PART IV

Pathogens: resistance, virulence, variation, and emergence

CHAPTER 10

The ecology and evolution of antibiotic-resistant bacteria

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Introduction

Nosocomial (hospital-acquired) infections are a severe and often underappreciated public health problem. In the United States alone, at least 200,000 people and probably far more suffer from a hospital-acquired infection every year. The associated mortality is considerable; the Center for Disease Control has estimated that 90,000 U.S. residents die each year from nosocomial infections. To place this number in context, AIDS/HIV kills approximately 17,000 per year in the United States, influenza 37,000 per year, and breast cancer roughly 40,000 per year.

As large as these numbers are, some estimates suggest that the actual magnitude of the problem could be up to tenfold higher. In 2004, the state of Pennsylvania instituted a mandatory reporting program for hospital-acquired infections (Volavka 2005). That year, Pennsylvania hospitals reported 11,668 hospital-acquired infections to the Pennsylvania Health Care Cost Containment Council (PHC4)—but they told a very different story to the insurance companies. By examining insurance billing records for claims and diagnoses that potentially resulted from nosocomial infections, PHC4 found that insurance claims were tenfold higher (115,631) than were the direct reports that they received. If one extrapolates from the Pennsylvania data to the entire United States, there could be over 2 million nosocomial infections per year in the United States. If the higher estimate is correct, roughly 7.5% of all hospital visits in the United States result in nosocomial infections, and dealing with these infections would be a major cause of antibiotic use in the clinical setting. These problems are not restricted to the United States. In Latin America, 6–10% of all hospital visits result in nosocomial infections (Salvatierra-González 2004). Worldwide, the WHO estimates that 8.7% of all hospital visits result in a nosocomial infection, with 1.4 million patients suffering from these infections at any given time (Tikhomirov 1987).

Nosocomial infections present such a great health challenge because they are often caused by antibiotic-resistant strains of bacteria well adapted to the hospital. Patients who are infected with antibiotic-resistant strains stay in the hospital longer, are more likely to die, and are more expensive to treat than are the patients who are infected with the drug-sensitive strains common outside of the hospital. For example, infection by *Enterobacter* strains resistant to third generation cephalosporins increases mortality fivefold, and the length and cost of stay by 50%, relative to infection by drug-sensitive strains (Cosgrove 2006). Methicillin-resistant *Staphylococcus aureus* (MRSA) infection doubles the mortality and increases the cost of care by nearly 40% relative to methicillin-sensitive *S. aureus* (MSSA) infection (Cosgrove 2006). The total economic burden of antibiotic resistance in clinical settings in the Untited States may be as high as $80 billion annually (S. Foster, personal communication).

In this chapter, we lay out what is known about the ecology and evolution of antibiotic resistance, with an emphasis on hospital-acquired strains of bacteria. We begin with a brief history of resistance to clinical antibiotics.

History of clinical antibiotic resistance

Although antibiotic-resistant bacteria are often characterized as emerging infectious diseases, physicians have been confronted with antibiotic resistance for as long as they have been using antibiotics. Modern antibiotics essentially began with penicillin, which was first used clinically in 1943 and was widely employed toward the end of the Second World War. Reports of penicillin resistance came within a year (Kirby 1949), and by 1945, a British hospital reported that nearly 8% of staphylococcal isolates were resistant to penicillin (see Table 10.1). Four years later, almost 60% of British clinical isolates were penicillin-resistant (Barber and Whitehead 1949). Similar patterns occurred in the United States (Rolinson 1971).

An arms race between drug development and resistance evolution ensued. In the 1950s cephalosporin C and its derivatives were introduced, and that, combined with the release of broad-spectrum penicillins in the early 1960s, selected for the plasmid-encoded broad spectrum β-lactamases, which confer resistance to penicillin as well as cephalosporins. The genes encoding the broad spectrum β-lactamases were often located on genetically mobile plasmids of *Escherichia coli* and thus were easily transferred both to other *E. coli* lineages and to other species that had previously lacked cephalosporin resistance (also see 'Mechanisms' below) (Matthew 1979; Medeiros 1997). Resistance to third generation cephalosporins was observed several years after their introduction and was widely disseminated by plasmid transfer in the following decade (Papanicolaou *et al*. 1990). During the mid-1980s, increasing use of carbapenems and monobactam also led to the evolution and widespread plasmid-mediated dissemination of carbapenem-hydrolyzing β-lactamases (Bush 2002). Finally, resistance to fourth generation cephalosporins, such as cefepime, is also increasing to the point where these β-lactamases ('CTX') have spread into bacteria from agricultural habitats, although this particular family of β-lactamases is still rare in the United States (Damjanova *et al*. 2006; Oteo *et al*. 2006). These emergent resistance loci make it all the more distressing that the U.S. Food and Drug Administration has not yet decided to prevent the approval of the agricultural use of cefquinome, a cephalosporin analogue of cefepime.

Another example is the rapid evolution of resistance to the macrolide antibiotic erythromycin. Introduced in 1952, erythromycin was heralded as a treatment of staphylococcal infections and used widely. In 1956, the first erythromycin-resistant staphylococci were isolated in France and the United States. By the late 1970s, *erm*-encoded

Table 10.1 The rapid evolution of antibiotic resistance in clinically important bacteria

Antibiotic	Year introduced	Year resistance observed
Penicillin	1943	1945
Chloramphenicol	1949	1950
Erythromycin	1952	1956
Methicillin	1960	1961
Cephalothin (1st generation cephalosporin)	1964	1966
Vancomycin[a]	1958[a]	1986
2nd & 3rd generation cephalosporins	1979, 1981	1987
Carbapenems	1985	1987
Linezolid	2000	2002

[a] Vancomycin was first released in 1958; however, it was not widely used until the early 1980s.

erythromycin resistance had successfully transferred to the respiratory pathogen *Streptococcus pneumoniae*. Currently, erythromycin resistance is extremely common in *S. pneunomiae* and should probably be viewed as the 'wild-type' phenotype: in parts of Asia, over 80% of *S. pneumoniae* isolates are resistant to erythromycin and other macrolides, and in many other countries erythromycin resistance ranges between 30 and 60% of clinical isolates (Bozdogan *et al*. 2003; Roberts and Sutcliffe 2005).

We have seen similar patterns for numerous other antibiotics. In response to increasing resistance to penicillin and erythromycin, physicians began using methicillin in 1960; resistance was observed within a year (Deresinski 2005). Methicillin-resistant strains, most notably methicillin-resistant *S. aureus*, exploded in epidemic proportions in hospitals during the 1980s. Vancomycin, though released in 1958, was not used heavily until the 1980s, when it became a common response to MRSA. In turn, vancomycin-resistant strains of enterococci (VRE) were observed in 1986 and spread rapidly in hospitals throughout the 1990s (Levine 2006). Linezolid, from a new class of antibiotics called oxazolidinones, offered a way to deal with certain VRE strains, and was released in the US in 2000—but by 2002, linezolid resistance was already being reported in vancomycin-resistant strains (Potoski *et al*. 2002). Because widespread use of a particular antibiotic has always led to a rapid evolutionary response, the increasing realization that there is a 'resistance problem' stems from the decrease in the availability of new drugs since the 1980s and not a fundamental change in the evolutionary response of microorganisms (Spellberg *et al*. 2004).

Thus we see that antibiotic resistance, which generates considerable mortality, morbidity, and economic cost, inevitably evolves rapidly and spreads broadly following widespread antibiotic use. What are the genetic mechanisms by which resistant phenotypes arise? Where do these phenotypes arise, and how do they make their way into human-associated bacterial strains? How do bacterial population genetics contribute to long-lasting, multidrug-resistant bacterial strains? What novel approaches to preventing or treating resistance can be derived from knowledge of ecology and evolutionary biology? These questions are addressed in turn.

Genetic mechanisms

From an evolutionary perspective, the mechanisms that facilitate antibiotic resistance arise in three distinct ways:

- by point mutation: single nucleotide changes alter the structure or function targeted by the antibiotic;
- by homologous recombination of existing point mutations: point mutations found in allelic variants are reassorted into a new allele containing the adaptive mutation; and
- by heterologous recombination of novel resistance loci: resistance loci, previously not present in the recipient strain, are acquired, often through the uptake of plasmids.

Point mutations

Point mutations are often the first genetic changes observed once a new drug is introduced. For example, a single base change in the structure of the peptide-binding groove of the ribosomal RNA can prevent some macrolide antibiotics from binding to their target (Hansen *et al*. 2002). Similarly, resistance to the quinolone antibiotics such as ciprofloxacin commonly results from point mutations in the gyrase and DNA polymerase subunits (Vila 2005).

While the odds are low that any particular mutation will occur in the right place in the genome to confer resistance in this way, bacterial population sizes are so large that it is quite likely that such a mutation will occur somewhere within the population of bacteria inhabiting a human host. For example, the human small intestine supports 10^{10} to 10^{11} bacterial cells per gram of fecal matter. Given mutation rates of roughly 2×10^{-3} per genome per replication and genome sizes on the order of 5×10^{6} base pairs, a single gram of fecal matter is likely to include at least one newly occurred instance of every single point mutation (Genereux and Bergstrom 2005). Moreover, even in large populations, allele frequencies can change substantially in as little as a day as a result of rapid generation times of bacteria and the strong selection for resistance in a population exposed to antibiotics. Population bottlenecks associated with

drug treatment or colonization events only further accelerate the process.

Homologous recombination

Homologous recombination is a second mechanism that can result in the evolution of resistance. A classic example is the evolution of penicillin resistance in *Neisseria gonorrhea*, the pathogen that causes gonorrhea. Changes in *penA*, which encodes penicillin-binding protein 2, can result in resistance to penicillin and other β-lactam antibiotics (Spratt 1994; Antignac *et al*. 2001). Interestingly, most resistant *Neisseria* do not appear to have acquired penicillin resistance via point mutations in *penA*, even though it is possible to generate such mutants in the laboratory. Rather, resistance is associated with 'mosaic' alleles derived from multiple recombination events with other *Neisseria* species (Spratt 1994). Sequence divergence within the recombinant regions of *penA* can reach up to 20%, indicating that the donor alleles diverged millions of years ago (Thulin *et al*. 2006).

Heterologous recombination

The most elaborate resistance mechanisms typically arise through a third process: heterologous recombination, the acquisition of novel resistance loci. Resistance genes move easily; one important route is plasmid transfer, where a plasmid 'minichromosome' containing resistance genes is transferred from one cell to another. Resistance genes may reside on plasmids either because of recent anthropogenic selection from heavy antibiotic use (Barlow and Hall 2002a) or because of long-term evolutionary associations lasting tens of millions of years (Barlow and Hall 2002b), making it all the more easy for them to reach human-associated bacterial strains. The frequent association of resistance genes with integrons and other highly mobile genetic elements facilitates movement of these genes between plasmid and chromosome (Levin and Bergstrom 2000).

Another route through which resistance genes move is via the cellular uptake of heterologous stretches of chromosome from some other source, either by evolved mechanisms of DNA uptake, known as active transformation (Lorenz and Wackernagel 1994), or by virally mediated transduction. While transformation rates—and with them heterologous recombination rates—vary dramatically across species (Maynard Smith *et al*. 1993; Maynard Smith and Smith 1999), there is good evidence of reasonable recombination frequencies even among those species once considered to be entirely clonal (Feil *et al*. 2001).

Heterologous recombination events typically involve the gain of a single locus or operon, such as the AcrAB efflux pump system in *E. coli*, which removes antibiotics by actively excreting them from the cell (George 2005). Efflux pumps are clinically problematic because they often confer resistance to multiple antibiotics and other antimicrobial compounds. For example, the AcrAB-TolC efflux pump found in *E. coli* confers resistance to chloramphenicol, tetracycline, erythromycin, novobiocin, fusidic acid, and various β-lactams. The same system also confers resistance to detergents, pine oil, fatty acids, bile salts, and organic solvents (Nikaido 1996). Because of its capacity to handle many substances, this efflux pump can now be maintained by selective pressures quite different from those that originally caused it to spread. This situation is not unique to therapeutic antibiotics and appears to apply to other antimicrobials, such as bacteriocins (Feldgarden and Riley 1999).

Enterococcus species provide an even more dramatic example of resistance via heterologous recombination. Vancomycin-resistant enterococci (VRE) occur at very high rates in hospital intensive care units, where they increase mortality, morbidity, and cost of care significantly (Lodise *et al*. 2002; Kaye *et al*. 2004). They were essentially untreatable prior to the development of the new antibiotics quinupristin-dalfopristin and linezolid early in this decade. VRE derive their resistance from an altered ligase that changes the structure of their cell wall (Marshall *et al*. 1997, 1998) and is encoded by one of six different gene clusters: VanA, VanB, VanD, VanE, and VanG, which have been acquired by lateral gene transfer, and VanC, which is native to several *Enterococcus* species (Depardieu and Courvalin 2005). The operons *vanA* and *vanB* can be found on plasmids or chromosomes, while the other four operons are exclusively chromosomal (Arthur

et al. 1996b; Casadewall and Courvalin 1999; Arias *et al*. 2000; Abadia Patino *et al*. 2002; Depardieu *et al*. 2003; Depardieu *et al*. 2004). The operons vary both in the conditions under which they are induced and in the level of resistance conferred to glycopeptides. Some operons confer resistance to vancomycin alone because the glycopeptide antibiotic teicoplanin does not induce resistance gene expression and consequently cannot provide resistance to teicoplanin; when induced in the laboratory, the products of these operons can confer resistance to teicoplanin**.** Other operons confer resistance of varying strength to both commonly used glycopeptide antibiotics (Arthur *et al*. 1996a; Quintiliani and Courvalin 1996; Depardieu *et al*. 2004).

VRE are an immediate clinical challenge that also pose an evolutionary threat, for they can serve as a source of vancomycin resistance genes for other hospital-associated bacteria. Most worryingly, vancomycin resistance has been transferred several times from VRE to MRSA, creating the specter of a highly resistant superbug (Flannagan *et al*. 2003). Fortunately, the infections were controlled in all of these cases and vancomycin-resistant MRSA has not yet taken off in hospitals or other environments.

Natural ecology

Not only do antibiotic resistance mechanisms arise by several different genetic processes; they enter human populations from a variety of ecological settings. In this section, we discuss three of the most important: natural soils, agricultural ecosystems, and patient care facilities such as hospitals and nursing homes.

Soil ecology

Most often, bacterial species that naturally produce antibiotics are the original source of laterally transmitted genes. Many soil microbes live in environments that are highly structured spatially, where they compete fiercely with other species for space and resources. Over millions of years of natural selection in these environments, bacteria have evolved an extensive repertoire of chemical weapons (antibiotics) and defenses (resistance mechanisms). Antibiotic resistance is not only used to withstand attacks from other strains and species; bacteria that naturally produce antibiotics have had to evolve antibiotic resistance mechanisms to protect themselves from their own antibiotic products.

A recent survey of resistance mechanisms in spore-forming soil bacteria revealed an astonishing preponderance of resistance phenotypes, with multidrug resistance in every one of the 480 strains screened and with no class of medical antibiotics effective against all of the strains (D'Costa *et al*. 2006). These antibiotic resistance mechanisms are not limited to soil-living bacteria; they may also be quite common in the enteric bacterial populations of wild animals. Sherley *et al*. (2000) sampled the enteric bacterial populations associated with Australian mammals. Even where human antibiotic use is rare to non-existent, many enteric bacteria were resistant to several drugs—and the genes conferring many of these resistant phenotypes predate the human use of antibiotics.

Agricultural use

Crucial as antibiotics are to human health, their use is by no means restricted to human or even veterinary medicine. In 2001, the Union of Concerned Scientists estimated that 70% of the antibiotic use in the United States was for non-therapeutic agricultural purposes (Mellon *et al*. 2001). Most of this non-therapeutic use was as a growth enhancer: it was not used to treat infections but rather at subtherapeutic doses to make animals larger. This is a particularly effective way to generate antibiotic resistance, because subtherapeutic doses allow for gradual step-wise evolution of resistance under a mild selective regime, whereas therapeutic dosing imposes a much stronger selective regime that requires a large phenotypic change if a bacterium is to survive. Therapeutic doses also eliminate virtually all of the antibiotic susceptible cells that could potentially acquire resistance genes through gene transfer, whereas subtherapeutic doses do not (Lees *et al*. 2006). For these reasons, the use of antibiotic growth enhancers in animal feed was banned in 2006 in the European Union.

Agricultural use has consequences. Resistance mechanisms that evolve in agricultural settings

can transfer into human populations, contributing to the overall problem of antibiotic resistance. Two of the most clearly documented cases of agricultural use leading to antibiotic resistance in humans are provided by nitrofurantoin resistance and vancomycin resistance.

Nitrofurantoin has been increasingly used in human medicine to treat urinary tract infections as resistance to cotrimoxazole (trimethoprim-sulfamethoxazole) has evolved. Nitrofurantoin can be carcinogenic, as its mode of action is to damage bacterial DNA, and thus was banned from agricultural use in the United States and the European Union (Prescott 2006). In 2002–3, there was an 'outbreak' of illegal nitrofuran antibiotic use in Portugese poultry farming (Antunes *et al*. 2006). Decreased susceptibility of *Salmonella* species to nitrofurantoin skyrocketed to 65% of all *Salmonella* isolates, and the prevalence in food animals of *Salmonella enteriditis* serogroup D isolates also increased (these particular salmonellae are very pathogenic in humans). Also, the frequency of nitrofurantoin resistance was indistinguishable between hospital isolates and poultry, while *Samonella* from other sources had much lower frequencies of resistance to nitrofurantoin. In addition, nitrofurantoin-resistant *Salmonella* were more likely to be resistant to other antibiotics.

Perhaps the most dramatic example of the effect of antibiotic use in agriculture occurred in the European Union, where the vancomycin analogue avoparcin was used extensively in feed. In Denmark, studies in the mid-1990s indicated that poultry and pigs on farms where avoparcin was used were three times as likely to carry vancomycin-resistant enterococci (Bager *et al*. 1997). Following a ban of avoparcin in 1997, the prevalence of VRE decreased both in farm animals and in the human population, with VRE in the human population dropping from 12% of isolates to 3% (Bager *et al*. 1999; Klare *et al*. 1999). While the ban of avoparcin was followed by a decrease in VRE, VRE remains more frequent in Denmark than in the United States (Aarestrup *et al*. 2001). The ban of avoparcin was unable to eliminate vancomycin resistance, probably because of linkage between vancomycin resistance genes and other antibiotic resistance genes on transmissible plasmids; this linkage has maintained VRE at low frequencies even in countries where avoparcin was never used in agriculture (Tomita *et al*. 2002; Lim *et al*. 2006). Given the burden of infection by VRE and other resistant bacteria, serious efforts to limit the spread of resistance in agriculture are worth undertaking.

Hospital transmission

Once antibiotic-resistant strains enter the human population and from there enter the hospital, they often spread rapidly through the hospital environment. To understand how and why, we need to understand the ecological circumstances that bacteria encounter within a hospital. Several features distinguish the ecology of hospital-acquired infection from the ecology of most community-acquired infectious diseases.

- Most of the resistant bacteria that cause problems in hospitals are species that are normally human commensals rather than pathogens (Bonten and Weinstein 1996). Many patients carry and transmit these bacteria asymptomatically, and patients often enter the hospital already colonized by sensitive strains of the species ultimately responsible for the resistance problem. If a patient is carrying a sensitive strain when admitted to the hospital, he or she may be less likely to be subsequently colonized by more dangerous hospital-associated strains.
- Antibiotics are used at very high rates in the hospital population in general and in intensive care units in particular. While some of the antibiotic use aims to treat pre-existing infections, more often antibiotics are used as prophylaxis to prevent infection of surgical incisions. This generates very strong selection for resistant variants, and it also clears sensitive populations, thereby making it easier for resistant strains to colonize a patient.
- Hospital staff can unwittingly act as disease vectors, shuttling bacterial strains from colonized to uncolonized patients (Stone *et al*. 2001).
- The patient population turns over at a very high rate. Unlike the population of a town or a country, the population of a hospital is rapidly changing, with patients staying only 5–10 days on average in a U.S. intensive care unit. Thus any given patient colonized by resistant bacteria may leave the hospital

before the resistant strains are cleared. This influences the dynamics of disease transmission considerably. To remain endemic within the hospital, a strain must transmit before the patient departs (rather than before the strain is cleared), and this imposes a time scale of days to weeks on the transmission dynamics necessary for endemicity.

• Once the resistant bacteria leave the hospital, they are not necessarily gone for good. Patients that move back and forth between hospitals and long-term care facilities serve as a particularly important reservoir of antibiotic-resistant strains, reintroducing those strains into the hospital even after a hospital outbreak has been controlled (Cooper *et al*. 2004; Smith *et al*. 2004).

Several mathematical models (reviewed by Bonten *et al*. 2001) attempt to determine the influence of some or all of these factors on the dynamics of nosocomial resistance. These models may be of limited value for making precise quantitative predictions about the course of evolution or the rate of spread, both because human-associated bacteria evolve and spread in an extremely complicated ecological milieu and because of the inherent historicity and stochasticity of the evolutionary process. Nonetheless, mathematical models of antibiotic resistance in hospitals can be useful in a number of ways (Lipsitch and Bergstrom 2002). First, mathematical models can help researchers identify phenomena relatively robust to the specific details of the system. For example, a number of independently designed mathematical models have confirmed the positive effect that general infection control measures such as hand-washing and barrier precautions have in reducing antibiotic resistance frequencies within hospitals—and these models usually (though not always) find that infection control reduces resistance irrespective of the precise parameters chosen.

Models can also provide mechanistic explanations for previously unexplained observations. For example, in hospitals—but not in larger communities—bacterial populations change rapidly in response to changes in antibiotic use and other interventions. Lipsitch *et al*. (2000) showed that this is a result of the high turnover rate at which patients leave the hospital; this, rather than bacterial clearance from individual patients or competition among bacterial strains, sets the time scale of change.

Mathematical models also help researchers generate testable hypotheses, and they can help select the most salient hypotheses for further evaluation in clinical trials. Given that clinical trials are extremely costly to run, this can be a great benefit. Similarly, models are useful in refining trial design. They can help researchers determine how to best assess the effect of an intervention and how to distinguish random fluctuations from meaningful (Cooper and Lipsitch 2004).

Population genetics

In all three of ecosystems considered above, resistance genes can persist beyond the period of antibiotic use due to population genetic processes: physical linkage of resistance genes on the chromosome and compensatory evolution to reduce the cost of resistance.

Linked genes

Once antibiotic resistance has evolved, reducing its frequency has obvious public health significance. The non-random association of multiple resistance and pathogenicity loci can allow resistance genes to be maintained by many different selective forces operating on several different mechanisms. First, as discussed previously in the case of efflux pumps (Nikaido 1996), one mechanism can confer resistance to many drugs. In many cases, resistance genes are associated in clusters on mobile genetic elements, such as plasmids and conjugative transposons (Leverstein-van Hall *et al*. 2002; Johnson *et al*. 2006). When a mobile genetic element is acquired, multiple resistance phenotypes will be gained. Finally, resistances can accumulate through a history of exposure to multiple antibiotics (Dagan and Lipsitch 2004).

Unfortunately, virulence genes and antibiotic resistance genes can be physically linked on the chromosome as well (Qin *et al*. 2006). A study of porcine *E. coli* found widespread correlations between dozens of virulence and resistance genes (Boerlin *et al*. 2005; Travis *et al*. 2006). These correlations also

appear to have a phylogenetic basis, with different *E. coli* lineages possessing different patterns of correlation (Johnson *et al*. 2003). These lineage-based correlations might result from a past history of selection in environments that simultaneously selected for virulence and resistance. Alternatively, biochemical mechanisms in different lineages could limit the acquisition of resistance plasmids. Finally, lineages differ in their mutation rates of antibiotic resistance mutations (Wirth *et al*. 2006). In addition, other phenotypes can also maintain antibiotic resistance genes even in the absence of antibiotics. In dairy cattle, vitamin D supplementation in feed can select for antibiotic resistance plasmids (Khachatryan *et al*. 2006a; Khachatryan *et al*. 2006b).

Compensatory mutation

One might think that when antibiotic treatment is withdrawn, resistant bacterial populations will revert to sensitivity. After all, resistance can be expensive, and in the absence of the antibiotic treatment, sensitive strains that do not pay the cost of resistance should replace the resistant strains. This is not necessarily true. One culprit is the occurrence of *compensatory mutations*: changes at other loci that reduce the fitness cost imposed by the resistant allele (Schrag and Perrot 1996; Schrag *et al*. 1997; Bjorkman *et al*. 1998). If the compensatory mutations that benefit the bacteria when coupled with the drug-resistant allele are harmful when coupled with the drug-sensitive allele, this process can lead a bacterial strain into an 'evolutionary lobster-trap': a genotype easy to reach by selection in one direction, but difficult to leave even when selection goes in the other direction.

We illustrate this process in Fig. 10.1, where we envision a two-locus model with wild type rc and evolved resistant strain RC. At the R locus, the R allele is drug-resistant and the r allele is drug-sensitive. At the C locus, the C allele compensates for the cost of resistance and the c allele is uncompensated. In the presence of antibiotics (left panel), there is no fitness valley between uncompensated sensitive and compensated resistant strains; resistance evolves easily by the pathway shown, from rc to Rc to RC. In the absence of antibiotics (right panel), a fitness valley appears between resistant compensated (RC) and sensitive uncompensated (rc) strains; the resistant RC genotype cannot easily evolve back to the sensitive rc genotype, because both of the intermediates, Rc and rC, have reduced fitness. While sensitive uncompensated rc individuals would be favored in the absence of antibiotics if they were produced, the repeated bottleneck structure of human-associated bacterial populations may largely preclude the emergence and fixation of the rc type from an RC population. This is because rc individuals may not arise within RC populations in time to reach high frequency and thus survive the ensuing bottleneck (Levin *et al*. 2000).

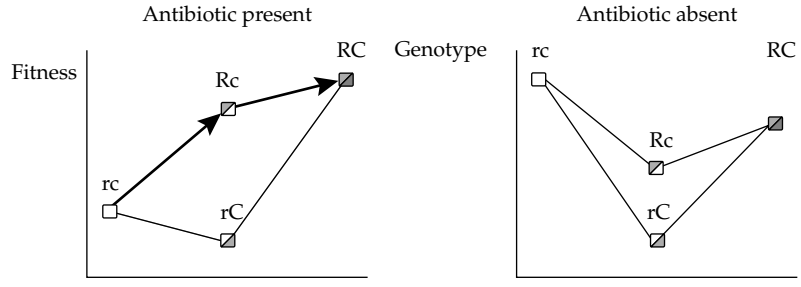

Figure 10.1 The lobster trap of compensatory mutation. When the antibiotic is present (left), resistance (R) is beneficial relative to sensitivity (r), and on a resistant background, compensation (C) is beneficial over the wild type (c). The resistant, compensated genotype RC is directly selected and can easily emerge. When the antibiotic is absent (right), the fitnesses change. Resistance is now costly relative to the sensitivity, but epistatic interactions between the loci create a fitness valley (Rc and rC genotypes) between the resistant compensated type (RC) and the highest-fitness type, sensitive uncompensated (rc). Thus a resistant compensated population does not readily revert to drug sensitivity.

Applying evolution/approaches for the future

So what can be done about antibiotic resistance? A two-pronged approach is needed. First, new drugs to which current strains are not resistant need to continue to be developed. This will provide a new set of responses to a future generation of 'untreatables,' much as quinupristin-dalfopristin and linezolid have offered a way to deal with previously untreatable VRE. Second, the growing understanding of the ecology and evolution of antibiotic resistance needs to be utilized in order to manage antibiotic use so that the evolution and spread of antibiotic-resistant strains can be slowed. These will be treated in turn.

Predicting resistance evolution

Evolutionary biology insights can be very helpful in drug design; one promising direction is the possibility of using *in vitro* evolution to predict the possible mechanisms by which antibiotic resistance can evolve. *In vitro* methods of generating sequence diversity, such as DNA shuffling and related approaches, can be used to identify candidate mutations or mutational combinations that give rise to resistance to a new drug (Stemmer 1994). Once candidate mutational combinations are identified by DNA shuffling, one can determine whether these combinations are likely to be reached by natural selection, using further *in vitro* evolution (Barlow and Hall 2002c, 2003), or even by exploring the full fitness surface defined by the various combinations of mutations at those candidate sites (Weinreich *et al*. 2006). In this way, pharmaceutical developers could screen drug candidates for the likelihood that resistance would rapidly evolve, and furthermore could search for drug combinations that hinder resistance evolution to the new therapy (Barlow and Hall 2003).

Narrow spectrum antibiotics

Historically, the trend in antibiotic development has been to broaden the range of targeted microorganisms by moving from 'narrow spectrum' to 'broad spectrum' drugs. For example, penicillin, the first β-lactamase, was only effective against some Gram-positive bacteria, while cefepime, a fourth generation cephalosporin, is active against most clinical species of Gram-positive and -negative bacteria. In part, this has been driven by the economic need to increase potential market share through wider potential use. However, widespread use of many antibiotics has promoted the evolution of resistance, for antibiotics target both the etiological agent and many other organisms that switch between commensal and pathogenic life histories (e.g., *E. coli*, *Enterococcus* spp.). Consequently, there is increasing interest in antibiotics that only target a few organisms (Gillor *et al*. 2005). Ironically, concerns about the MRSA epidemic and organisms such as VRE and *Acinetobacter* have renewed interest in narrow spectrum antibiotics (Talbot *et al*. 2006). The 'market share' (i.e., the disease load) of these pathogens is so great that developing drugs to combat just one of these organisms is profitable. We now turn to one promising avenue for narrow spectrum antibiotics: bacteriocins.

Bacteriocins

One class of antibiotics that has not been used therapeutically is bacteriocins. These are antibiotics produced by bacteria that typically only kill closely related bacteria, often within the same species or genus, narrowing the set of 'non-target' organisms that could evolve resistance. Because bacteriocins do not affect eukaryotic cells, these compounds could have far fewer toxic side effects than many other classes of compounds. One class of bacteriocins currently being investigated is the colicins: plasmid-encoded protein antibiotics active against *E. coli* and related species. Colicins bind to a receptor molecule located on the surface of the cell and are then transported through the cell membrane into the interior of the cell (James *et al*. 1996), where they kill bacteria through several mechanisms, including DNA and RNA endonuclease activity and disruption of the integrity of the cell membrane (Pugsley 1984).

Two mechanisms provide defense against colicins: 'resistance' and immunity. Colicin resistance involves the alteration of either the receptor or the transport systems so that the colicin cannot enter the cell. A single point mutation in either of these systems will confer resistance to multiple

colicins (Feldgarden and Riley 1999). In addition, some mutations confer costs in certain habitats. These costs result from the multiple functions of the receptors and transport systems, including interactions with efflux pumps (e.g., *tolC*), maintaining the integrity of the cell membrane, and nutrient uptake (Davies and Reeves 1975). For example, mutations in the FepA receptor confer resistance to colicins B and D, but also limit growth in iron-limited environments (Pugsley and Reeves 1976). Colicin immunity functions like a poison–antidote system. It is due to an immunity protein produced together with the colicin protein (Kleanthous *et al*. 1998). Immunity proteins are specific to the particular colicin with which they are produced and are not thought to have significant effects on sensitivity to other colicins.

Because colicins must bind to a receptor and then be compatible with the uptake systems of the host cell, they often have a limited host range and can be used to target particular pathogens, such as extraintestinal pathogenic *E. coli*. In addition, if colicin resistance results in trade-offs, such as low growth under iron-limited conditions or the inability to withstand environmental stress, it can be disadvantageous in such habitats, limiting the spread of these resistant genotypes.

Although in natural environments the frequency of colicin resistance is quite high (Feldgarden and Riley 1998), some pathogenic *E. coli* appear to be highly susceptible to colicins (Murinda *et al*. 1996). Recent work has attempted to modify colicins and other bacteriocins to be able to evade naturally occurring resistance mechanisms, often by altering receptor and transport targets (Gillor *et al*. 2005).

Quorum sensing disruptors

Another promising approach to non-traditional antimicrobial chemotherapy is to alter bacterial behavior rather than eliminating bacteria outright. Many of the most harmful activities that bacteria engage in—including toxin production and biofilm construction—are social activities that result from coordinated behavior. Coordination is often achieved using quorum sensing signals, which allow bacteria to regulate their activities in a density-appropriate manner and engage in social behavior only when densities are high enough (when a 'quorium' is present) for this to be effective (Miller and Bassler 2001). If we could disrupt the quorum sensing systems that bacteria use to turn on toxin and biofilm production, we could potentially reduce the impact of many bacterial infections and possibly also hasten clearance by conventional antibiotics; thus quorum sensing disrupters are of considerable interest in antibacterial drug development (Finch *et al*. 1998; Hartman and Wise 1998; Hentzer *et al*. 2003).

At first glance, this strategy may seem to have limited potential. Bacteria use quorum sensing to make certain gene products inducible rather than constitutive. Simple point mutations could presumably switch these gene products to be constitutively expressed. Thus one might think that resistance to quorum sensing disrupters—in the form of constitutive expression—would evolve rapidly. But in an elegant application of evolutionary modeling, André and Godelle (2005) point out that bacterial social behavior, once disrupted, may be extremely slow to return.

Their argument is essentially this: bacterial social behavior requires cooperation from many reproductively distinct individuals. Thus social behavior, including biofilm formation and toxin production, requires that bacteria somehow solve the collective action problem so that free-riders do not cause cooperation to break down. Where bacterial cooperation occurs, it is not an unavoidable consequence of direct individual selection as antibiotic resistance usually is, but rather a finely balanced consequence of multilevel selection. Thus if bacterial cooperation is disrupted, it may not return as readily as individually selected traits. To see how this might work, imagine a population of bacteria in which social behavior has been halted by disrupting quorum sensing. Whereas with conventional antibiotics the first antibiotic-resistant mutant has a substantial growth advantage, with quorum sensing disrupters the first resistant mutant has a growth disadvantage. It provides a public good by producing constitutively, but it receives no benefits from the other members of the population who are not producing due to the quorum sensing disrupter. Moreover, because these behaviors are selected at the population level, if resistance does evolve it is likely to do so on the time scale of populations, rather than on the time scale of individuals. While a bacterium may

reproduce in a matter of hours, populations often turn over on scales of weeks to months and thus resistance to quorum sensing disruptors is likely to evolve much more slowly that does resistance to conventional antibiotics.

Ecological modeling

Population biology also provides a framework for thinking about ways to alter the hospital environment and to manage drug use so as to minimize the evolution and spread of resistance. Here, mathematical models of disease accord with proven clinical strategies (Lipsitch *et al*. 2000). Handwashing reduces the rate of transmission within hospitals and thus makes it harder for hospital-adapted strains to persist endemically within the hospital. Clinically, this has been known for nearly fifty years as an effective response to antibiotic resistance outbreaks (Barber *et al*. 1960). Barrier precautions, increased staff-to-patient ratios, and other basic hygiene have a similar effect. A one-time shift in the formulary reduces the strength of selection favoring resistant strains; repeated experience reveals the value of this approach (Lilly and Lowbury 1978).

Antibiotic cycling

More recently, several authorities have speculated that antibiotic cycling—in which drug classes are rotated on a scheduled basis—may constitute an effective way for hindering resistance evolution and spread. The basic logic parallels that underlying crop rotation and HIV drug rotation: by confronting bacteria with a changing environment, their ability to track the environmental conditions may be reduced. Unfortunately, the clinical trials conducted thus far have been mostly disappointing, as revealed in a meta-analysis (Brown and Nathwani 2005).

Mathematical modeling helps one see the reason for these results: antibiotic cycling does not necessarily reduce the environmental heterogeneity at the scale relevant to bacterial clones spreading through the hospital (Fig. 10.2) (Bergstrom *et al*. 2004). While antibiotic cycling may introduce longer-term heterogeneity in the hospital, it does not increase the local heterogeneity generated when different patients receive different drugs according to patient history, indications, and physician preference. In fact, by standardizing drug therapy at any given time, a cycling program may even decrease

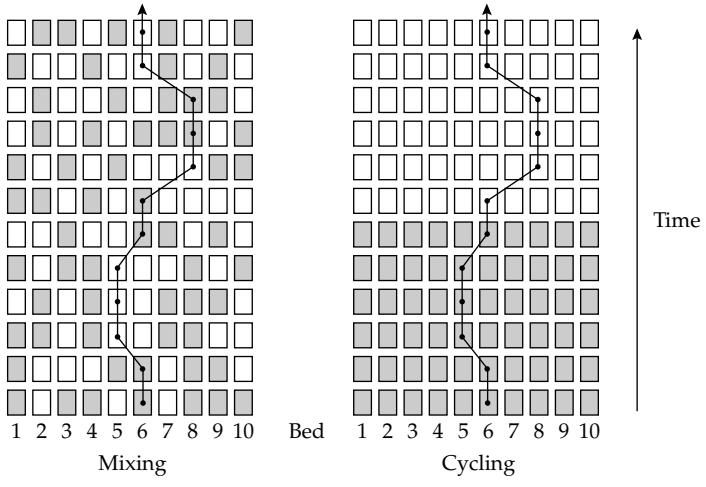

Figure 10.2 Paradoxically, cycling provides a more homogeneous environment for bacteria than does 'mixing,' in which different patients receive different drugs but the proportions of drug use in a ward stays roughly constant over time. The black line indicates the trajectory of a bacterial lineage as it passes from patient to patient and bed to bed. Shaded rectangles indicate patients receiving one drug, open rectangles indicate patients receiving an alternative drug. The bacterial lineage in the mixing ward faces more heterogeneous selective conditions (antibiotic types) than does the bacterial lineage in the cycling ward. From Bergstrom *et al.* (2004).

local heterogeneity. Because successful hospital-associated clones pass among multiple patients within the span of a single antibiotic 'cycle,' local heterogeneity turns out to be a more important restriction on the bacterial population's ability to track its environment than is the longer-term temporal heterogeneity introduced by cycling.

Conclusions

As grave as the public threat posed by antibiotic resistance may be, we should maintain perspective on which aspects of our lives are directly threatened and on which are not. Even if we lose much of our ability to resolve bacterial infection using antibiotics, we will not necessarily return to Dark Ages rates of infectious disease mortality—or even to nineteenth-century rates.

Figure 10.3 illustrates infectious disease mortality rates, measured as deaths per 100,000 individuals per year, across the twentieth century. Over that century, infectious disease mortality declined dramatically, from roughly 800 deaths per 100,000 per year in 1900 to roughly 60 deaths per 100,000 per year in 1996. Remarkably, the vast majority of this decline had nothing to do with the development and use of antibiotics!

By 1935, when the first antibacterials, known as sulfonamides, were released, disease mortality had already declined almost threefold from its 1900 rate. By the time that penicillin was first used clinically in 1943, infectious disease mortality had dropped to less than a quarter of its 1900 rate. Thus at most a third of the overall decline could conceivably be attributed to antibiotics—and even this is likely to be a considerable overestimate, given that the decreasing trend in disease mortality continued steadily from 1900 to 1950, rather than accelerating with the advent of antibiotics. Presumably, most of the decline in infectious disease mortality over the twentieth century was instead a consequence of increased sanitation, improved public health infrastructure and practice, improvements in food handling, storage, and preparation, and improvements in nutrition (Genereux and Bergstrom 2005).

Thus if we fall behind in the race against the evolution of antibiotic-resistant bacteria, we are unlikely to return to the sort of infectious disease mortalities that plagued humankind in 1900. What

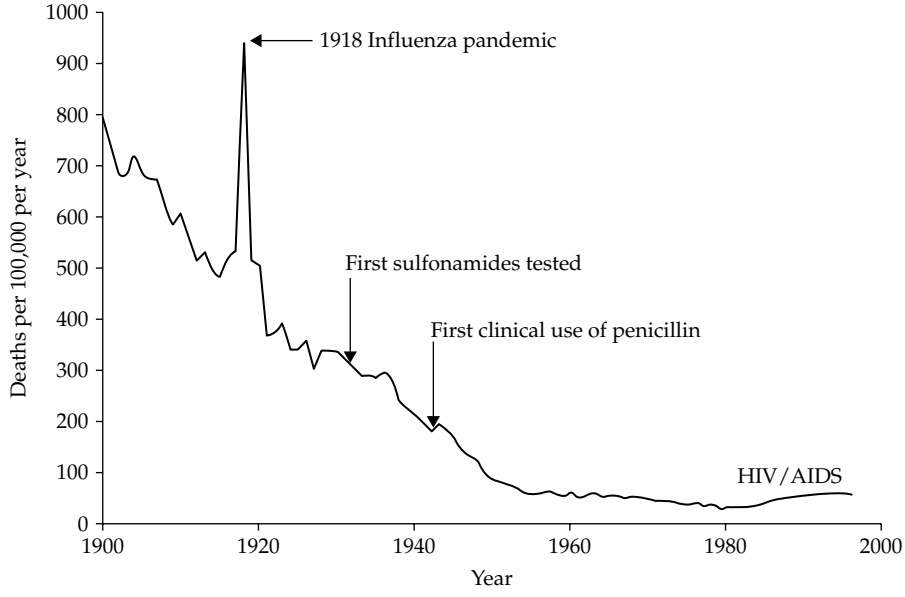

Figure 10.3 Infectious disease mortality rate in the United States (Armstrong *et al.* 1999; Genereux and Bergstrom 2005). Redrawn with permission from Armstrong *et al.* (1999).

would we lose if antibiotic resistance becomes even more widespread and we have fewer options for treating multidrug-resistant infections? With the rise of HIV (which is responsible for most of the post-1980 increase in infectious disease mortality shown in Fig. 10.3) and increasing numbers of patients who are immunocompromised for other reasons, we are dealing with increasing populations of patients for whom antibiotics are a necessity. Furthermore, prophylactic use of antibiotics is critical for our ability to perform invasive surgical procedures without a serious risk of infection. Thus the loss of effective antibiotics would indeed by an enormous setback to medical practice.

Summary

1. The evolution of resistance to a clinical antibiotic occurs with near certainty after several years of widespread use.
2. Antibiotic resistance can evolve by a variety of genetic mechanisms and spread throughout and between species via gene transfer.
3. Resistance mechanisms that evolve in agricultural settings where antibiotics are used can transfer into human populations, contributing to the overall problem of antibiotic resistance.
4. Associations among resistance genes, and the process of compensatory evolution can result in the retention of resistance genes, even in the absence of selection favoring resistance.
5. Novel approaches to antimicrobial therapy—including narrow spectrum antibiotics, bacteriocins, and disruptors of quorum sensing—may provide alternatives to traditional broad spectrum antibiotics for which resistance is less quick to evolve.
6. To eradicate antibiotic resistance from a hospital setting, researchers need a thorough understanding of the underlying ecology. For example, ecological models have shown that antibiotic cycling, the hospital equivalent of crop rotation, does not necessarily reduce the environmental heterogeneity at the scale relevant to bacterial clones spreading through the hospital and thus may be ineffective at reducing the frequency of resistant strains in a hospital setting.

Acknowledgments

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