The widespread use and misuse of antibiotics imposes immense selective pressures for the emergence of antibiotic-resistant bacteria, and as a consequence the development of antibiotic resistance is inevitable. For those antibiotics that are derived from natural products, resistance is most commonly due to the acquisition of genes encoding enzymes that inactivate the antibiotic, modify its target, or result in active efflux of the antibiotic (1). These resistance genes are believed to have evolved hundreds of millions of years ago in soil bacteria, either as protection from antibiotics produced by other soil bacteria or as protection for antibiotic-producing soil bacteria against their own antibiotics (2). Enzymes that inactivate synthetic antibiotics such as quinolones, sulfonamides, and trimethoprim have not been found, and for these antibiotics and those natural products where inactivating or modifying enzymes have not emerged (for example, rifampicin, resistance may involve the acquisition of new genes, almost invariably carried on plasmids or transposons, that result in enzymatic modification of the normal target so that it no longer binds the antibiotic (for example, resistance to macrolide antibiotics by methylation of 23S ribosomal RNA (rRNA)) (8). Alternatively, resistance may result from mutational (or recombinational) events that lead to the development of antibiotic-resistant forms of the normal targets.

Here, I will focus on this latter mechanism, and particularly on resistance due to the development of altered forms of the normal targets that have increased resistance to antibiotics. Such resistance may involve the acquisition of new genes, almost invariably carried on plasmids or transposons, that result in enzymatic modification of the normal target so that it no longer binds the antibiotic (for example, resistance to macrolide antibiotics by methylation of 23S ribosomal RNA (rRNA)) (8). Alternatively, resistance may result from mutational (or recombinational) events that lead to the development of antibiotic-resistant forms of the normal targets.

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Several types of target modification are found in antibiotic-resistant clinical isolates of enteric bacteria (5).

However, the most common mechanism of resistance is the development of altered forms of the normal targets that have increased resistance to antibiotics. Such resistance may involve the acquisition of new genes, almost invariably carried on plasmids or transposons, that result in enzymatic modification of the normal target so that it no longer binds the antibiotic (for example, resistance to macrolide antibiotics by methylation of 23S ribosomal RNA (rRNA)) (8). Alternatively, resistance may result from mutational (or recombinational) events that lead to the development of antibiotic-resistant forms of the normal targets.

The development of enzymatic targets with reduced affinity for antibiotics is a major mechanism of resistance when inactivating or modifying enzymes are absent. This includes resistance to rifampicins and quinolones (3, 6) and to β-lactam antibiotics in species where β-lactamases are absent (7). Most of my review here will focus on resistance to β-lactam antibiotics, for this provides a particularly instructive example of resistance that is the result of modification of enzymatic targets.

### References


65. The "intrinsic resistance" mechanism of P. aeruginosa, probably based on the MxaF-Mxb-OprK system, seems to handle any of about a dozen β-lactam compounds tested. Although imipenem appeared to be an exception, its efflux might have been hidden by its very rapid influx through the OprD channel, as described above [X.-Z. Li and H. Nikaido, unpublished results].


80. I thank K. Poole, K. Lewis, and M. Saeier Jr. for access to unpublished data and preprints and D. Thanassi for useful comments. Studies in the author's laboratory were supported by U.S. Public Health Service grants AI-06644 and AI-33702.
PBP-Mediated Resistance to β-Lactam Antibiotics

Penicillin and other members of the β-lactam family of antibiotics kill bacteria by inactivating a set of transpeptidases that catalyze the final cross-linking reactions of peptidoglycan synthesis (9). Penicillin inhibits these enzymes by acting as a structural analog, forming an irreversible penicilloyl-enzyme complex that is analogous to the transient acyl-enzyme formed during the normal transpeptidation reaction. Transpeptidases are difficult to assay and are usually detected and studied as penicillin-binding proteins (PBPs). Bacteria possess multiple transpeptidase-PBPs that have different functions in the synthesis of peptidoglycan during the cell cycle (9). Besides these physiologically important PBPs [PBPs with a high relative molecular mass (high-Mr, PBP)], bacteria contain one or more low-Mr PBPs that function as D-alanine carboxypeptidases. Inactivation of low-Mr PBPs is not thought to affect the killing action of β-lactam antibiotics (9).

Resistance to penicillin in clinical isolates is most commonly due to hydrolysis of the antibiotic by a β-lactamase (1, 7). In the relatively few bacterial species in which β-lactamas are unknown to exist, the development of resistance to penicillin can occur by two mechanisms. In Gram-negative bacteria, reductions in the permeability of the outer membrane, or the development of high-Mr PBPs that have decreased affinity for the antibiotic, can provide increased resistance (7). Only the latter can occur in Gram-positive species. PBP-mediated resistance to β-lactam antibiotics is well documented in several species, including Hae-mophilus influenzae and Neisseria gonorrhoeae among Gram-negative pathogens, and Streptococcus pneumoniae, viridans group streptococci and enterococci, and Staphylococcus aureus and S. epidermidis among Gram-positive pathogens (7).

The rarity of PBP-mediated resistance is probably due to several factors. One major reason is that such resistance is difficult to achieve because β-lactam antibiotics have multiple killing targets. Reduction in the affinities of each of these targets for the antibiotic is necessary for the development of high-level resistance (10). For example, N. gonorrhoeae (the gonococcus) possesses three PBPs (11): PBP 1 and 2 are essential enzymes, and inactivation of either of these is a lethal event, whereas PBP 3 is a low-Mr enzyme that is not thought to be a lethal target of β-lactam antibiotics. Development of resistance in this bacterium requires a reduction in the affinity of the high-Mr, PBP with the highest affinity (PBP 2), as inhibition of this enzyme causes the bacteria to be killed at the minimal inhibitory concentration (MIC) of penicillin. But reduction in the affinity of PBP 2 only increases the level of resistance to the concentration of penicillin that results in the lethal inactivation of PBP 1. Gonococci that produce a low-affinity form of PBP 2 must therefore develop a low-affinity form of PBP 1 to achieve higher levels of resistance (12). PBP-mediated resistance in clinical isolates of this Gram-negative bacterium also involves reductions in the permeability of the cell envelope (12). In other bacteria there may be three or more PBPs whose affinities need to be reduced to achieve high-level resistance. In S. pneumoniae (the pneumococcus) for example, the development of high-level resistance to penicillin requires the reduction in the affinities of at least four high-Mr, PBPs (13).

A further impediment to PBP-mediated resistance is the fact that penicillin is a substrate analog, and reductions in affinity require a subtle restructuring of the active center of the transpeptidase domain of high-Mr PBPs, so that they decrease their affinity for penicillin without impairing their ability to recognize the normal substrate (10, 14). In gonococci and other Gram-negative bacteria, high levels of resistance can be achieved by relatively small reductions in the affinities of PBPs as reductions in the permeability of the outer membrane also occur. In pneumococci, the extent of reduction in the affinities of PBPs relates more directly to the level of resistance. The most highly resistant pneumococci are >1000-fold more resistant to benzylpenicillin than truly susceptible isolates. At least some of the high-Mr PBPs have to reduce their affinities so that they fail to beacylated by penicillin concentrations that are 1000-fold greater than those that acylate these enzymes in susceptible isolates. Laboratory studies suggest that large changes in the ability of a PBP to discriminate between the binding of an inhibitor and the structurally analogous substrate cannot be accomplished by single amino acid substitutions (14). The development of PBP-mediated resistance is therefore likely to be a gradual process, involving the introduction of multiple amino acid substitutions into multiple, high-Mr, PBPs (7).

The three-dimensional structures of high-Mr, PBPs are unknown, although high-resolution structures of several serine β-lactamases, and low-Mr, PBPs, have been determined (9). The recognition of conserved sequence motifs within the transpeptidase domains of high-Mr, PBPs, and in serine β-lactamases and low-Mr, PBPs, suggests that these enzymes have similar structures. The sequence motifs are located around the active center of these penicillin-interacting enzymes (9), and as expected the amino acid changes that reduce the affinity of PBP 3 of Escherichia coli for cephalaxin are within, or close to, these conserved motifs (14).

Recombination and Development of Altered PBPs in Pathogenic Neisseria

Gonococci isolated in the preantibiotic era are inhibited by about 0.004 μg of benzylpenicillin per milliliter of medium. Isolates that required higher MICs of penicillin were detected in the 1950s, and overtly resistant gonococci (MICs > 1 μg/ml) were found in the Far East in the 1960s and 1970s and are now encountered worldwide (15). Gonococci harboring plasmids that express TEM β-lactamase only emerged in 1976 (15). Meningococci are extremely closely related to gonococci, but surprisingly plasmids expressing TEM β-lactamase have not yet become established. PBP-mediated resistance in meningococci is developing but so far has not reached a level that results in the clinical failure of penicillin therapy (16). These "penicillin-resistant" meningococci contain altered forms of PBP 2 with a decreased affinity for penicillin, but there is no evidence that they possess altered forms of PBP 1 (17).

Analysis of the PBP 2 genes of penicillin-susceptible and penicillin-resistant gonococci and meningococci provided the first evidence that low-affinity PBPs arise by recombination rather than by mutation (18). The PBP 2 genes of penicillin-susceptible isolates of each species are very uniform in sequence, whereas those of resistant isolates have a mosaic structure, consisting of regions that are essentially identical to those in susceptible isolates and regions that are 14 to 23% divergent in sequence (Fig. 1). In most resistant isolates, the divergent regions have been derived from the PBP 2 gene of Neisseria flavescens, whereas in others they are from N. cinerea, and some isolates contain regions derived from both of these human commensal species (18). Gonococci and meningococci are naturally transformable, and the mosaic structure is believed to have arisen by the replacement, by homologous recombination, of regions of the PBP 2 genes of susceptible gonococci and meningococci with the corresponding regions from the PBP 2 genes of the commensal Neisseria species (18). The result of these interspecies recombinational events is the production of altered forms of PBP 2 that contain numerous amino acid substitutions and insertions compared to PBP 2 of susceptible isolates. For reasons that are discussed below, the hybrid PBPs encoded by mosaic PBP 2 genes have decreased affinity for penicillin.
PBP-Mediated Resistance to β-Lactam Antibiotics in *Streptococcus pneumoniae*

The most spectacular example of PBP-mediated resistance is found in *S. pneumoniae*. Pneumococci that produce β-lactamase have never been reported, and their resistance to penicillins and cephalosporins is entirely due to alterations of PBPs (7). Pneumococci possess five high-Mr PBPs (1A, 1B, 2A, 2B, and 2X) and the low-Mr PBP 3, which has not been implicated in the killing action of β-lactam antibiotics (13, 19). The most highly penicillin-resistant isolates (MICs of 2 to 16 μg of benzylpenicillin per milliliter) produce altered forms of PBP 1A, 2X, 2B, and 2A that have reduced affinity for the antibiotic (7, 13, 19). As in the pathogenic *Neisseria*, low-affinity forms of the high-Mr pneumococcal PBPs have arisen by recombination rather than mutation. The PBP 1A, 2X, and 2B genes of resistant isolates contain highly divergent regions (Fig. 2), in contrast to the genes of truly susceptible isolates (that is, isolates from the preantibiotic era), which are very uniform in sequence (20). Pneumococci are naturally transformable, and the mosaic structure in their PBP genes is believed to have arisen by inter-species homologous recombination, although it has proven difficult to identify the source or sources of the divergent regions. At least three different sources have been implicated, and recently one of these has been identified as *S. mitis* (21). In addition, in the naturally transformable viridans group species *S. oralis* and *S. sanguis* (which are close relatives of pneumococci), PBP-mediated resistance appears to have emerged by the replacement of their normal PBP genes with those from penicillin-resistant pneumococci (22).

The altered PBPs in pneumococci have greatly decreased affinity for almost all β-lactam antibiotics, including the third-generation cephalosporins. Penicillin-resistant pneumococci therefore show cross-resistance to other β-lactam antibiotics. In most resistant isolates, the MICs of third-generation cephalosporins are equal to, or slightly less than, the MICs of penicillin. Third-generation cephalosporins may still be effective in treating pneumococcal infections caused by penicillin-resistant pneumococci because large amounts of these antibiotics can be maintained in the blood and cerebral spinal fluid. However, high-level resistance to third-generation cephalosporins has recently emerged (23). In contrast to high-level resistance to penicillin, which requires alterations of four PBPs, clinical pneumococcal isolates with ceftriaxone MICs as high as 16 μg/ml have alterations of only PBPs 1A and 2X (24), as the other PBPs have inherently low affinity for third-generation cephalosporins and therefore are not involved in the killing of pneumococci by clinically relevant concentrations of these compounds (25). Because altered forms of PBPs 1A and 2X with reduced affinity for both penicillins and cephalosporins are now common in the pneumococcal population, it is not surprising that isolates with overt resistance to third-generation cephalosporins have emerged.

**Interspecies Recombination as a Mechanism for Producing Low-Affinity PBPs**

Penicillin-resistant gonococci, meningococci, and pneumococci invariably possess mosaic high-Mr, PBP genes, whereas penicillin-susceptible isolates do not. These mosaic genes have been shown to encode PBPs with decreased affinities, which contribute to the penicillin resistance of the bacteria. However, the sequence of events that has led to resistance is in most cases unclear. The simplest idea (18) is that transformable species can exchange genes with those of their close relatives at a frequency that declines with increasing sequence divergence, until with about 25 to 30% sequence divergence homologous recombination is no longer possible. Variations in the amino acid sequences of closely related PBPs will result in variations in their affinity for penicillin. In this scenario, after the introduction of penicillin in the 1940s rare recombinational events (such as those that result in the replacement of a PBP 2 gene of a pathogenic *Neisseria* species with a homologous gene from a related species that, by chance, had a lower affinity PBP 2) became strongly selected (19).

This scenario provides an adequate explanation of the origins of the mosaic PBP 2 genes that contain *N. flavescens* sequences. Isolates of *N. flavescens* from the preantibiotic era possess a PBP 2 that has a much lower affinity for penicillin than PBP 2 of either *N. gonorrhoeae* or *N. meningitidis*, and the interspecies recombinational events that are believed to have occurred in nature have been simulated in the laboratory (26). The development of low-affinity forms of PBP 2 in pathogenic *Neisseria* by replacement of their PBP 2 genes (or the relevant parts of them) with the PBP 2 gene from *N. flavescens* is therefore well established.

It has not, however, been possible to show that replacement of the PBP 2 gene of *N. meningitidis* with the PBP 2 gene of *N. cinerea* provides increased resistance to penicillin (26). A crucial difference between
the *N. cinerea* sequence in a resistant meningococcus and that in typical *N. cinerea* isolates is the presence of an additional aspartic acid codon in the former (26). The same insertion is found in PBP 2 of all penicillin-resistant gonococci, where it is known to contribute to their reduced affinity for penicillin (27). A similar problem occurs in those pneumococcal PBP 2A genes that contain regions of the *S. mitis* PBP 2B gene (21). The *S. mitis* regions in resistant pneumococci possess an alteration adjacent to the SXN motif (where S is Ser, N is Asn, and X is any amino acid) (Fig. 3) that is known to reduce affinity for penicillin, whereas this is not found in typical *S. mitis* isolates (21). One possible explanation is that some *N. cinerea* and *S. mitis* isolates in the preantibiotic era possessed these crucial amino acid differences as polymorphisms, and these were the donors in the interspecies recombinational events. Alternatively, the crucial amino acid differences may have been introduced recently, resulting in increased penicillin resistance in the commensal species, with subsequent transfer of the genes into pneumococci and pathogenic *Neisseria*. Neither of these explanations is entirely satisfactory, however.

Because the well-documented examples of PBP-mediated resistance have occurred in species that are naturally transformable, it is tempting to suggest that transformation, with its ability to promote interspecies recombination, provides a more efficient route to resistance in these species than the sequential introduction of multiple amino acid substitutions by the expected process of mutation and selection. Interestingly, resistance to sulfonamides appears to have arisen in meningococci by interspecies homologous recombinational events that produce altered forms of dihydropteroate synthase (28).

It is perhaps surprising that the development of altered PBPs and dihydropteroate synthase have occurred by interspecies recombination rather than by mutation. Recombination appears to be frequent in natural populations of both gonococci and meningococci, such that alleles in the population are at, or close to, linkage equilibrium (29). However, interspecies recombination might be expected to occur at much lower frequencies than intraspecies recombination because of differences in restriction systems and the ability of mismatch repair systems to abort mismatched heteroduplexes (30). But differences in restriction systems have little or no effect on transformation, and (at least in pneumococci) mismatch repair systems become saturated when heteroduplexes are formed between extensively mismatched DNA molecules (31). Similarly, in *Neisseria* recombination between highly divergent outer membrane protein genes contributes to the generation of antigenic diversity (29), and the mismatch repair system may act mainly for correction of errors during replication, rather than as a barrier to interspecies recombination. The limited effects of restriction and mismatch repair systems may allow interspecies recombination to occur in transformable species at frequencies that are not very much lower than those of intraspecies events. Indeed, interspecies recombination has been detected in the recA and argF genes of natural populations of *N. meningitidis*, as well as in the PBP and dihydropteroate synthase genes, where the resulting phenotypes provide strong selection for these events (29).

The large number of amino acid differences between the PBPs of penicillin-resistant and penicillin-susceptible meningococci and pneumococci have made it difficult to identify the amino acid changes that reduce affinity for penicillin. In PBP 2B of pneumococci, the substitution Thr445 → Ala at the residue after the SXN motif, as well as substitutions in a small region between the active-site serine motif and the SXN motif (Fig. 3), has been shown to be a major contributor to the reduced affinity of PBP 2B for penicillin (9, 32). The additional aspartic acid residue in PBP 2 of all penicillin-resistant gonococci is also within this small region (27). Similarly, substitutions in this region, and within the SXN motif, were found in PBP 3 of laboratory mutants of *E. coli* (14). In the absence of a known three-dimensional structure, any attempt to explain the effects of these amino acid changes on the affinity of PBPs for penicillin would be pure speculation.

**Methicillin-Resistant Staphylococcus aureus**

Methicillin-resistant *S. aureus* (MRSA) provides another example of PBP-mediated resistance by an unexpected mechanism.

Staphylococcus aureus is not naturally transformable, and the mechanism of its resistance is entirely different from that in pathogenic *Neisseria* and *S. pneumoniae*. MRSA produces unaltered forms of the normal four staphylococcal PBPs but also contains an additional high-Mr PBP (PBP 2') with a very low affinity for essentially all ß-lactam antibiotics (7, 33). The PBP 2' gene (meCA) is absent from normal *S. aureus* isolates and is part of a transposon (33). The mecA gene is also found in methicillin-resistant coagulase-negative staphylococci (for example, *S. epidermidis*). The acquisition of the mecA gene appears to have happened only once, and the gene has subsequently spread both within- and between staphylococcal species (34).

The ability of a single PBP to take over the functions of the three normal high-Mr, PBPs of staphylococci is surprising as multiple high-Mr PBPs are thought to be required for the normal growth and morphogenesis of bacteria (9). PBP-mediated resistance in MRSA is analogous to bacterial resistance to sulfonamides and trimethoprim, in which alternative target enzymes with low affinity for these substrates are acquired (4, 5). Because ß-lactams are natural products, it is possible that the mecA gene originated in a ß-lactam-producing soil bacterium. There is, however, no evidence for this view, and the source of the PBP 2' gene is unknown.

**Target Changes in Rifampicin- and Quinolone-Resistant Clinical Isolates**

The rifamycins and quinolones both inhibit single enzymatic targets and are not believed to act as substrate analogs (3, 6). Resistance to these antibiotics by the development of altered target enzymes should therefore arise much more easily than resistance to ß-lactam antibiotics. This appears to be the case, because for both groups of antibiotics single amino acid substitutions in their target enzymes...
can provide clinically significant resistance. The rifamycins interact with the β subunit of RNA polymerase (6). Resistance because of the acquisition of inactivating enzymes has not been reported in clinical isolates (35), which is unusual for antibiotics that are natural products. Resistance because of reductions in the affinity of RNA polymerase emerges in clinical isolates at a sufficiently high frequency to limit the use of rifamycins as a monotherapy for most bacterial infections. Rifampicin binds to the β subunit at a site distant from the active site (36), and the relatively rapid development of resistance appears to be due to the existence of numerous amino acid substitutions and insertions or deletions that can provide large decreases in affinity for the antibiotic (Fig. 3) (37–39). In laboratory mutants of E. coli, resistance to rifampicins results from amino acid substitutions, or small insertions and deletions, within three short highly conserved regions of the β subunit of RNA polymerase (37). Most of the alterations occur within one of these regions (residues 507 to 534) (37), and alterations within the corresponding region have recently been found in rifampicin-resistant clinical isolates of Mycobacterium tuberculosis (38) and M. leprae (39). Eighteen different substitutions (involving eight different residues) and two small deletions have been found in a collection of over 80 rifampicin-resistant M. tuberculosis (38). In nine resistant M. leprae, three amino acid substitutions at a single residue, and a two–amino acid insertion, were found within this region (39).

The older quinolones (for example, nalidixic acid) and the newer fluoroquinolones (for example, ciprofloxacin) inhibit DNA gyrase (3). Laboratory mutants selected for increased resistance to quinolones usually have mutations in the gyrA gene encoding the A subunit of DNA gyrase. Almost all of these mutations alter amino acids in a short region that has been termed the quinolone resistance–determining region (40). Resistance to fluoroquinolones in the clinical setting has emerged in several species, notably in MRSA (41). In this species, single amino acid substitutions at Ser83 or Glu87 of the A subunit of gyrase can provide high-level resistance to ciprofloxacin (MICs of 16 to 128 μg/ml); increased activity of an efflux system can also contribute to resistance in some isolates (3, 41). Clinically significant resistance in E. coli is less common, as this species is inherently more susceptible to fluoroquinolones than S. aureus. Single amino acid substitutions at, or around, Ser83 (corresponding to Ser80 of S. aureus) provide 20-fold increased resistance, but high-level resistance requires double amino acid substitutions in the A subunit or additional mutations that reduce the permeability of the outer membrane (40).

Conclusion

We can make some tentative conclusions about the features of antibiotics that make target-mediated resistance emerge relatively quickly and those that may delay the development of resistance. One important feature that has undoubtedly slowed the development of PBP-mediated resistance is the ability of β-lactams to inactivate multiple targets. Some antibiotics that act on the ribosome (for example, chloramphenicol) may also interact with multiple targets because resistance by mutation has never been achieved either in the laboratory or in nature (8). This is believed to be due to the fact that these antibiotics interact primarily with rRNA, which is encoded by multiple gene copies (8). Unfortunately, resistance to these derivatives of natural products has emerged as a result of the acquisition of genes that protect the ribosome or that result in antibiotic efflux.

A second feature of antibiotics that may delay the emergence of resistance is a close structural analogy to the substrate, because bacteria develop resistant target enzymes by exploiting differences between the binding of substrate and antibiotic. For antibiotics that do not show structural analogs (for example, rifampicin), numerous amino acid substitutions may provide large reductions in affinity (34–36), whereas for close analogs only highly specific amino acid substitutions that provide slightly increased discrimination between substrate and inhibitor are likely (14). Finally, it may be an advantage for antibiotics to be irreversible inhibitors and to be derived synthetically rather than from natural products of soil microorganisms, for inactivating or modifying enzymes probably exist in soil microorganisms even for those classes of naturally occurring antibiotics that have yet to be discovered.

In many ways, β-lactam antibiotics come close to these ideal features, except that they are derived from natural products. The widespread occurrence of β-lactamases negates the fact that β-lactams irreversibly inactivate multiple targets by acting as an analogs of substrate. In bacterial species that lack β-lactamases, PBP-mediated resistance appears never to have arisen by the accumulation of multiple amino acid substitutions in target enzymes but has depended on unexpected events involving horizontal gene transfer. High-Mr PBPs have an added advantage as targets; their active sites are outside the cytoplasmic membrane, which reduces the possible mechanisms by which resistance due to decreased access to targets can emerge.

One approach to the production of the ideal antibiotic would be to have irreversible synthetic inhibitors of high-Mr PBPs that are close analogs of substrate but that are inherently resistant to β-lactamases because they are not based on β-lactam structures. Whether such ideal antibiotics can be produced by rational design is unclear (42). Furthermore, we should not be too sanguine, as in the last 50 years bacteria have exploited the remarkable facility to develop resistance to every antibiotic that has been developed, often by quite unexpected mechanisms.

REFERENCES AND NOTES

32. C. G. Dowson, unpublished results.
43. B.G.S. acknowledges support from the Wellcome Trust and the U.K. Medical Research Council.