 (10) are an important facet of the mucosal-epithelial defense against pathogens. We investigated the effects of several antimicrobial peptides on the mucosal pathogen *Bordetella pertussis*, which is the gram-negative bacterium that colonizes the trachea of the human host and causes whooping cough.

The susceptibilities of *B. pertussis* isolates to various commercially available antimicrobial peptides were assessed by a modification of the radial diffusion assay described by Lehrer et al. (11). In addition, the concentration of peptide required to kill or inhibit the growth of 50% of the organisms (C-50%) was determined for each peptide. For the radial diffusion assay, wild-type *B. pertussis* BP338 (22) was grown overnight on Bordet-Gengou medium (BBL, Cockeysville, Md.) supplemented with 15% sheep blood (Cocalico Biologicals, Reamstown, Pa.) as described previously (22). The bacteria were harvested in modified Stainer Sholte (SS) broth (24) to an optical density at 600 nm of approximately 0.2, and 0.2 ml of this suspension was added to 10 ml of molten (52°C) 1% agarose (type I; low electroendosmosis; catalog no. A 6013, Sigma Chemical Co., St. Louis, Mo.) in SS containing supplements and 0.15% bovine serum albumin (Sigma). Tween 20, which is used in other systems to facilitate diffusion (11), was not added since it was found to inhibit the growth of *B. pertussis*. The agarose was dispensed into an Integrid square petri dish (Falcon; Becton-Dickinson, Lincoln Park, N.J.) and was allowed to harden. Holes (3 mm in diameter) were made with an aspirator punch (ICN Biomedicals, Costa Mesa, Calif.), and 5 μl of peptide (Sigma) (Table 1) serially diluted in RPMI medium (Whittaker Bioproducts, Walkersville, Md.) was placed therein. Following a 4-h incubation at room temperature to allow for diffusion, a 10-ml overlay of SS-agarose without bacteria was added. The resultant zones of inhibition were read 24 to 48 h later with a Bausch & Lomb metric scale and a stereomicroscope (×7 magnification). The Student *t* test was used to analyze the data. The C-50% was determined as follows. Serial twofold dilutions of the different antimicrobial peptides were made in 100 μl of RPMI medium in a microtiter plate. To each well was added 10 μl of *B. pertussis* (see above) in SS broth resulting in a final concentration of approximately 2×10^6 CFU/ml. Following a 2-h incubation at 37°C, 100 μl from each well was diluted in 900 μl of SS broth, and the number of surviving bacteria was ascertained by plating out 10-fold serial dilutions on Bordet-Gengou agar. As a control, organisms were similarly added to wells containing RPMI medium but no peptides. The C-50% was determined and was adjusted according to the purity of the peptide stated by the manufacturer.

As shown in Table 1, the susceptibility of *B. pertussis* BP338 to the different antimicrobial peptides varied with the type of peptide and was dose dependent. In order of decreasing potency, the C-50%'s of the peptides are ranked as follows: cecropin B > cecropin A >> melittin > cecropin P1 > (ala^{8,13,18})-magainin II amide > mastoparan = defensin HNP1 > protamine ≥ magainin II = magainin I. In general, similar results were obtained by the radial diffusion assay.

We next determined whether avirulent mutants of *B. pertussis* had different susceptibilities to killing by antimicrobial peptides. In *B. pertussis* isolates, virulence is controlled by the *bvg* locus, the products of which enable the bacterium to sense its environment and respond by coordinately activating and repressing a number of genes (for a review, see reference 19). *bvg* mutants do not express any adhesins, toxins, or other virulence factors and are completely avirulent (23). *B. pertussis* BP347 is

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TABLE 1. Susceptibility of *B. pertussis* BP338 to antimicrobial peptides

Antimicrobial peptide	Radial diffusion assay				C-50%	
	Amt (μg/μl)	Concn (μM)	No. of assays[a]	Zone size (mm)[b]	No. of assays	Concn (μM)
Cecropin A	1	0.2	2	24.3 ± 1.6	7	0.56–0.94
	0.12	0.02	4	17.6 ± 1.4		
Cecropin B	1	0.2	2	21.3 ± 4.4	5	0.12–0.26
	0.12	0.02	4	13.2 ± 1.4		
Cecropin P1	1	0.25	10	14.6 ± 2.8	7	≤6.1
	0.12	0.03	10	4.5 ± 2.7		
Defensin HNP1	2.5	0.58	2	6.0 ± 1.4	1	>46.5
	1	0.23	2	<3[c]		
Magainin I	10	3.1	3	6.4 ± 3.2	1	>249
	1	0.3	3	<3		
Magainin II	10	3	3	9.7 ± 2.2	1	>243
	1	0.3	3	<3		
(ala^{8,13,18})-Magainin II amide	5	1.6	2	17.2 ± 0.5	7	4.1–7.9
	0.56	0.18	3	9.0 ± 1.0		
Mastoparan	1	0.48	6	13.0 ± 2.0	4	>47.7
	0.12	0.06	6	<3		
Melittin	1	0.3	6	16.5 ± 1.2	5	1.8–3.9
	0.12	0.04	6	9.3 ± 0.9		
Protamine	10	2.4	2	19.5 ± 0.7	1	122–244
	1	0.2	2	<3		

[a] Number of independent assays.
[b] Values are means ± standard deviations.
[c] No detectable zone size.

one such mutant (22, 23). Exposure of BP347 to the different antimicrobial peptides resulted in three types of responses. Compared with the wild type, strain BP338, strain BP347 was significantly more susceptible to killing by cecropin P1 (Fig. 1) and, to a lesser extent, (ala^{8,13,18})-magainin II amide (data not shown), suggesting that *bvg*-activated genes can mediate some resistance to killing by these peptides. In contrast, the *bvg* mutant strain BP347 was markedly resistant to killing by defensin HNP1 and protamine at concentrations of peptide that killed the wild-type strain efficiently (Fig. 2). Finally, both BP338 and BP347 were equally susceptible to killing by cecropin A, cecropin B, magainin I, magainin II, mastoparan, and mellitin (data not shown).

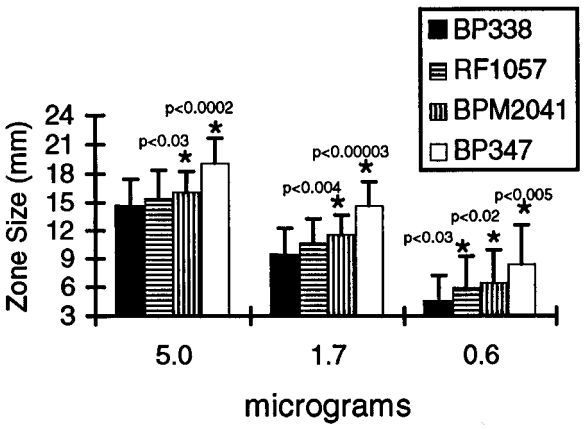

FIG. 1. Susceptibilities of *B. pertussis* strains to cecropin P1. Each bar represents the mean and standard deviation of 10 separate experiments. Significant differences between the wild-type strain and the different mutants are indicated. BP338 is the wild-type strain; RF1057, BPM2041, and BP347 are the *brkB*, *brkA*, and *bvg* mutants, respectively.

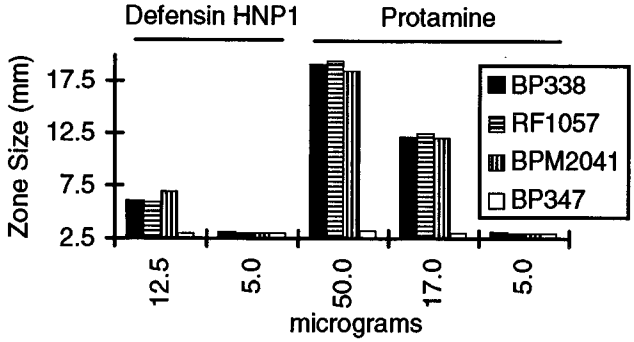

FIG. 2. Susceptibilities of *B. pertussis* strains to defensin HNP1 and protamine. Each bar represents the mean of at least two experiments. BP338 is the wild-type strain; RF1057, BPM2041, and BP347 are the *brkB*, *brkA*, and *bvg* mutants, respectively.

We have recently identified a *bvg*-regulated virulence locus called *brk* (for *Bordetella* resistance to killing) which encodes resistance to killing by the classical pathway of complement (6). Two divergently transcribed genes, *brkA* and *brkB*, are required for resistance. BrkA is predicted to be an outer membrane protein which is processed at its C terminus and shares sequence identity with two other *B. pertussis* proteins, pertactin (2) and tracheal colonization factor (7). BrkB is predicted to be a cytoplasmic membrane protein. The mechanism of resistance of the Brk proteins to complement killing has yet to be determined; however, lysis of gram-negative bacteria by complement is due to the insertion of the C9 component of the membrane attack complex into the cytoplasmic membrane (20). This is analogous to the mechanism of killing by antimicrobial peptides in that lysis is also dependent on insertion of the peptide into the cytoplasmic membrane (17). Given this analogy, we decided to examine the susceptibilities of BrkA and BrkB mutants to killing by antimicrobial peptides. Of the peptides in Table 1, only cecropin P1 was able to discriminate between the wild-type strain BP338 and the BrkA mutant strain BPM2041 (6) (Fig. 1). Thus, BrkA mediates resistance to killing by both the classical pathway of complement (6) as well as resistance to killing by the antimicrobial peptide cecropin P1. While the zone sizes of the BrkB mutant RF1057 (6) exposed to cecropin P1 were slightly larger than the zone sizes produced by the wild-type strain, these were statistically significant only at the lowest concentration of peptide used (Fig. 1). It is also evident from Fig. 1 that, in addition to *brk*, there must be other *bvg*-activated genes in the wild-type strain that participate in the resistance to killing by cecropin P1, since BP347 is more susceptible than the *brk* mutants to cecropin P1.

Intrinsic resistance to antimicrobial peptides in bacteria can be imparted by peptide (14) and potassium (15) transporters, as illustrated by the recently identified *sap* (sensitive to antimicrobial peptides) regulon in *Salmonella* spp. or by components of the outer membrane (e.g., lipopolysaccharide or outer membrane proteins) which, in general, act as a barrier (12, 16, 21). BrkA does not resemble the Sap proteins either in terms of the magnitude of the protection from killing or in terms of

sequence comparisons. Instead, BrkA, by virtue of its predicted location on the surface of the bacterium and by the predicted negative charge (pI 5.89) of its 73-kDa processed form, may serve as a barrier in the outer membrane to impede the penetration of the cationic cecropin P1 peptide. Whether the mechanisms of resistance mediated by BrkA to killing by complement or cecropin P1 are similar awaits further investigation. The resistance of the avirulent *bvg* mutant strain BP347 to killing by protamine and defensin HNP1 compared with the susceptibility of the wild type or *brk* mutants (Fig. 2) is intriguing. Possible explanations might be the presence of a crystalline porin structure which is unique to avirulent (i.e., *bvg* mutant) strains of *B. pertussis* (9), or this might be mediated by the product of a *bvg*-repressed gene.

In summary, we have shown that *B. pertussis* isolates display broad spectra of susceptibilities to a number of antimicrobial peptides. For one of these peptides (the pig intestine-derived cecropin P1), the wild-type *B. pertussis* strain was relatively more resistant to killing compared with the *brkA* mutant and the *bvg* mutant strain, suggesting the possibility that resistance to killing by some antimicrobial peptides may play a role in the pathogenesis of whooping cough. In this regard, in the future it would be of interest to examine peptides (e.g., tracheal antimicrobial peptide) (3, 4) that may be present in the human trachea, which is the site of colonization by *B. pertussis.*

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