Chapter eight

The genetic basis of resistance to antimicrobial drugs

The development of safe, effective antimicrobial drugs has revolutionized medicine in the past 70 years. Morbidity and mortality from microbial disease have been drastically reduced by modern chemotherapy. Unfortunately, micro-organisms are nothing if not versatile, and the brilliance of the chemotherapeutic achievement has been dimmed by the emergence of microbial strains presenting a formidable array of defences against our most valuable drugs. This should not surprise us, since the evolutionary history of living organisms demonstrates their adaptation to the environment. The adaptation of micro-organisms to the toxic hazards of antimicrobial drugs is therefore probably inevitable. In Chapter 7 we saw that in many species of bacteria a degree of intrinsic resistance to toxic chemicals is conferred by cellular permeability barriers and low levels of expression of a range of multidrug efflux pumps. Both elements of defence can be enhanced in acquired drug resistance by genetic changes in response to higher levels of drug challenge. The extraordinary speed with which antibiotic resistance has spread among bacteria and certain viruses such as HIV during the era of chemotherapy is due, in large measure, to the remarkable genetic flexibility of these organisms. Figure 8.1 provides an example in the alarming rise of a drug-resistant pathogen, methicillin (or multidrug)-resistant *Staphylococcus aureus*, in part of the United Kingdom in just nine years.

The first account of microbial drug resistance was given by Paul Ehrlich in 1907, when he encountered the problem soon after the development of arsenical chemotherapy against trypanosomiasis. As the sulfonamides and antibiotics were brought into medical and veterinary practice, resistance against these agents began to emerge. Resistance to antibacterial and antimalarial drugs is now widespread, and increasing resistance to antifungal and antiviral drugs is also a major concern. Our intention in this chapter is to give an outline of the genetic background to the problem of drug resistance; in Chapter 9 we describe the major biochemical mechanisms that give rise to resistance.

The tremendous advances made in the science of bacterial genetics over the past 60 years have found a most important practical application in furthering our understanding of the problem of drug resistance. As a result, we now have a fairly complete picture of the genetic factors underlying the emergence of drug-resistant bacterial populations. Although the study of the genetics of resistance in pathogenic fungi and protozoa is less developed, advances in DNA sequencing technology should bring about significant improvements in our understanding of these organisms. The depressingly rapid emergence of drug-resistant variants of the human immunodeficiency virus during the chemotherapy of AIDS has given a powerful impetus to the study

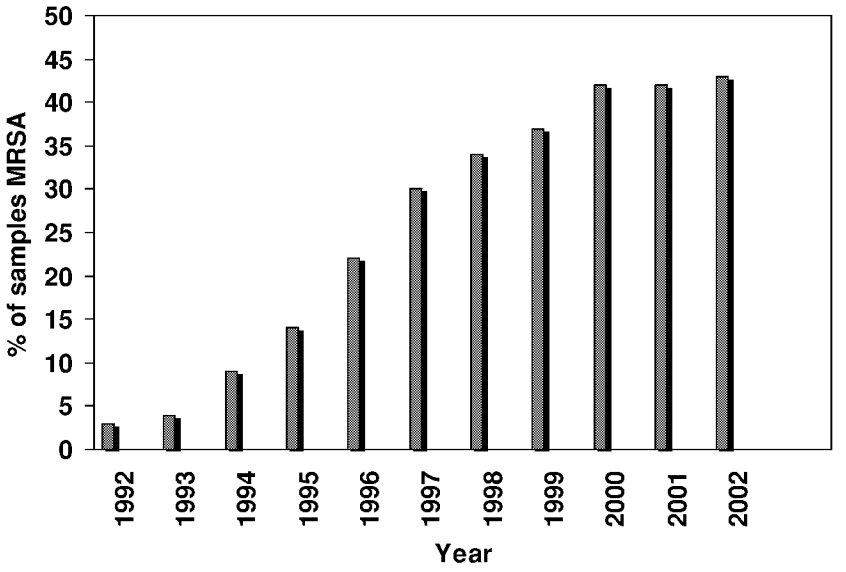

FIGURE 8.1 The recent rise in the incidence of multidrug-resistant *Staphylococcus aureus* in England and Wales. The vertical axis indicates MRSA as a percentage of all samples of *Staphylococcus aureus* examined in clinical laboratories. (Source: Health Protection Agency.)

of the genetic and biochemical basis of resistance to antiviral drugs.

The early studies on the genetics of drug resistance were bedeviled by an exhausting controversy. On the one hand were those who believed that the development of a resistant cell population could be explained by the phenotypic adaptation of the cells to an inhibitory compound without significant modification in their genotype. The opposing faction took the view that any large population of cells which was sensitive overall to a drug was likely to contain a few genotypically resistant cells. The continued presence of the drug resulted in the expansion of the numbers of resistant cells by a process of selection.

Evidence gathered over the years strongly supports the second of these two theories. As we shall see, there are examples of phenotypic adaptation to antimicrobial drugs, but such cells are usually genotypically different from wild-type cells. When the selective pressure applied by an antimicrobial drug is removed, the resistant microbial population may revert to drug sensitivity if the resistant cells are at a selective disadvantage compared with drug-sensitive cells in a drug-free environment and could therefore eventually be outnumbered by the sensitive cells.

8.1 Mutations and the origins of drug-resistance genes

Once it was accepted that drug-resistant organisms are genetically different from the wild types, it was natural to consider how such differences might arise. One obvious possibility is that of spontaneous mutations. These can arise in several ways:

1. Damage to the genome caused by adverse environmental factors, including ionizing radiation and chemical mutagens.
2. Base-pairing errors during genomic replication.
3. Frameshift mutations caused by the deletion of segments of DNA which frequently occur at short DNA repeat sequences.
4. Frameshift mutations caused by the intragenic insertion of mobilizable genetic material, such as transposons, which corrupt the correct flow of information from the wild-type genome.

Spontaneous mutations are relatively rare—on the order of one mutation per 10^{7}–10^{11} cells per generation—although in organisms lacking a proofreading

mechanism during genomic replication, as in HIV, the mutation rate is much higher. There is also a mutator phenotype in some bacteria which ensures a much higher mutation frequency than normal. An interesting example is that of *Helicobacter pylori,* the bacterium associated with peptic ulcer disease and gastric cancers. As many as 33% of *Helicobacter pylori* strains show an abnormally high rate of mutation to antibiotic resistance. The nature of this high mutation frequency is not known at present, but it is of potential relevance to the clinical challenge of eliminating *Helicobacter pylori* from the stomach by treatment with antibiotics. When the vast numbers of organisms in microbial populations are considered, the probability of even low mutation rates causing drug resistance is quite high. The simple and elegant technique of replica plating convincingly demonstrates that spontaneous mutations to drug resistance can occur in drug-sensitive bacterial populations in the absence of drugs (Figure 8.2). A spontaneous mutation may occasionally cause a large increase in resistance, but resistance often develops as a result of numerous mutations, each giving rise to a small increase in resistance. In this situation, highly resistant organisms emerge only after prolonged or repeated exposure of the microbial population to the drug.

8.1.1 Spontaneous mutations and drug resistance in HIV

A major challenge to the effective treatment of AIDS is the unique and alarming speed with which HIV becomes resistant to every drug deployed against it, including inhibitors of viral reverse transcriptase and HIV protease. The origins of drug resistance in HIV lie in the high rate of viral replication and the ease with which spontaneous mutations arise in its RNA genome. As a single-stranded RNA virus, HIV lacks a proofreading mechanism to eliminate sequence errors resulting from the low fidelity of HIV reverse transcriptase. As a result, mutations occur with high frequency. During the course of an infection, the combination of high replication and mutation rates permits rapid and extensive evolution of the viral population in response to immunological and chemotherapeutic challenges to its survival. For example, within weeks of starting treatment with the reverse transcriptase inhibitor lamivudine, spontaneous mutation results in the replacement in the reverse transcriptase of the circulating viruses of methionine-184 by valine, a change associated with high-level resistance to lamivudine.

Resistance to some other drugs, such as azidothymidine, develop through successive mutations which progressively reduce the drug sensitivity of the target enzyme. The loss of sensitivity to an inhibitor can be associated with reduced catalytic efficiency, which places the virus particles at a competitive disadvantage compared with viruses with unimpaired enzyme. However, the reduction in enzymic efficiency may be compensated by further mutations which progressively restore enzymic activity. A recent alarming discovery is that under laboratory conditions, AZT and lamivudine adversely affect the fidelity of reverse transcriptase, thus further increasing the frequency of mutations. The clinical significance of this finding has yet to be explored. As discussed in Chapter 4, the clinical approach to coping with the rapid acquisition of drug resistance by HIV is to treat patients with a combination of drugs. In this way, the emergence of resistant viruses can be delayed by months or even years. Even so, the eventual emergence of viral populations resistant to multidrug therapy may be inevitable.

8.1.2 Origin of clinically important resistance genes in bacteria

Originally it was believed that spontaneous mutations followed by the selection of resistant organisms in the presence of a drug explained the emergence of drug-resistant populations. However, while this appears to be true in the case of HIV, the discovery that bacteria can acquire additional genetic material by conjugation, transformation and transduction led to the conclusion that spontaneous mutations make an important but not exlusive contribution to the emergence and spread of drug resistance in bacteria. Mutations underlie the up-regulation of drug efflux pumps and the reduction or loss of porin function in many bacteria as well as the increased expression of constitutive β-lactamases in pathogens such as *Enterobacter cloacae* and *Citrobacter freundii.* Spontaneous mutations also lead to the progressive modification of β-lactamases, enabling

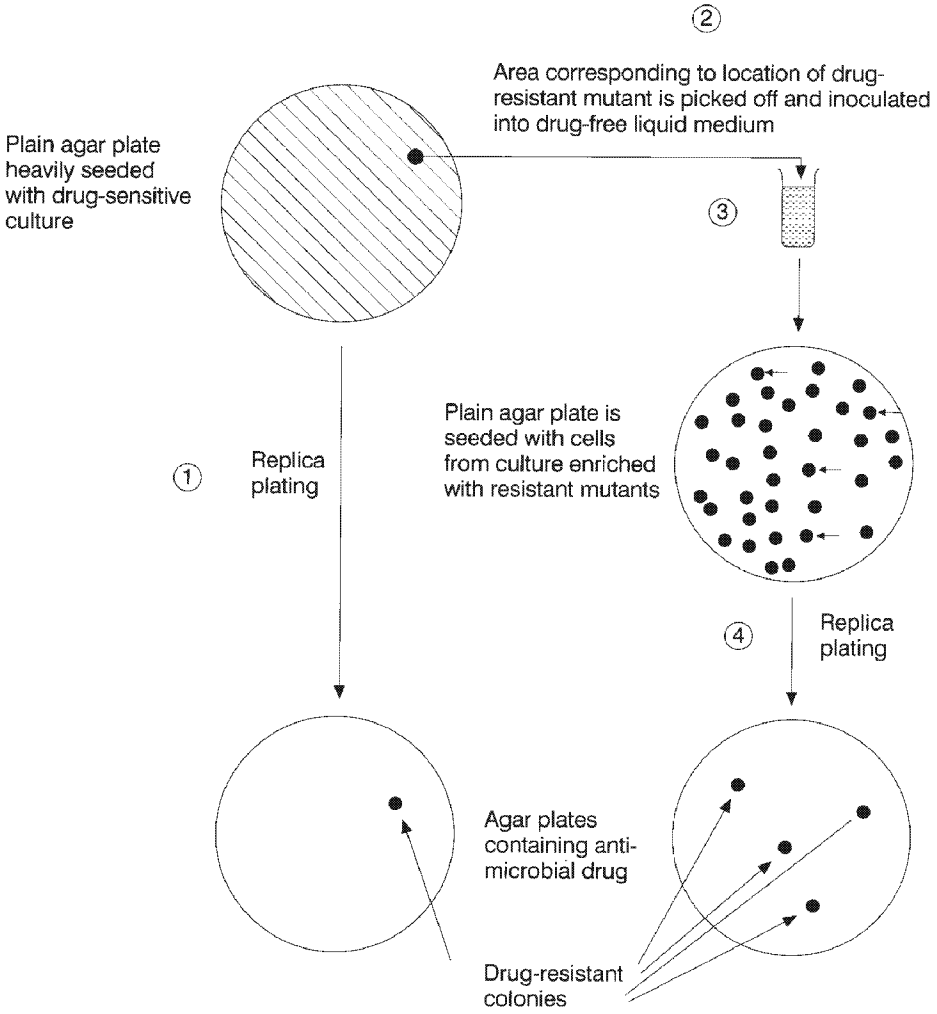

FIGURE 8.2 The technique of replica plating reveals the existence of drug-resistant cells in a population that is overall drug-sensitive. A plain agar plate is heavily seeded with cells from the drug-sensitive culture and incubated until growth occurs. Cells are transferred by a velvet pad to a plate containing the antibacterial drug; this plate is then incubated and the position of any colonies noted. The area on the drug-free plate corresponding to the location of the resistant colony on the drug plate is picked off and cultured in drug-free medium. Although still contaminated with sensitive cells, this culture will contain many more resistant cells than the original culture. Plating out of the 'enriched' culture on a plain plate followed by replication to a drug plate therefore reveals a higher number of drug-resistant colonies. The experiment shows that drug-resistant mutants occur in a bacterial population not previously exposed to the drug.

them to cope with the many novel chemical variants of β-lactams. Strains of *Mycobacterium tuberculosis* resistant to isoniazid, rifampin, pyrizinamide, ethambutol and streptomycin can all be explained by mutations in the genes encoding the target sites for these drugs. Resistance to the quinolones regularly arises through mutations in genes encoding the target DNA gyrase enzyme.

As we shall see in the next chapter, the biochemical machinery conferring bacterial resistance to drugs of major importance in medicine can be complex. Understandably, therefore, there is considerable interest

in the origins of the genes that variously encode drug-inactivating enzymes, drug efflux pumps, enzymes that depress drug sensitivity by the covalent modification of drug targets and proteins that block the binding of drugs to their targets. Antibiotic-producing bacteria, such as the streptomycetes, protect themselves against the toxic effects of their own antibiotics with enzymes that inactivate aminoglycosides, chloramphenicol and β-lactams. In addition, many streptomycetes express β-lactamases even though they do not produce β-lactams, presumably as a protective measure against β-lactams synthesized by other organisms in the microenvironment. Genes encoding the pumped efflux of tetracyclines, proteins that protect ribosomes against tetracyclines, and the enzymic modification of ribosomal RNA associated with resistance to erythromycin, have all been identified in streptomycetes. Nucleic acid and protein sequence data support the suggestion that genes for aminoglycoside-inactivating enzymes found in aminoglycoside-resistant clinical isolates may have originated from streptomycetes.

Mosaic genes

Although bacterial genes which encode antibiotic-inactivating enzymes and drug efflux pumps almost certainly evolved in the very distant past, antibacterial drug resistance mediated by mutations is generally thought to have emerged during the modern era of chemotherapy. In addition to point mutations, deletions and insertions, there is also the remarkable phenomenon of mosaic genes which arise by interspecies genetic recombination. By far the most common mechanism of resistance to β-lactam antibiotics is that of antibiotic hydrolysis by β-lactamases, which are probably of ancient origin. However, β-lactam resistance in several important pathogens, including *Haemophilus influenzae, Neisseria gonorrhoeae, Streptococcus pneumoniae, Staphylococcus aureus* and *Staphylococcus epidermidis,* can also be caused by penicillin-binding proteins (Chapter 2) with reduced affinity for β-lactams. This type of resistance is relatively rare because the killing action of β-lactams depends on drug interactions with several high-molecular weight PBPs, and resistance therefore necessitates reductions in β-lactam affinity in each PBP. Although it is conceivable that such reductions in affinity could have arisen gradually from incremental changes in protein structure that were due to the accumulation of mutations in the PBP genes, it is clear that recombination amongst PBP genes from different species is a major cause of the low-affinity PBP phenotype in bacteria.

Analysis of the sequences of genes for PBP2 from penicillin-sensitive and penicillin-resistant meningococci and gonococci reveals that whereas the sequences from penicillin-sensitive bacteria are uniform, the resistant gene sequences have a mosaic structure. The mosaics are created when regions essentially identical with those from penicillin-sensitive bacteria recombine with regions that have significantly divergent sequences. The mosaic genes encode PBP2 variants with decreased affinity for penicillin. Sequence information obtained from bacterial DNA databases show that the divergent regions in the mosaic genes originate from *Neisseria flavescens* and *Neisseria cinerea.* PBP2 prepared from specimens of *Neisseria flavescens* preserved from the preantibiotic era has a much lower affinity for penicillin than PBP2 from either *Neisseria gonorrhoeae* or *Neisseria meningitidis.* The mosaic genes are thought to have arisen by interspecies recombination following transformation by DNA released from lysed cells (see later discussion) amongst these bacteria. Mosaic genes encoding low-affinity PBPs 1a, 2x, 2b and 2a have been isolated from *Streptococcus pneumoniae* resistant to both penicillins and cephalosporins. Pneumococci are also readily transformable, and the divergent regions of the mosaic genes appear to have originated from several other bacterial species. However, not all low-affinity PBPs are the result of mosaic gene formation. An important example is that of PBP2a, which is encoded by the *mecA* gene responsible for the notorious methicillin-resistant *Staphylococcus aureus.* The *mecA* gene is located on a large (~50 kilobases) DNA element inserted into the bacterial chromosome. This so-called resistance island encodes proteins that are homologous with transposases and integrases (see later discussion) which probably catalyze both excision and integration of the *mecA* gene. *Staphylococcus aureus* may have acquired *mecA* by gene transfer from another organism.

Interspecies recombination amongst meningococci also resulted in mosaic genes that encode sulfon-

amide-resistant dihydropteroate synthase (Chapter 4). Allelic variations in the *tetM* gene, which determines the ribosome protection form of resistance to tetracycline (Chapter 9), are due to recombination amongst the distinct *tetM* alleles found in *Staphylococcus aureus* and *Streptococcus pneumoniae*. *TetM* genes are widely distrbuted in both Gram-positive and Gram-negative bacteria. Finally, it should be noted that the generation of mosaic genes by interspecies recombination in bacteria is not limited to resistance genes. The phenomenon is widespread in bacteria and underlies, for example, the highly divergent genes that encode the proteins of the outer membranes of *Neisseria* spp.

8.2 Gene mobility and transfer in bacterial drug resistance

The spread of drug resistance amongst bacterial pathogens owes much to the remarkable ability of bacteria to mobilize genes in both chromosomal and plasmid DNA and to transfer and exchange genetic information. Evidence that drug resistance could be transferred from resistant to sensitive bacteria came from combined epidemiological and bacterial genetic studies many years ago in Japan. The first clue was provided by the isolation, from patients suffering from dysentery, of strains of shigella resistant to several drugs, including sulfonamides, streptomycin, chloramphenicol and tetracycline. Even more striking was the discovery that both sensitive and multiresistant strains of shigella could occasionally be isolated from the same patient during the same epidemic. Most patients harbouring multiresistant shigella also had multiresistant *Escherichia coli* in their intestinal tracts. This suggested that drug resistance markers might be transferred from *Escherichia coli* to shigella and vice versa. Subsequently it was confirmed that Gram-negative bacteria can indeed transfer drug resistance not only to cells of the same species but also to bacteria of different species and genera. As we shall see, the phenomenon of horizontal gene transfer, as it is now termed, is not confined to Gram-negative bacteria but also occurs in Gram-positive organisms. However, before we describe the transfer of drug-resistance genes between bacterial cells, we must first consider the movement of genes within the bacterial genome itself.

8.2.1 Transposons and integrons

For many years the movement of genes among plasmids and chromosomes was believed to result from classic recombination dependent on the product of the bacterial *recA* gene and the reciprocal exchange of DNA in regions of considerable genetic homology. This permits the exchange of genetic information only between closely related genomes. However, such a restricted phenomenon seemed unlikely to explain the widespread distribution of specific resistance determinants. It is now clear that the acquisition of genetic material by plasmids and chromosomes in both Gram-negative and Gram-positive bacteria is not limited by classic *recA*-dependent recombination. Replicons, known as transposons, can insert themselves into a variety of genomic sites that often have little or no homology with the inserting sequence, although such transposition events are rare, one in 10^5–10^7 cells per generation. Because there are many possible transposon, insertion sites in the bacterial genome, a higher frequency of insertion would probably result in too great a rate of gene disruption and mutation. In the simplest transposons, the whole of the genetic information is concerned with the insertion function. Insertion sequences (IS elements) are sequences of approximately 750–1600 base pairs encoding a specific endonuclease called a transposase. The IS elements are flanked by inverted repeats of 15–20 base pairs that are characteristic of individual transposons. Immediately adjacent to the inverted repeats are short direct repeats (5–11 base pairs) whose sequences depend on the target site where the transposon is inserted.

The genes for drug resistance are carried by composite transposons designated by the prefix Tn. In the class 1 transposon, Tn9, the gene encoding the enzyme that confers resistance to chloramphenicol, chloramphenicol acetyl transferase (Chapter 9), is flanked by two IS elements. These genes are again bounded by inverted repeats which in turn are flanked by short direct repeats (Figure 8.3a). Tn3 (Figure 8.3b) is a class 2, complex transposon which contains the genes for the transposase and for resolvase, an enzyme that catalyzes recombination between the insertion sequences. These genes, together with the gene for β-lactamase, are flanked by the inverted repeats. Transposons carrying arrays of drug-resistance genes have been

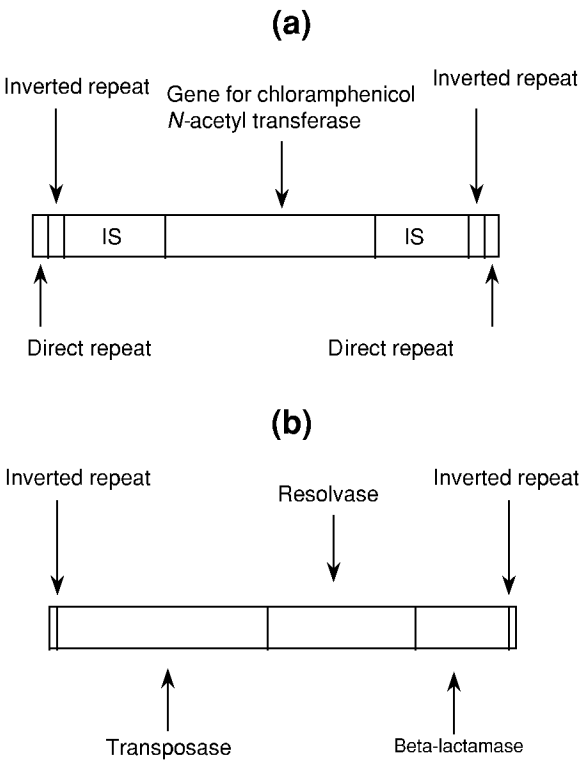

FIGURE 8.3 The structure of transposons: (a) Class 1 transposon Tn9, which includes the gene for bacterial resistance to chloramphenicol. IS, insertion sequence. (b) This class 2 complex transposon, Tn3, confers resistance to β-lactam antibiotics. The gene dimensions are not drawn to scale.

identified in both Gram-positive and Gram-negative bacteria.

In some transposons the drug-resistance genes are arranged within structures called integrons. These consist of an *int* gene that encodes for a site-specific recombination enzyme or integrase, an integron receptor site, *attI*, and one or more gene cassettes. Usually each gene cassette contains a single drug-resistance gene and a specific recombination site, called a 59-base-pair element, located downstream of the gene. The association of the integrase function with the specific recombination site confers mobility on gene cassettes and the ability of integrons to capture and integrate whole arrays of cassettes. Cassette excision is the reverse of integration and generates a circularized form of the cassette which may exist independently for extended periods. There are multiresistance integrons that confer various combinations of resistance to β-lactams, aminoglycosides, trimethoprim, chloramphenicol, antiseptics and disinfectants. More than 40 gene cassettes and three classes of integrons are known, and the reader is referred to reviews listed under 'Further reading' for detailed descriptions of this complex field. Although integrons are found in transposons, they also occur frequently as independent entities.

Mobilization of class 1 transposons along with their complement of drug-resistance genes occurs by nonreplicative transposition; that is, the transposon copy number is not increased during transposition. The transposon is excised from its original site and reinserts into a new site virtually anywhere within the bacterial genome, including chromosomal and plasmid sites. Class 2 transposons, on the other hand, are mobilized by a replicative process. The replicated copy of the transposon inserts into a new site. In both cases the transposase first introduces staggered cuts, nine base pairs apart, at the donor site in the transposon and at the intended recipient site. The recipient site, 4–12 base pairs in length, is then replicated to form noninverted, or direct repeats on either side of the inserted transposon. In contrast, as described earlier, gene cassette excision and capture in integrons is accomplished by site-specific recombination, although there are rare examples of cassettes that integrate into nonspecific sites.

To summarize, therefore, drug-resistance genes in bacteria are subject to two major modes of intragenic mobilization that promote a continual flux of resistance determinants around bacterial DNA:

1. Resistance genes associated with transposons, whether or not as cassettes, are mobilized along with the rest of the transposon and can be inserted essentially anywhere in the bacterial genome, either chromosome or plasmid.
2. Both transposon-associated and independent integrons containing resistance gene cassettes exchange and capture cassettes by site-specific recombination.

Conjugative transposons

The transposons described so far are by themselves unable to promote gene transfer by conjugation between

bacterial cells, although they participate as passengers during R-plasmid transfers. However, there is another type of transposon, referred to as conjugative transposons. These are discrete DNA elements normally integrated into bacterial chromosomes which encode proteins that enable excision of the transposon from the chromosome and its transfer to recipient bacteria by intercellular conjugation. Conjugative transposons occur widely in Gram-positive bacteria and contribute to the spread of drug resistance among major pathogens such as *Streptococcus* spp. and *Enterococcus* spp. In Gram-negative bacteria, conjugative transposons were first identified in the genus *Bacteroides*, which accounts for 25–30% of the microbial flora of the human intestinal tract. Subsequently, conjugative transposons carrying drug-resistance genes have been found in other Gram-negative species, including *Salmonella*, *Vibrio* and *Proteus*.

The potential for conjugative transposons to spread drug resistance was highlighted when the first conjugative transposon to be discovered, Tn916, was found to carry resistance to tetracycline. Originally Tn916 was detected on the chromosome of a multiresistant isolate of *Enterococcus* (previously called *Streptococcus*) *faecalis*, and it was also observed to integrate readily into coresident plasmids and into many sites of the chromosomes of bacterial recipients of Tn916. A closely related conjugative transposon, Tn1545, found in *Streptococcus pneumoniae*, also mediates tetracycline resistance as well as resistance to erythromycin and kanamycin.

The mobilization of conjugative transposons from bacterial chromosomes involves the following steps, although details of the initial signals for mobilization are not fully defined:

1. Staggered cuts are introduced at each end of the transposon, leaving 6-nucleotide, single-stranded stretches of DNA, known as coupling sequences.
2. The noncomplementary coupling sequences are then ligated to generate covalently closed, double-stranded circular intermediates.
3. During the insertion stage, the coupling sequences form temporary non-base-pairing interactions with the target site, which can be in either a coresident plasmid or the chromosome of a recipient bacterium after conjugation. It is not yet clear how correct base pairing is subsequently established, but it may involve either replication through the insertion region or repair of a mismatch. The insertion process of conjugative transposons differs from that of 'true' transposons in that the recipient or target site is not replicated.

The intercellular conjugation process promoted by conjugative transposons is not well understood. Unlike the process mediated by R-plasmids in Gram-negative bacteria (see next section), surface pili do not appear to be involved. It is clear that only single-stranded copies of the transposons are transferred to recipient cells during conjugation.

The intercellular traffic of conjugative transposons is highly regulated. Many *Bacteroides* transposons carry the *tetQ* gene for tetracycline resistance, which is dependent upon ribosomal protection. Remarkably, tetracycline is a highly effective stimulant of conjugative transposon-mediated mating in these species. A suite of transposon genes activated by tetracycline promotes transposon mobilization and self-transfer as well as mobilization of coresident plasmids sharing the same donor cells. Tetracyclines can therefore stimulate the spread of the resistance genes throughout the bacterial population of the intestinal tract, including many other bacterial species.

Conjugative transposons comprise a highly variable group of mobilizable DNA elements, and the reader is directed to references under 'Further reading' for more detailed descriptions.

8.2.2 R-plasmids

Cellular conjugation mediated by R-plasmids is the major mechanism for the spread of drug resistance through Gram-negative bacterial populations. R-plasmids usually exist separately from the bacterial chromosome. They consist of two distinct but frequently linked entities:

1. The genes that initiate and control the conjugation process, and
2. A series of one or more linked genes, often found within transposon-integron com-

plexes, which confer resistance to antibacterial agents.

The conjugative region is closely related to the F-plasmid, which also confers on Gram-negative bacteria the ability to conjugate with cells lacking an F-plasmid. A complete R-plasmid resembles the F-prime plasmid (F′) in carrying genetic material additional to that which controls conjugation.

A great variety of R-plasmids have been described which carry various combinations of drug-resistance genes. Other phenotypic characteristics conferred by R-plasmids can be used in systems of classification. These include the ability (fi^+) or inability (fi^-) to repress the fertility properties of an F-plasmid coresident in the same cell; the type of sex pilus (see later discussion) that the R-plasmid determines; the inability of R-plasmids to coexist in a bacterium with certain other plasmids, which permits the division of R-plasmids into several incompatibility groups; and finally, the presence of genes in the R-plasmid that specify DNA restriction and modification enzymes. R-Plasmids are usually defined by a combination of these properties.

Molecular properties of R-plasmids

R-Plasmids can be isolated from host bacteria as circular DNA (Figure 8.4) in both closed and nicked forms, and both forms coexist in the cell. The closed circular structure is probably adopted by R-plasmids not engaged in replication. The contour lengths and thus molecular weights of isolated R-plasmids depend very much on the host bacterium and the culture conditions prevailing immediately before the isolation procedure. The R-plasmid may sometimes dissociate into its conjugative and resistance determinants. This is more common in some host species, e.g. *Proteus mirabilis* and *Salmonella typhimurium*, than in *Escherichia coli*, where dissociation is rare. Dissociation seems to depend on the activity of a simple transposon that may be inserted at the junction of the two regions. The molec-

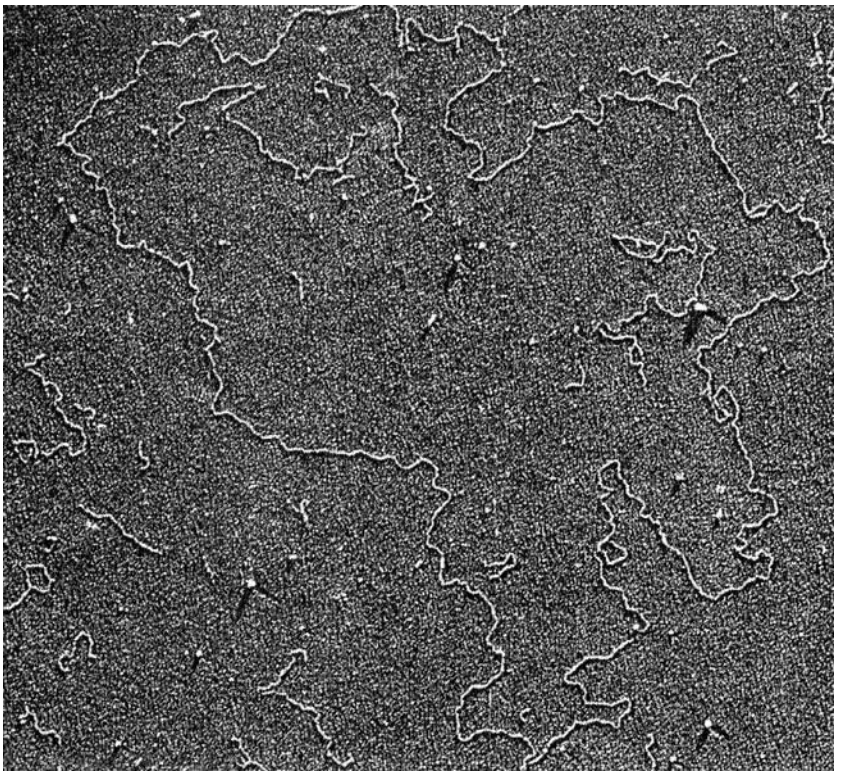

FIGURE 8.4 Electron micrograph of R-plasmid DNA isolated from *Proteus mirabilis* harbouring an R-plasmid with resistance markers to streptomycin, sulfonamides and chloramphenicol. The circular DNA has a total length of 28.5 μm. [This photograph is reproduced from *J. Bacteriol.* **97**, 383 (1969) by kind permission of Dr. Royston Clowes and the American Society for Microbiology.]

ular masses of between 50×10^6 and 60×10^6 kDa of the conjugative regions from R-plasmids are much greater than those of the drug-resistance genes. For example, the genes for chloramphenicol, streptomycin, spectinomycin and sulfonamide resistance have a combined molecular mass of only 12×10^6 kDa.

The copy number of R-plasmids harboured by individual bacteria is determined by the properties of the plasmid and its hosts as well as the culture conditions. As a general rule (to which there are exceptions), the larger R-plasmids are present in only a limited number of copies (between one and four) per chromosome in *Escherichia coli*, whereas in *Proteus mirabilis* the number is much more variable and even varies during the growth cycle. Conditions that give rise to an increased number of R-plasmid copies are sometimes associated with enhanced resistance. However, the level of cellular resistance does not always reflect the number of resistance gene copies. For example, although the number of R-plasmid copies is frequently greater in *Proteus mirabilis* than in *Escherichia coli*, the level of resistance to several drugs expressed in the former organism is usually lower than in *Escherichia coli*.

Cellular conjugation and R-plasmid transfer

Cells bearing an R-plasmid (R^+) are characterized by their ability to produce surface appendages known as sex pili. The sex pili of R^+ bacteria resemble those produced by F^+ organisms. When R^+ cells are mixed with sensitive R^- cells, mating pairs are immediately formed by surface interaction involving the sex pili. The transfer of a copy of the R-plasmid from the R^+ to the R^- cell begins, and the acquisition of the R-plasmid by the recipient cell converts it to a fertile, drug-resistant cell that can in turn conjugate with other R^- cells. In this way drug resistance spreads rapidly through the bacterial population. Uncovering the details of bacterial conjugation and the transfer of DNA has challenged investigators for many years. Although a wealth of information has emerged, several critical steps in the process remain to be defined. What may appear superficially to be a fairly simple phenomenon is in fact highly complex, and here we provide only an outline of the process.

The conjugal pair is brought into close surface contact by the attachment of the pilus of the donor cell to the recipient and its subsequent retraction by a process of 'reeling in'. The interaction between the cells triggers cleavage of a specific strand of the donor R-plasmid in the origin-of-transfer site (*oriT*) within a protein-DNA complex called the relaxosome, which contains the DNA strand-cleaving, or relaxase, enzyme. Only one strand, the T-strand, which is unwound following plasmid cleavage at the *oriT*, is transferred in a 5′ to 3′ direction from the donor to the recipient cell. Determining how the T-strand leaves the donor cell and penetrates the recipient has been a major research challenge. The extrusion of DNA from donor cells has some features in common with the secretion of toxins and virulence proteins referred to as the type IV secretory process. So-called coupling proteins are involved in transferring the exported proteins across the complex cell envelope of Gram-negative bacteria into the external environment. A recent suggestion is that in a typical plasmid such as R388, after generation of the T-strand of DNA, the relaxase protein, TrwC, serves as a pilot to guide the T-strand into the type IV secretory pore. A coupling protein, TrwB, then 'pumps' the DNA strand through the transporter-pore system, which perhaps involves ATP hydrolysis as an energy source. The details of the final transfer into the recepient cell remain shrouded in uncertainty, although the model suggests a possible role for the surface pili in breaching the permeability barriers of the recipient cells. It must be emphasized, however, that this proposal is speculative and is supported mainly by circumstantial evidence on the nature of the coupling and pilot proteins and the effects of loss-of-function mutations in these proteins on the DNA transfer process. Once inside the recipient cell, the ends of the transferred strand are ligated to produce covalently closed circular DNA. Finally, DNA replication, catalyzed by DNA polymerase III, generates double-stranded plasmid DNA from the single-stranded molecules in both donor and recipient cells.

Fortunately, the frequency of R-plasmid transfer is much lower than that of F transfer. Following the infection of an R^- cell with an R-plasmid, a repressor protein accumulates which eventually inhibits sex pilus formation. The ability to conjugate is therefore restricted to a short period immediately after acquisition of the R-plasmid. Sex pilus production in F^+ cells, in contrast, is not under repressor control and conjugal

activity is therefore unrestricted. Mutant R-plasmids without the ability to restrict sex pilus formation exhibit a much higher frequency of R-plasmid transfer.

It is also worth noting that certain R-plasmids, and other self-mobilizing plasmids without drug-resistance genes, can promote the intercellular transfer of coresident plasmids that lack the genetic information for conjugation and transfer. Such mobilizable plasmids achieve transfer either by using the conjugal apparatus furnished by the self-mobilizing plasmids (*trans* mobilization) or by integration with these plasmids (*cis* mobilization). Cooperative interactions amongst plasmids add significantly to the genetic flexibility of bacteria and to their ability to spread drug resistance through microbial populations.

Clinical importance of R-plasmids

It is generally agreed that R-plasmids existed before the development of modern antibacterial drugs. Clearly though, the widespread use and abuse of these drugs led to a vast increase in drug resistance caused by R-plasmids. This has been especially noticeable in farm animals, which in many countries receive clinically valuable antibacterial drugs, or compounds chemically closely related to them, in their foodstuffs as growth enhancers. The animals act as a reservoir for Gram-negative bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, harbouring R-plasmids potentially transferable to man. Fortunately, some countries have restricted the growth-enhancer application of clinically valuable antibiotics, although contravention of the regulations is not unknown.

The adverse contribution of R-plasmid-mediated drug resistance to human morbidity and mortality is undeniable. For example, the major requirement in the treatment of neonatal diarrhea caused by certain pathogenic strains of *Escherichia coli* (a potentially dangerous condition) is the prevention of fatal dehydration. Even so, elimination of the pathogenic organisms may also be important, but this is often difficult in the face of multiple resistance to commonly used antibacterial agents. In one notorious outbreak, the children were infected with a pathogenic strain of *Escherichia coli* resistant to β-lactams, streptomycin, neomycin, chloramphenicol and tetracyclines. The infection eventually responded to gentamicin, which was the only drug of those tested to which the pathogenic bacteria were sensitive. Another potentially alarming development has been the appearance of the typhoid organism, *Salmonella typhi*, carrying an R-plasmid with genes for resistance to chloramphenicol and cotrimoxazole, the drugs most commonly used to treat this disease.

Certain ecological factors probably limit the clinical threat posed by R-plasmids. In the environment of the gastrointestinal tract, the conjugal activity of R^+ bacteria may be less than that in the ideal culture conditions of the laboratory. The emergence of an R^+ population of bacteria during antibiotic therapy is more likely to result from selection of resistant cells than from extensive conjugal transfer of resistance. After cessation of antibiotic treatment, the numbers of R^+ bacteria in the feces fall, although usually not to zero. Low-level antibiotic contamination of the environment and/or a previously unsuspected persistence of drug-resistance and resistance-transfer genes in bacterial populations may contribute to this potentially serious situation.

8.2.3 Conjugative plasmids in Gram-positive bacteria

Although the existence of conjugative plasmids which carry drug-resistance genes in Gram-positive bacteria has been recognized for some time, their role in the dissemination of drug resistance is only now being more thoroughly investigated. As described earlier, the physical contact between Gram-negative bacteria necessary for the conjugal transfer of genes is largely attributable to the sex pili. A similar mechanism has not been identified in major Gram-positive pathogens. Major differences in the cell envelopes of Gram-positive and Gram-negative bacteria suggest that the modes of intercellular DNA transfer may also differ substantially. However, sequencing studies on several Gram-positive conjugative plasmids reveal homologies with proteins of the type IV secretion system involved in Gram-negative R-plasmid transfer. Furthermore, the relaxosome of Gram-positive conjugative plasmids is similar to that of the R-plasmids.

8.2.4 Nonconjugal transfer of resistance genes

Transduction

During the two distinct processes of phage transduction, which occurs in both Gram-positive and Gram-negative bacteria, genetic information is transferred by phage particles from one bacterium to a related phage-susceptible cell.

Generalized transduction may occur during the lytic phases of both virulent and temperate phages. Fragments of degraded host chromosomal and plasmid DNA, which may carry drug-resistance determinants, can become packaged into newly generated phage particles, leaving behind some or all of the phage DNA. Lytic release of the phages enables them to inject both phage and donor host DNA into other bacteria, some of which is integrated into the recipient genome, although between 70 and 90% of the transferred DNA is not integrated in this way. Nevertheless, nonintegrated DNA may also survive in the recipient and replicate as a plasmid. In abortive transduction, none of the transferred DNA is integrated into the recipient genome, but again, the nonintegrated DNA survives and replicates as a plasmid. The drug-resistant phenotype in recipient bacteria is maintained in both types of transduction.

The process of specialized transduction depends on an error in the lysogenic cycle. Excision of the phage DNA from the host genome during induction of the lytic phase is insufficiently precise and carries some of the bacterial DNA along with phage DNA. The resulting phage genome contains up to 10% of the bacterial DNA next to the phage integration site in the bacterial genome. Clearly this process has the potential for generating infectious phage particles that carry bacterial genes for drug resistance. Although recombinant or defective phages arising from specialized transduction are able to inject their DNA into new hosts, they cannot reproduce independently, nor are they lysogenic. Specialized transduction has been most thoroughly studied with lambda phage, and the reader is referred to a relevant text on bacterial genetics for a detailed account of the mechanisms involved in lambda phage transduction. In general terms, relatively little of the injected recombinant phage DNA is integrated into the bacterial genome unless the phage population contains normal phages as well as the defective ones. The normal phages insert into the bacterial genome at a specific *att* site that resembles the phage *att* site. The insertion process generates two hybrid bacterial-phage *att* sites where the defective recombinant phage DNA can insert. The presence of the normal phage renders the bacteria susceptible to the induction of phage-mediated lysis. The resulting lysate, containing roughly equal amounts of defective recombinant phages and normal phages, is highly efficient in transduction.

Although transduction of drug-resistance determinants is readily demonstrated under laboratory conditions, its contribution to the spread of drug resistance in natural and clinical settings is difficult to quantify.

Transformation

Under certain conditions most genera of bacteria can absorb, integrate and express fragments of 'naked' DNA containing intact genetic information, including that for drug resistance. The phenomenon of transformation of bacteria by DNA is more complex than it may appear at first glance. It has been most thoroughly investigated in the species in which it was first discovered more than 60 years ago, *Streptococcus pneumoniae*. Only bacteria in a state of competence are able to absorb and integrate exogenous DNA into their own genome. *Streptococcus pneumoniae* becomes competent during exponential growth when the population density exceeds 10^7–10^8 cells ml^{-1}. Under these conditions the bacteria secrete a competence factor that stimulates the synthesis of up to 10 other proteins essential for transformation. Competent cells bind double-stranded DNA, provided that its molecular mass is at least 500 kDa. One strand of the DNA is hydrolyzed by an exonuclease associated with the cell envelope and the remaining strand enters the cell while bound to competence-specific proteins. Integration into a homologous region of the recipient genome probably occurs by nonreciprocal general recombination.

While competent *Streptococcus pneumoniae* can take up DNA from a range of bacterial species, the Gram-negative opportunist pathogen *Haemophilus influenzae* is more fastidious and only accepts DNA from closely related species. Furthermore, *Haemo-*

philus influenzae does not produce a competence factor but absorbs double-stranded DNA encapsulated in membrane vesicles. Although transformation is a widespread phenomenon, it is not surprising to find important differences in the details of the actual mechanism among the bacterial species. The complexity and diversity of the transformation system indicates its evolutionary importance in the exchange of genetic information in the bacterial world. The frequency of transformation of genetic markers can be as high as 10^{-3} under laboratory conditions when artificially high levels of DNA are added, i.e. one cell in every thousand takes up and integrates a particular gene. Transformation is probably therefore a significant contributor to the spread of drug-resistance genes. A specific example of the relevance of transformation to drug resistance is illustrated by the existence of the mosaic genes for PBPs with diminished affinity for β-lactam antibiotics, for sulfonamide-resistant dihydropteroate synthase and for the *tetM* form of tetracycline resistance referred to previously. Unfortunately, the extent to which transformation occurs in relevant environments, such as hospital wards and the intestinal tract, cannot be quantified with any certainty.

8.3 Global regulators of drug resistance in Gram-negative bacteria

We have seen how the capture of several determinants for resistance to individual drugs by mobile and transferable genetic elements can result in bacteria acquiring the multidrug-resistant phenotype. However, Gram-negative bacteria have yet another means of achieving a similar end. Genes called regulons exert transcriptional control over several chromosomal genes that confer resistance to many antibiotics by restricting access to their molecular targets. The *marA* locus in *Escherichia coli* contains an operon, *marRAB*, whose expression is inducible by at least two antibiotics, tetracycline and chloramphenicol, and by uncouplers of oxidative phosphorylation. Resistance to these and to many other drugs is increased by the induction process. The MarA protein encoded in the operon belongs to a family of transcriptional regulators and controls the expression of numerous other genes, probably in concert with MarR, a regulator protein, and the MarB protein, whose function is currently unknown. The *mar* operon, when induced, upregulates the multidrug efflux pump AcrB and its linked outer membrane component, TolC (Chapter 7). The *mar* operon also increases the expression of *micF*, an RNA molecule which slows the expression of the OmpF porin channel protein. Enhanced drug efflux combined with diminished outer membrane permeability caused by a reduction in the expression of OmpF underlies the increase in resistance to a range of structurally unrelated drugs that is mediated by the *marA* locus.

Another global regulator of drug resistance, *ramA*, has been found in the Gram-negative pathogen *Klebsiella pneumoniae*. The *ramA* gene encodes a transcriptional activator protein, RamA, that is distantly related to the MarA protein of *Escherichia coli*. Like MarA, RamA confers resistance to a wide range of structurally unrelated drugs by upregulating the expression of several genes. RamA-mediated resistance also appears to depend upon a combination of drug efflux and a reduction in the level of the OmpF protein in the outer membrane.

Genetic loci resembling *marA* and *ramA* are widespread among Gram-negative bacteria, and the regulation of multidrug resistance by these operons is likely to be a significant contributor to the overall problem of drug resistance in Gram-negative bacteria.

8.4 Genetic basis of resistance to antifungal drugs

The yeast pathogen *Candida albicans* exists exclusively as a diploid organism, i.e. it has two copies of every gene. Allelic differences between the two gene copies are commonly encountered among clinical isolates of *Candida albicans*. Resistance to the azole antifungal drugs is often caused by mutations in the target enzyme 14α-demethylase (Chapter 3) encoded by *erg11*. For example, there is a point mutation (R467K) which replaces lysine with arginine at position 467. Analysis of azole-resistant clinical isolates of *Candida albicans* showed that resistance was high when both gene copies carried the R467K mutation compared with heterozygotes in which only one allele was mutated. Resistance to the nucleoside analogue, 5-fluorocytosine in *Candida albicans* is also

modulated by allelic differences. As described in Chapter 4, 5-FC is first converted in the cells to 5-fluorouracil by the enzyme cytidine deaminase. A mutation in one allele for this enzyme results in partial resistance to 5-FC because the unaffected allele continues to express the wild-type enzyme. The generation of cells homozygous for the disabled gene via mitotic recombination causes high-level resistance to 5-FC. In contrast, the haploid yeast *Candida neoformans* can acquire high-level resistance to 5-FC in a single step because there is only one copy of the gene for cytidine deaminase. This situation is reflected in the clinic, where high-level resistance to 5-FC is more commonly detected in *Candida neoformans* than in *Candida albicans*.

Transposable genes and plasmids occur widely in fungi, especially in yeasts. However, at present there is no evidence that these elements contribute to the spread of drug resistance among fungal pathogens such as *Candida* spp., and there is no phenomenon in these organisms comparable with that of mobilizable and transmissable drug-resistance genes in bacteria.

8.5 Genetic basis of drug resistance to antimalarial drugs

As described in Chapter 9, the biochemical basis of resistance to antimalarial drugs can be due either to reduced drug uptake by the parasite, as in the case of chloroquine and possibly other quinoline drugs, or to the loss of drug sensitivity in target enzymes, including dihydrofolate reductase and dihydropteroate synthase. Spontaneous mutations resulting in the selection of drug-resistant parasites in response to sustained drug challenge appear to be a common pattern throughout the malarious regions of the world. Sexual reproduction and genetic recombination in the malarial parasite provide additional opportunities for the spread of drug resistance.

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