### Review

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# Arming the enemy: the evolution of resistance to self-proteins

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A remarkable range of novel antibiotics is attracting increasing interest as a major new weapon in the campaign against bacterial infection. They are based on the toxic peptides that provide the innate immune system of animals, and it is claimed that bacteria will be unable to evolve resistance to them because they attack the 'Achilles' heel' of bacterial membrane structure. Both experimental evidence and theoretical arguments suggest that this claim is doubtful. If so, the introduction of these substances into general use may provoke the evolution of resistance to our own defence proteins and thus compromise our natural defences against infection.

#### Background

When antibiotics were first introduced into general practice in the 1940s, the evolution of resistance was foreseen but discounted, because it would depend on a rate of beneficial mutation that was certain to be extremely low. This optimism has not been justified by events (Tenover, 2001). Resistance to all antibiotics in current use has evolved, often within a few years of their introduction (Palumbi, 2001). Many strains of pathogenic bacteria are resistant to several different antibiotics, often through plasmid-borne genes. Cross-resistance is common. Resistance evolves in settings where the antibiotics are used most heavily, such as hospitals and farms, and the level of resistance is generally correlated with the level of antibiotic administered. The unexpected capacity of bacterial populations rapidly to evolve strong and specific resistance to antibiotics has gone a long way towards annulling the advances in antibacterial chemotherapy that have been the basis of clinical practice for the last 50 years. In an attempt to keep ahead of bacterial evolution, new antibiotics based on the cationic antimicrobial peptides (AMPs) produced by all multicellular organisms are being developed (Andreu & Rivas, 1998; Hancock & Chapple, 1999; Schroder, 1999; Zasloff, 2002). They have great promise. At the same time, they may, in our opinion, pose a serious and unprecedented threat to public health.

#### **Diversity of AMPs**

Peptide antibiotics fall into two broad classes whose evolutionary biology is very different. The first is a large and heterogeneous category of peptides that are synthesized on very large, modular enzyme complexes by bacteria and fungi. They often incorporate unusual amino acids, and may be modified by glycosylation or ring formation or in other ways. This group includes the gramicidins, bacitracins, polymyxins, streptogramins and their derivatives. The second category is quite different. It comprises linear peptides consisting almost entirely of conventional amino acid residues that are produced by all major kinds of organisms (including microbes). These are translated using ribosomes in the usual fashion of protein synthesis, and we therefore call them RAMPs, for Ribosomally synthesized AntiMicrobial Peptides (Hancock & Chapple, 1999) to distinguish them from the non-RAMPs of the first category. RAMPs include the defensins, indolicidins and cathelicidins in mammals; bombinin, magainin and buforin in frogs; cecropin and melittin in insects; the thionins in plants; and the bacteriocins, epidermidins and nisin in bacteria. They are extremely diverse. There is no clear homology between the RAMPs produced by different species, even within the same class, and structurally different RAMPs are produced in different tissues of the same individual. The non-RAMPs are used like conventional antibiotics, and share their strengths and weaknesses while having some of their own. RAMPs have not yet been used as extensively, and it is they that offer a new opportunity for antimicrobial chemotherapy, while presenting a unique threat to longterm public health.

The deadliness of RAMPs, and of non-RAMPs such as the polymyxins, is attributable to their interaction with very general biochemical characteristics of bacterial cell membranes. In particular, the negatively charged phospholipid head groups on the outer surface of bacterial membranes render them highly vulnerable to electrostatic and hydrophobic interactions with RAMPs, whereas eukaryotic cell membranes, with little or no net charge, are almost immune. Consequently – it is argued – it will be difficult for bacteria to evolve resistance to RAMPs. Zasloff (2002)

points out that RAMPs have remained effective against bacterial infection for millions of years, 'confounding the general belief that bacteria, fungi and viruses can and will develop resistance to any conceivable substance.' Thus Schroder (1999) writes that '.. it seems to be difficult for micro-organisms to acquire resistance, making these peptides very attractive for therapeutic use as antibiotics', while Hancock & Chapple (1999) claim that 'It is also very difficult to raise mutants resistant to these cationic peptides, and there are very few naturally resistant bacteria'.

It is very important that these claims turn out to be true. The evolution of resistance to any antibiotic makes it less useful in treating disease, of course. Quite incidentally, it also deprives any organism that produces it of part of its antibacterial armoury. This would not normally be a matter for concern; but in the case of antimicrobial peptides, *we ourselves are the producers*. The evolution of resistance to human antimicrobial peptides, therefore, may have much more serious consequences than the evolution of resistance to conventional antibiotics, because our ability to resist infection might be permanently compromised. Before these substances are released for general use, it is, in our view, important to be quite sure that we are justified in dismissing the possibility that bacteria will evolve resistance to them.

#### **Resistance to RAMPs**

The key issue is whether or not resistance to RAMPs will evolve when they are used therapeutically, or industrially in agriculture or food processing. Despite claims that resistance will seldom or never arise, or if it does will be intolerably costly, there is a steadily increasing body of evidence documenting a range of mechanisms that provide protection against RAMPs (Peschel & Collins, 2001). Bacteria that produce RAMPs such as nisin and epidermin must be self-immune, of course; in fact, plasmids that encode bacteriocins also bear self-immunity genes. Species that are chronically exposed to RAMPs are often highly resistant to them, and where RAMPs are used industrially novel resistance readily evolves. Nisin is already widely used as a food preservative, for example, and nisin-resistant strains have been obtained in common food-spoiling organisms such as Listeria, Clostridium, Bacillus and Staphylococcus (Ming & Daeschel, 1993). In more natural circumstances, resistant genotypes can be identified by careful studies. Plant defensins inhibit fungal growth by binding to specific receptors on the cell surface. Thevissen et al. (2000) isolated mutants of Saccharomyces with reduced binding of the defensin Dm-AMP1, which enabled them to grow at 40 µM whereas wild-type strains are inhibited by  $1-2 \mu M$ .

Resistance to RAMPs seems to vary greatly in specificity: in some cases it is highly specific and protects bacteria against only a narrow range of host peptides, whereas other cases involve mechanisms that confer broad resistance to many types of RAMP. The yeast mutants studied by Thevissen *et al.* (2000), for example, were cross-resistant to other defensins – which are structurally similar to insect RAMPs – but not to chemically unrelated antifungal agents. In a similar fashion, plasmid pSK1 of *Staphylococcus aureus* confers resistance to human platelet microbicidal protein 1 via the efflux protein encoded by *qacA*, but not to nisin or neutrophil  $\alpha$ -defensin (Kupferwasser *et al.*, 1999). Genes that mediate resistance to epidermin in *Staphylococcus epidermidis* also mediate resistance to the similar peptide gallidermin from *Staphylococcus gallinarum*, but not to the less similar lantibiotic nisin or the insect RAMP melittin (Otto *et al.*, 1998). Nisin-resistant *Listeria* and *Clostridium*, on the other hand, are cross-resistant to chemically unrelated bacteriocins (Crandall & Montville, 1998).

#### Mechanisms of resistance

Resistance to RAMPs is mediated by a range of mechanisms that prevent the proteins from entering the membrane, expel them from it, or destroy them in the cytoplasm.

#### Modification of outer cell layers

The incorporation of components with reduced anionic charge obstructs the original aggregation of RAMPs on the cell membrane. In Gram-positive bacteria such as staphylo-cocci, substitution of positively charged alanine residues into cell wall teichoic acids reduces deposition of RAMPs onto the cell surface. Transposon inactivation of the *dlt* operon creates strains with reduced alanine content that are highly susceptible to human defensin, several animal RAMPs, and even to bacterial RAMPs such as nisin (Peschel *et al.*, 1999).

#### Failure to penetrate the outer membrane

The cell membrane can readily be modified so as to diminish the effectiveness of RAMPs. The most obvious route is that lower concentrations of anionic phospholipids will enhance resistance. Substitution of lysine into the membrane phospholipids of *Staph. aureus* reduces the net negative charge and causes reduced loading of RAMPs; inactivating the locus responsible, *mprF*, greatly increases susceptibility to killing by neutrophils (Peschel *et al.*, 2001). Similar genes are found in many other bacteria. An increased content of aminoarabinose also decreases the charge on the membrane and confers a certain level of resistance to RAMPs (Shafer *et al.*, 1984). Resistance of *Staph. aureus* to human platelet microbicidal protein is caused by elevated levels of long-chain unsaturated lipids that cause greater membrane fluidity (Bayer *et al.*, 2000).

The secretion of RAMPs is often induced by host recognition of Gram-negative bacteria mediated by the binding of receptors such as CD14 to lipid A of the bacterial outer cell membrane; in turn, bacteria have systems for detecting the presence of host tissues and modifying their cell membrane so as to evade recognition. In *Salmonella*, the signal transduction pathway PhoP/PhoQ mediates resistance to RAMPs and survival within host tissues. It controls the expression of a large group of genes, including about 15 that encode outer-membrane proteins. PhoPactivated genes are responsible for the survival of the cell in environments such as host macrophages, principally through modifying the composition of lipid A and thus changing the conformation of the membrane surface, making it less vulnerable to detection and destruction by the host. There is also a group of PhoP-repressed loci, which encode proteins involved in host tissue invasion. PhoP/PhoQ is essential for pathogenesis, and strains in which the system is inactivated are avirulent (Miller et al., 1990). The variety of loci controlled by the system and the complexity of their interactions provides a rich field for the evolution of different kinds of resistance to RAMPs. Many and perhaps all other Gram-negative bacteria have PhoP/PhoQ homologues and are virulent because of their ability to modify their cell membranes (Oyston et al., 2000).

#### Export of peptides from the cell

Efflux systems may remove peptides from the cytoplasm. Those described so far fall into four main categories: ATP-binding proteins, 'major facilitator' proteins, resistancenodulation-division proteins and small multidrug resistance proteins. Many organisms can express more than one efflux system. All of these efflux systems can provide the basis of resistance to RAMPs. In staphylococci, the plasmid pSK1 confers resistance to several classes of antibiotics. It includes the gacA locus, which encodes a member of the major facilitator group of proteins that is capable of exporting a broad range of structurally dissimilar organic cations from the cell. The plasmid confers resistance to platelet microbicidal protein 1, a RAMP expressed in human neutrophils (Kupferwasser et al., 1999). Moreover, strains isolated from endovascular infections are more resistant to tPMP-1 than strains isolated from soft-tissue abscesses (Bayer et al., 1998), suggesting that resistance can evolve in bacterial populations within the body. Lantibiotics such as epidermin are expelled from the cytoplasmic membrane of Staph. epidermidis by ATPdependent translocases encoded by the three genes epiE, epiF and epiG (Otto et al., 1998). These are similar to the ABC transporters with conserved ATP-binding cassettes responsible for the uptake or excretion of a broad variety of substrates in a wide range of organisms. Specific resistance to lantibiotics in staphylococci is also conferred by small membrane-associated proteins such as PepI, although their mechanism of action remains obscure (Pag et al., 1999). Efflux pumps that confer resistance to RAMPs have been reported from a range of other organisms. Resistance to protegrin in Neisseria gonorrhoeae, for example, is associated with a plasmid-encoded efflux pump similar to those involved in resistance to other antibiotics (Shafer et al., 1998).

#### Proteolysis

Once inside the cell, RAMPs are difficult to target specifically for destruction. Nevertheless, a few instances have been reported. The *pgtE* locus of *Salmonella typhimurium*, for example, is activated in host tissues by PhoP and encodes an outer-membrane protease that cleaves  $\alpha$ -helical peptides and thus protects the cell against RAMPs such as defensins (Guina *et al.*, 2000).

It seems possible that resistance to RAMPs, far from being unlikely and uncommon, is widespread in nature and readily induced in the laboratory. Indeed, it has become clear in the last few years that resistance to RAMPs is a normal and necessary component of pathogenesis in *Salmonella* (Groisman *et al.*, 1992) and *Staphylococcus* (Peschel & Collins, 2001). Resistance does not invariably evolve. The widespread use of nisin as a food preservative or of polymyxin B as a topical antibiotic has not led to any dramatic increase in levels of resistance. Rather, the evidence suggests caution in accepting claims that resistance will not evolve.

#### The evolution of antibiotic resistance

There is a sharp qualitative difference between two kinds of mechanism that have nothing in common. On the one hand, there are the processes, briefly reviewed above, by which individuals survive in the presence of toxins. These involve molecular interactions such as efflux pumps and proteolytic enzymes. On the other hand, there are the processes by which populations adapt to the presence of toxins, the most important of which is continued selection. It is conceivable, of course, that RAMPs are so intransigent that no molecular mechanisms exist that are capable of resisting them, in which case no adaptation can occur. Because this does not seem to be the case, however, we must enquire whether population processes are likely to lead to the evolution of resistance.

The simplest case would be a single chromosomal allele encoding resistance to a single substance. The population comprises resistant cells (symbolized R) that bear the allele, and susceptible (S) cells that do not. The environment, in an equally simple manner, consists of toxic (T) patches, where the substance is present, and permissive (P) patches, where it is not. At regular intervals cells are redistributed randomly among patches, with cells from the toxic patches contributing some fixed fraction K of the total population. The frequency of the resistant allele at equilibrium is then

$$f_{\rm R}^* = [K(w_{\rm RT}w_{\rm SP} - w_{\rm RP}w_{\rm ST}) + w_{\rm ST}(w_{\rm RP} - w_{\rm SP})]/(w_{\rm ST} - w_{\rm RT}) (w_{\rm RP} - w_{\rm SP})$$

where  $w_{ij}$  is the fitness of the i-th type in the j-th patch, provided that  $0 < f_R^* < 1$ ; otherwise the allele is maintained at very low or very high frequency by mutation-selection balance. Now, suppose that the susceptible allele is lethal in toxic patches  $(w_{ST}=0)$ , set  $w_{SP}=1$  without loss of generality, and define the cost of resistance to be  $C=w_{SP}-w_{RP}$ . Then we have the simple result

$$f_{\rm R}^* = K/C$$

showing how the evolution of resistance depends on the relationship between the frequency of exposure to the toxin and the cost of resistance. The frequency of resistance alleles will increase with K, that is, as the frequency (or more precisely the productivity) of toxic sites increases, and will reach fixation if  $K \ge C$ . This might be caused by the clinical or agricultural use of antibiotics, for example. Resistance will also be favoured if the cost C is reduced. This might happen through compensatory mutations in the original resistant background, or more simply through the general intoxication of the environment as a whole through the release of large volumes of the antibiotic, which by favouring the spread of weakly resistant types reduces the relative disadvantage of strongly resistant types. Resistance alleles will always segregate at appreciable frequencies if  $K \gg 0$ , because they have a refuge in toxic patches, and are fixed if K > C.

#### **Evolution of resistance to RAMPs**

This simple picture will be affected by mutation, plasmid transmission, the occurrence of cross-resistance, transmission among hosts and many other factors, so that more realistic models of infection are necessarily more complicated. Sophisticated mathematical theories describing the evolution of resistance to antibiotics have been developed in population genetics (Levin et al., 1997; Stewart et al., 1998) and epidemiology (Austin et al., 1999; Lipsitch et al., 2000). Models that refer explicitly to the use of antibiotics in medical practice, including variables such as patient turnover, are summarized by Levin (2001). The essential features of these models can be used to construct computer simulations that track the evolution of resistance to conventional antibiotics or RAMPs (Fig. 1). A reasonable scheme is to begin with a pristine environment inhabited by a population of susceptible bacteria that are able to grow and to migrate onto a population of normal (uninfected) hosts, where they live as commensals. As they do so, resistant types arise at low frequency by mutation. These may have a lower growth rate, reflecting the existence of a cost of resistance. From time to time, however, a host may become infected as the result of a chance cut or graze. If an antibiotic is applied, resistant bacteria will have an advantage and will thereby tend to spread. The frequency of resistance at equilibrium will depend on the fraction of the bacterial population that grows on infected hosts and on the cost of resistance, in the manner caricatured in the previous section.

A model that simulates the evolution of resistance to RAMPs differs from a conventional model in one important respect. In a conventional model, the probability of infection ( $P_{infect}$  in Fig. 1) depends on the total bacterial

population at the site of injury, whether susceptible or resistant (to the conventional antibiotic), since the antibiotic has yet to be applied. In a model specific to RAMPs, on the other hand, whether or not a host individual becomes infected after a chance injury depends primarily or entirely on the number of resistant bacteria that it harbours, since bacteria susceptible to RAMPs grow poorly if at all. Chemotherapy will then facilitate the evolution of resistance if it magnifies the difference in growth rates between susceptible and resistant bacteria.

Given that the RAMP treatment is effective, so that susceptible bacteria are inhibited in treated hosts, the evolution of resistance depends in the first place on the balance of two kinds of cost. The first is the cost of resistance in pristine habitats or on normal (non-treated) hosts, as before, which can be defined as  $C_{\rm RP} = (G_{\rm SP} - G_{\rm RP})/$  $G_{\rm SP}$ , where G is a growth rate. [For explicit definitions of parameters, see legend to Fig. 1.] The second is the cost of susceptibility in infected hosts,  $C_{\rm SI} = (G_{\rm RI} - G_{\rm SI})/$  $G_{\rm RI}$ . At one extreme, suppose that  $G_{\rm SI} = 0$ , so that the native RAMP defences are fully effective in killing susceptible bacteria. This may provoke the evolution of resistance, but the situation is not worsened, or changed much, by the introduction of RAMP chemotherapy. At the other extreme, suppose that  $G_{SI} = G_{RI}$ , so that native RAMPs are completely ineffective. In this case, RAMP chemotherapy will cause the evolution of resistance in much the same way as conventional antibiotics. It is in the region between these two extremes that administering RAMPs to infected patients may provoke the evolution of resistance that would not otherwise occur.

For example, suppose that we set  $C_{SI} = 0.25$ , so that the growth rate of susceptible types is 25% less than that of resistant types in infected hosts. There are roughly equal numbers of bacteria living on hosts and in the general environment, with moderate rates of movement between the two. Other parameters are chosen so as to be biologically reasonable (see legend to Fig. 1); for example, susceptible bacteria are barely able to grow in infected hosts ( $G_{SI} = 1.125$ ), and the growth of resistant bacteria is less in treated hosts ( $G_{RT} = 1.2$ ) than in untreated hosts  $(G_{\rm RI} = 1.5)$ , although it exceeds that of susceptible bacteria in both ( $G_{ST}=0$ ,  $G_{SI}=1.2$ ). With no RAMP treatment, resistance is maintained at very low frequency by recurrent mutation if  $C_{\rm RP} > 0.33$  about, and spreads to high frequency if  $C_{\rm RP} < 0.38$  about. Between the two is a region where the outcome is heavily influenced by the stochastic nature of infection, and where the evolution of resistance may be long-delayed, if it occurs at all. The threshold value of  $C_{\rm RP}$ that permits the evolution of resistance is thus about 0.35. We can then investigate the effect of allocating each infected individual, immediately after the onset of infection, to a course of RAMP therapy with a probability of 0.5. In this case, the critical value of  $C_{\rm RP}$  is about 0.65. Thus the effect of the therapy is to enlarge the class of resistant mutants able to invade the population and be



Fig. 1. Model of a bacterial population challenged by an antibiotic. The parameter values given are those used in the example cited in the text.

(a) Genetic model. Bacteria are Susceptible or Resistant, with each type arising from the other by mutation (forward and backward rates equal,  $u = 10^{-6}$ ).

(b) Environmental model. There are five kinds of site, each capable of supporting a maximum of K bacteria:

Pristine sites in the general environment ( $K_{\text{pristine}} = 100\,000$ )

Polluted sites, contaminated by antibiotics ( $K_{polluted} = 0$ )

Normal hosts, colonized by bacteria but asymptomatic ( $K_{normal} = 1000$  per host, population of n = 100 individuals) Infected hosts, in which bacteria cause disease symptoms (unlimited)

Toxic hosts, treated with antibiotic (unlimited)

(c) Growth model. The factor by which each genotype in each site grows in one cycle:

Toxic host:  $G_{ST} = 0$   $G_{RT} = 1.2$ 

(d) Dispersal model. A fraction D of the population at each site disperses from that site at the end of each cycle. Of the bacteria living on hosts, a fraction T are transmitted to other hosts (T=0.5). The remaining bacteria, with all of those from pristine and polluted sites, are redistributed among sites according to the target size S of the sites.

Pristine sites:  $D_P = 0.1 S_P = 10000$ 

Polluted sites:  $D_L = 0.1$   $S_L = 0$ 

Normal host:  $D_N = 0.1 S_N = 1$  per individual

Infected host:  $D_1 = 0.1$   $S_1 = 1$  per individual

Toxic host:  $D_T = 0.1 \ S_T = 1$  per individual

(e) Infection model. Transition between host states is a stochastic process governed by the bacterial load L borne by an individual. In conventional models, this would be the total number of bacteria; in RAMP models, it is the number of resistant bacteria.

Transition from Normal to Infected:

 $P_{\text{infect}} = I_{\text{limit}} [1 - \exp(-i^* L)] : I_{\text{limit}} = 1, i = 0.01$ 

Transition from Infected to Toxic:

 $P_{\text{treat}} = \text{constant} (0 \text{ or } 0.5)$ 

Transition from Toxic to Normal if number of bacteria drops below threshold for infection. Transition from Toxic to death  $P_{\text{death}} = T_{\text{limit}}[1 - \exp(-t^* L)]$ :  $T_{\text{limit}} = 1$ , t = 0.0001

A dead host is immediately replaced by a new Normal host free of bacteria.

maintained at high frequency. This effect is quite a large one. Without therapy, only mutants able to grow at about two-thirds the rate of the susceptible types can invade. When half of infected hosts receive AMP therapy, on the other hand, resistant types that grow at about a third of the rate of the susceptible types can invade. Thus administering RAMPs to infected hosts can greatly relax the conditions for the evolution of resistance.

Whether RAMP therapy causes the spread of resistance in circumstances where it would otherwise not evolve depends on the combination of costs (Fig. 2). Without RAMP therapy, resistance spreads readily when  $C_{\rm RP}$  is low and  $C_{SI}$  is high. Beyond a certain limit, it fails to spread at all. When RAMP therapy is applied, the evolution of resistance becomes almost insensitive to C<sub>SI</sub> and instead depends only on  $C_{RP}$ ; resistance will spread provided that  $C_{\rm RP}$  is sufficiently small, for almost any value of  $C_{\rm SI}$ . This is because a sufficiently small value of  $C_{\rm RP}$  causes the frequency of resistance at mutation-selection equilibrium to be sufficiently high to create a few infected individuals; when these are treated they will incubate populations of resistant bacteria that are then transmitted to uninfected hosts. The effect is to create a region in which  $C_{\rm RP}$  and  $C_{\rm SI}$ are both relatively low, in which resistance spreads only when RAMP therapy is administered. The size and shape of this region depends in detail on the parameter values chosen for the model, but its existence under reasonable combinations of values argues that the possibility that resistance may evolve should be taken seriously. The main ways in which the conclusion will be modified by changing parameter values are as follows.



**Fig. 2.** Evolution of resistance to RAMPs. For 10 values of the cost of susceptibility in Infected hosts ( $C_{\rm SI}$ ), the value of the cost of resistance in Pristine sites ( $C_{\rm RP}$ ) that just permitted the invasion and establishment of Resistant types was determined by trial and error using the model specified in Fig. 1. In the area between the two lines, resistance spreads if RAMP therapy is used, but not otherwise. The thickness of the lines reflects the stochastic nature of the process.

#### (a) The genetic model

The mutation rate affects the frequency of resistant bacteria in the general environment at mutation-selection equilibrium, which in turn governs the probability that a host individual will become infected. Reducing the mutation rate retards the evolution of resistance because it retards the appearance of infected and, consequently, treated hosts; it does not affect the final frequency of resistance. Plasmid carriage, not permitted in this model but very common in natural systems, would greatly accelerate the evolution of resistance through horizontal transfer.

#### (b) The environmental model

If the number of bacteria that inhabit hosts is small relative to the number growing in the general environment, then the advantage of resistance is reduced because resistant bacteria are likely to be dispersed away from the hosts where they evolved. This does not affect the range of genotypes that spread, or the rapidity with which resistance evolves, but it reduces the final frequency of resistance. For example, if ten times as many bacteria grow in the general environment as on the host population, the final frequency of resistance falls from about 0.5 to about 0.1. The general environment might become polluted by the habitual use of RAMPs, however, creating patches in which resistant types have an advantage. This might occur, for example, through the use of RAMPs in agriculture or food processing. High levels of resistance to the non-RAMP bacitracin, for example, have been found in isolates from poultry, pigs and other domestic stock (Aarestrup et al., 1998) and from farm soil (Jensen et al., 2001), from where it has spread to less likely environments such as bottled mineral water (Massa et al., 1995). The mechanism of resistance involves an ABC transporter system that expels the peptide from the membrane (Podlesek *et al.*, 2000; Neumuller et al., 2001). Suppose that polluted sites are only one-tenth as frequent as pristine sites, and that susceptible bacteria are 25% less fit in these sites. With the same combination of parameters as before, the threshold cost of resistance in pristine sites rises from 0.65 to 0.75. Thus environmental pollution by particular RAMPs may extend the range of resistant genotypes that are able to spread.

#### (c) The transmission model

In many circumstances, it is likely that the bacteria dispersing from a host individual will encounter another host, rather than passing to the general environment, regardless of the relative numbers of bacteria in each. This promotes the evolution of resistance in the same way that habitat choice promotes local adaptation in simple models of heterogeneous environments. RAMP therapy magnifies this effect because of the proliferation of resistant bacteria in treated individuals. Moreover, because sick people (bearing different kinds of bacteria able to exchange genes through plasmids) tend to be aggregated in hospitals, the degree of transmission in such places is far higher than is assumed by supposing a random distribution.

#### (d) The infection model

The model describes a stochastic model of infection governed by a rate parameter. This parameter translates the number of resistant bacteria colonizing a host into a probability of infection, and consequently its effect on the evolution of resistance is essentially the same as that of the mutation rate.

#### (e) The treatment model

When infected individuals are more likely to be treated, the overall relative fitness and thus the final frequency of resistance increases.

Although the detailed behaviour of the model depends on the parameter set used, the evolution of resistance to RAMPs as a consequence of their use in therapy occurs over a broad range of parameter values. Moreover, natural populations seem likely to lie within this range. It is clear that resistance is not currently segregating at high frequency in most populations of pathogenic bacteria. Thus  $C_{\rm SI}$  must be relatively low, whereas  $C_{\rm RP}$  might be either low or high. The cost of resistance to conventional antibiotics is often surprisingly low, and can be further reduced by the spread of compensatory mutations after the establishment of resistance (Andersson & Levin, 1999). We do not know much about the cost of RAMP resistance, but there is no reason to suppose that it is out of line. Nisin-resistant strains of Listeria and Clostridium grow more slowly than wild-type on a range of standard media (Mazzotta et al., 2000), for example, but the effect is not a large one. This would place populations in the region of the  $C_{\rm SI} - C_{\rm RP}$ phase space where RAMP therapy is most likely to trigger the evolution of resistance. Certainly, the argument that this is inherently unlikely to occur is without foundation.

If resistance is likely to evolve as a consequence of the widespread use of RAMPs, why are bacterial strains resistant to the great range of peptides produced by living organisms so rare? This is the strongest reason for believing that resistance will not evolve after all (Zasloff, 2002). The great diversity of RAMPs, which is one of the most striking features of this class of substances, could be interpreted in two ways, however. In the first place, RAMPs may have evolved independently in each species, or small group of closely related species. They would then provide a vast reservoir of antibiotics with almost unlimited potential to control bacterial populations. This seems very unlikely, because the systems for the induction and expression of RAMPs are strikingly similar in Drosophila and mammals (Hoffmann et al., 1999). The second possibility is that bacteria evolve specific resistance rather easily, so that rare peptides are likely to be more effective. This would generate

negative frequency-dependent selection driven by hostpathogen coevolution that would lead to rapid evolution at RAMP-encoding loci and thus great diversity among species. The population frequency of resistant types depends in part on the diversity of host defences. Wild populations of grasses are usually infested at low levels by a variety of pathogens that rarely cause serious disease, whereas the large-scale planting of cereal monocultures often elicits epidemics; natural communities of fungi produce a diversity of antimicrobial agents, but resistance remains low because a lineage of bacteria is rarely exposed for long enough, or in large enough numbers, for selection to be effective. If this interpretation be correct, then the therapeutic use of insect or fungal RAMPs might not be of great concern, any resistance that evolves as a consequence being highly specific, whereas the use of mammalian or human RAMPs would be correspondingly risky.

## Consequences of the evolution of resistance to human RAMPs

Drug development is driven by commercial (people trying to make money) and social (people trying to get well) pressures. For reasons that are easy to understand, regulatory procedures (for example, those developed by the US Food and Drug Administration: Guidance for Industry, HEW (FDA) 77-3046; document available at http://www.fda.gov/cder/guidance/index.htm) emphasize the efficacy and safety of a substance that is administered directly to individuals. It is very likely that RAMPs, including human RAMPs, can satisfy regulatory criteria and will be introduced into clinical practice in the near future. They will probably be effective in controlling infection, and will thereby directly enhance the health and well-being of millions of people. Less fortunately, regulatory procedures include no provision for estimating the effects on the health of populations in the future, nor do they require, or ever involve, participation in trials by qualified population or evolutionary biologists. Consequently, they are poorly designed to detect even grave and highly probable risks to public health arising from the population biology of microbes. We should be prepared, therefore, for the less desirable side effects that will follow from the evolution of resistance to RAMPs. For epidermal systems, these might include the frequent failure of minor cuts and scrapes to heal properly, and the increased risk that they will develop into serious bacteraemias. For neutrophil systems, we might experience a higher incidence and greater severity of diseases caused by chronic infection or subsequently associated with it, such as certain kinds of heart disease and cystic fibrosis. Instead of dismissing the possibility that widespread resistance will evolve, we should use the bitter experience that we have gained from conventional antibiotics to plan for it. The impact of resistance can be reduced by a range of procedures involved in the prescription of drugs and the treatment of patients (Levin, 2001). A more ambitious approach would be to supplement the traditional medical practice of treating individual

patients by attempting to manage populations of bacteria so as to control and direct the evolution of resistance. At all events, the status of infectious bacterial disease in 10 or 20 years' time will depend as much on our ability to understand evolutionary mechanisms and manipulate population processes as it will on our mastery of pharmacology.

#### Coda

Susceptibility to RAMPs has been called the 'Achilles' heel' of bacteria (Zasloff, 2002). The reference is to the Greek hero whose whole body, except for the right heel, was made invulnerable by being dipped in the Styx or (more plausibly) burned on a sacred fire. After prevailing in many combats, he was at last killed before the walls of Troy when Paris shot him in the heel with an arrow. This is encouraging; but there are darker myths. Deianeira the wife of Heracles wove a fine new shirt for her husband on his return from Trachis. When it was complete, she rubbed it with a piece of wool soaked in the blood of Nessus, a centaur whom Heracles had killed for attempting to ravish her. This was intended as a love-charm to ensure Heracles' fidelity; but instead the poison in the blood burned off his skin and killed him. Thus a garment that was intended to protect its wearer concealed the agent of his destruction. One moral of the story might be that any fine new clothes should be carefully tested for any trace of centaur's blood.

#### Acknowledgements

G. B. is funded by the Natural Sciences and Engineering Research Council of Canada, and by the Fonds pour les Chercheurs et a l'Aide de la Recherche du Québec.

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