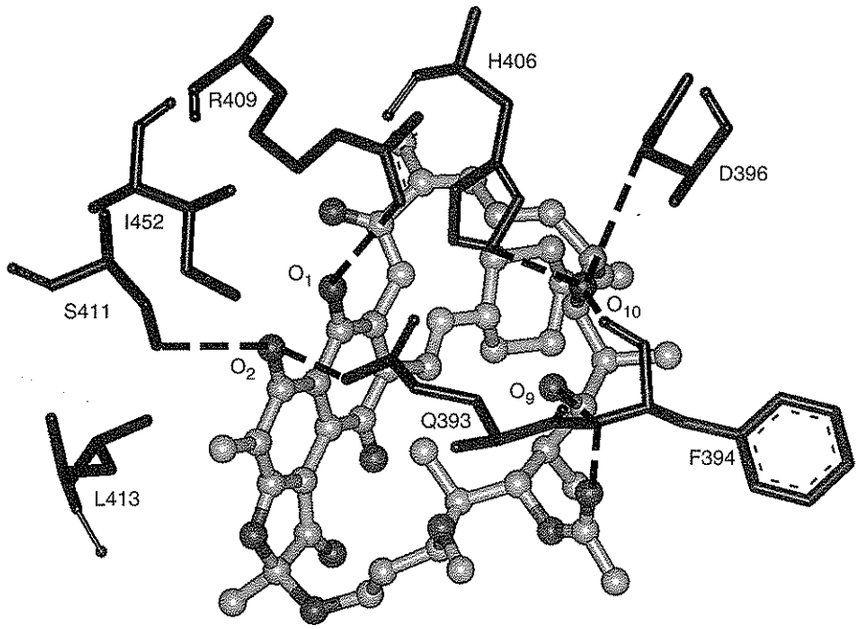


Figure 6.10 Binding site for rifamycin on its target protein, the β subunit of RNA polymerase. (From Campbell et al. [2001].)

and 411 (Spratt, 1994). Resistance development is relatively rapid if rifampin is used as a single agent (Heep et al., 2000), explaining one of the reasons combination therapy is used for tuberculosis (see chapter 11 of Scholar and Pratt, 2000). The 50% inhibitory concentration against mycobacterial RNA polymerase is in the range of 0.005 to 0.1 μg/ml and eukaryotic RNA polymerases are at least 100-fold less sensitive.

section III

Antibiotic Resistance

SECTION III EXAMINES RESISTANCE TO THE ANTIBACTERIAL DRUGS that have been discussed in section II. Chapter 7 introduces the topic of resistance in antibiotic-producing microbes that must have developed self-protection mechanisms to avoid self-destruction during antibiotic production. The three main intrinsic mechanisms in antibiotic producers are (i) inactivation of the antibiotic, (ii) efflux of the antibiotic, and (iii) modification of the susceptible molecular target. These mechanisms presage the resistances that are acquired as susceptible bacterial pathogens become resistant. Chapter 8 takes up inactivation of antibiotics in the context of both β-lactam hydrolysis and aminoglycoside covalent modification. Chapter 9 details the families of efflux pumps found in pathogens. Chapter 10 describes protein modifications in penicillin-binding proteins that underlie the methicillin-resistant *Staphylococcus aureus* (MRSA) phenotype, 23S rRNA methylation prevalent in erythromycin resistance, and reprogramming of peptidoglycan intermediates in vancomycin resistance.

Bacteria	Case mortality	% of isolates resistant to: Methicillin	Vancomycin
Coagulase-negative *Staphylococcus*	21%	80%	—
Staphylococcus aureus	25%	30%	—
Enterococcus faecalis	25%	—	20%

Incidence and severity of methicillin- and vancomycin-resistant infections. —, not determined.

chapter 7

Natural and Producer Immunity versus Acquired Resistance

During the five to six decades that antibiotics have been in ever-widening therapeutic use, the development of antibiotic resistance has followed. The historical observations are that whenever a new antibiotic, broader-spectrum forms of an existing antibiotic, or a new class of antibiotics is introduced into widespread use in people, clinically significant resistance appears. It may be a matter of months (penicillin resistance was detected as early as 1945) or it could take years: vancomycin resistance took almost 30 years (1987) after clinical introduction (1954). The long delay was probably due to limited use of vancomycin in the first 25 years, but as it advanced to the front of the therapeutic line, resistance emerged. Also, as we shall note in chapter 10, while clinically significant resistance to β-lactams can occur through the action of one gene product, the hydrolytic β-lactamase, the vancomycin-resistant enterococcus (VRE) phenotype requires a five-gene resistance cassette to have been assembled. The outbreaks of resistance can be geographically scattered and to all major antibiotic classes. **Figure 7.1** shows a map of outbreaks in the United States over the 11-year period from 1983 to 1994 of pathogens resistant to both penicillins and cephalosporins, to the fluoroquinolone ciprofloxacin, to the macrolide erythromycin, and to the glycopeptide vancomycin. Similar patterns have continued since 1994, as noted in chapter 17.

Antibiotic-resistant bacteria are selected for in hospital settings much more rapidly than in the outside community. In hospitals there is intensive and essentially constant exposure of bacteria to antibiotics. In these microenvironments there is selective pressure for antibiotic-resistant bacteria to maintain those determinants, survive, and even dominate the bacterial populations. In a large population of bacteria, say 10^8, exposed to a drug there is a competition between death of all the bacteria and the development of rare mutations that confer resistance. Given the short replication time for bacterial division (as short as 20 to 30 minutes) and a typical frequency of one error per 10^7 bases as their DNA polymerases copy DNA, then the 100 million bacteria will contain about 10 mutants in the population. If these mutations are randomly dispersed in the

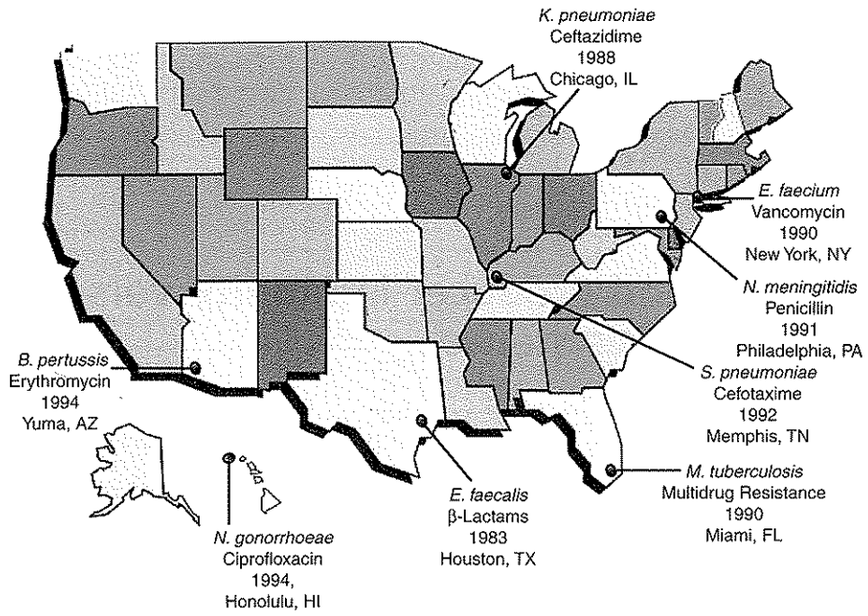


Figure 7.1 Recent outbreaks of antibiotic-resistant bacteria in the United States over the 11-year period 1983–1994.

genome of a bacterium the size of *Escherichia coli*, with 3,000 genes, then 0.3% (10/3,000) of the genes will have one mutation. If one of these gene mutations is in a target for an antibiotic and the mutation confers some degree of resistance that renders that bacterium less sensitive, then it will have a selective survival advantage. As its sensitive neighbors die, it will persist and have space to grow and dominate the culture and may disseminate effectively.

In addition to the independent evolution of point mutations to create resistant genes, such genes can be collected on transposable DNA elements (transposons), which are mobile genetic elements that can transpose between DNA sequence elements on both larger mobile elements (plasmids) and on bacterial chromosomes (conjugative transposons) (see Whittle et al., 2001). Thus the genes responsible for the VanA phenotype of vancomycin resistance are typically on a transposon embedded in a plasmid in VRE cells. Analogously, the TEM-1 β-lactamase is often carried by the transposon TN3 (Amyes, 2001). Plasmid-sized DNA elements can integrate into specific attachment sites on chromosomes to create antibiotic resistance islands, as found in *Salmonella enterica* serovar Typhimurium DT104 (chapter 17) and methicillin-resistant *Staphylococcus aureus* (MRSA) (this chapter). This allows multiple resistance genes to be maintained together. All these routes ensure rapid spread and stable maintenance of collections of antibiotic resistance genes through bacterial populations. Jain et al. (1999) have suggested that extensive horizontal gene transfer is a continuing process among bacteria.

The resistant strain will continue to be selected for by the continuing presence of antibiotic in the microenvironment, e.g., in a hospital ward. **Figure 7.2**

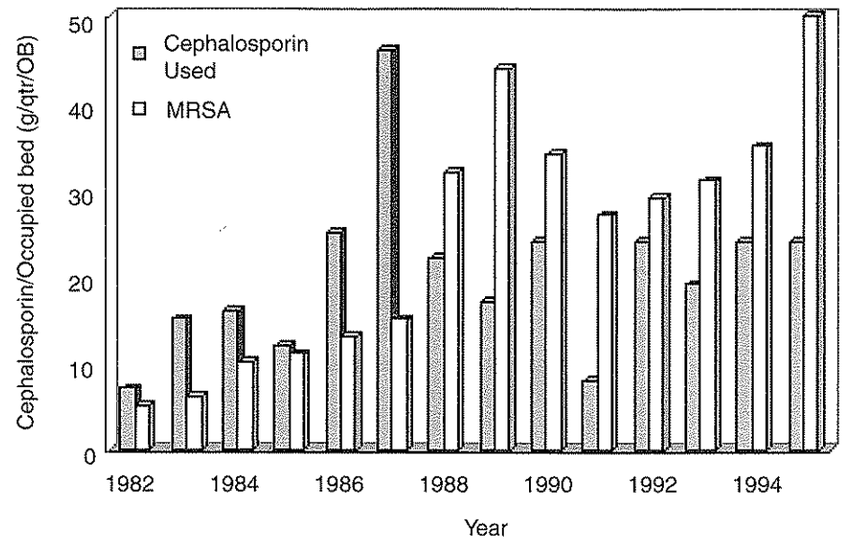
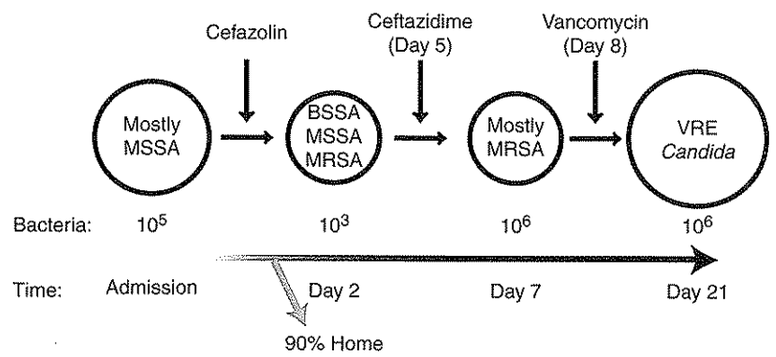


Figure 7.2 Time course for development of resistance to cephalosporins by MRSA.

shows a clear temporal relationship over a 12-year period between the use of cephalosporins and the emergence of MRSA (Hiramatsu et al., 2001). The effects can also occur in a compressed period of time. For example, patients admitted for surgery are given antibiotics prophylactically and then for a few days post-surgery to reduce the probability of infectious complications. A study of nasal culture history in such a patient population indicated (**Fig. 7.3**) that on preadmission, a given aliquot size contained 10^5 *S. aureus* bacteria, essentially all sensitive to methicillin (MSSA) (Schentag et al., 1998). Following surgery and medication with cefazolin (a cephalosporin), 90% of the patients were sent home at 2 days postsurgery, without infectious complications. The nasal bacterial count was down 100-fold to 10^3 and was a mixture of sensitive, borderline-resistant, and resistant staphylococci. For those patients who remained in the hospital and

Figure 7.3 Progression of bacterial populations in surgical patients from MSSA to MRSA to VRE in a three-week time frame. (From Schentag et al. [1998], with permission.)



were switched on day 5 to a second cephalosporin (ceftazidime), when assayed on day 7, the bacterial count was up 1,000-fold to 10^6 and was mostly MRSA. These patients were then switched to a 2-week course of vancomycin, and for those still in the hospital on day 21, the nasal cultures revealed 10^6 VRE as well as some *Candida*. Because of the short division time of bacteria, the selection for drug-resistant, life-threatening pathogens can be remarkably rapid.

By the above argument, bacterial resistance to antibiotics is not a matter of if but only a matter of when. This logic argues there will be a constant need for cycles of new antibiotic discovery and development. As soon as an antibiotic is introduced for widespread clinical use, the selection of resistant organisms will start and a finite therapeutic lifetime will occur before resistance of pathogens becomes sufficiently widespread to lessen that drug's efficacy. Then one needs the next generation (e.g., we are at fourth-generation cephalosporins and third-generation erythromycins) or whole new classes of antibiotics. This is further emphasized by the list of bacteria in Table 7.1 that are resistant to different classes of antibiotics used clinically. A recent review of methods to assess antimicrobial resistance is provided by Cockerill (1999).

Many antibiotics that have been developed into approved antibacterial drugs have MICs or sometimes 50% inhibitory concentrations, often in Petri plate assays, in the range of 1 μg/ml. For antibiotics with molecular weights $\leq$500

Table 7.1 Bacterial resistance to various classes of clinically used antibiotics

Antibiotic	Structural class	Target	Mutant/ plasmid	Efflux	Porin	Inact.	Target alteration
Ampicillin	Penicillin	E	+/+	√	√	√	√
Ceftriaxone	Cefalosporin	E	+/+	√	√	√	√
Imipenem	Carbapenem	E	+/+	√	√	√	√
Fosfomycin	Phosphonic acid	E	+/+		√	√	
Gentamicin	Aminoglycoside	R	+/+	√		√	√
Chloramphenicol	Phenylpropanoid	R	+/+	√		√	√
Tetracycline	Polyketide (II)	R	+/+	√		?	√
Erythromycin	Macrolide	R	+/+	√		√	√
Clindamycin	Lincosamide	R	+/+			√	√
Synercid	Streptogramin	R	+/+	√		√	√
Telithromycin	Ketolide	R	+/+	√		√	√
Ciprofloxacin	Fluoroquinolone	D	+/+	√			√
Vancomycin	Glycopeptide	E	+/+				√
Sulfisoxazole	Sulfonamide	M	+/+				
Trimethoprim	—	M	+/+				
Rifampin	Ansamycin	P	+/+			√	√
Fusidic acid	Steroid	T	+/+	√			√
Linezolid	Oxazolidinone	R	+/−				√
Novobiocin	Coumarin	D	+/+				√
Isoniazid	—	M	+/−				
Pyrazinamide	—	M	+/−				
Nitrofurantoin	Nitrofuran	M	+/−			(√)	
Polymyxin	Peptide	E	+/−	√			√
Capreomycin	Peptide	R	+/−			√	√
Mupirocin	Pseudomonic acid	T	−/+				√

Inact., inactivation; D, replication; E, envelope; M, metabolism; P, RNA polymerase; R, ribosome; T, translation; —, nonstandard structural class.
Adapted from a poster on Mechanisms of Antibiotic Action and Resistance, C. Walsh, J. Trauger, P. Courvalin, J. Davies (2001), *Trends in Microbiology, The Lancet Infectious Disease, Current Opinion in Microbiology, Trends in Molecular Medicine.*

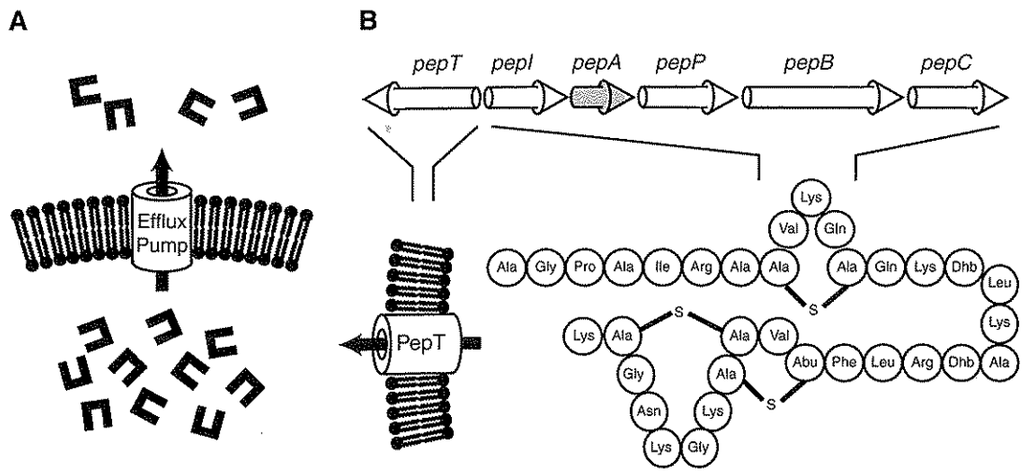
g/mol, this would be in the ~2 μM concentration range. For example, 50% inhibitory concentrations of oxazolidinones for susceptible organisms are in the 1-μg/ml range, while tetracycline MIC around 0.1 μg/ml may be typical. Vancomycin MICs are often in the range of 0.1 to 0.5 μg/ml; MICs of quinolones such as ciprofloxacin are reported in the range of 0.05 to 0.5 μg/ml for sensitive organisms. Macrolides of the erythromycin family may have MIC in the 0.01- to 1-μg/ml range. The potencies have translated into useful dosing ranges for treatment of infections in humans. As bacteria develop antibiotic resistance determinants and mechanisms, when MIC reach or exceed 8 μg/ml the organisms may be classified as moderately resistant. Organisms for which MICs are above 32 μg/ml are generally viewed as clinically resistant to the antibiotic.

How do antibiotic producers escape their own destruction?

Many observers have noted that antibiotic producers could be vulnerable to their own chemical weapons of destruction and must have worked out strategies for their own protection and immunity. This has led to the general concept that antibiotic resistance genes and mechanisms must have coevolved with antibiotic biosynthetic capability for a just-in-time self-protection scheme. In the next section of this chapter we shall examine some examples that give insights into the variety of autoprotection mechanisms in play in antibiotic-producing bacteria and the timing that switches on the protection as antibiotic production is geared up.

As a general rule, though, natural product antibiotics are made by enzymatic machinery within the bacterial cell and the mature antibiotics are secreted molecules. The armamentarium of almost every antibiotic producer examined includes one or more transmembrane protein pumps presumably dedicated to pumping out the antibiotics as they are made and before they accumulate to harmful concentrations within a producing cell (Fig. 7.4A). When clusters of

Figure 7.4 Schematics of antibiotic efflux protein pumps: (A) general scheme; (B) function for lantibiotic efflux pumps.



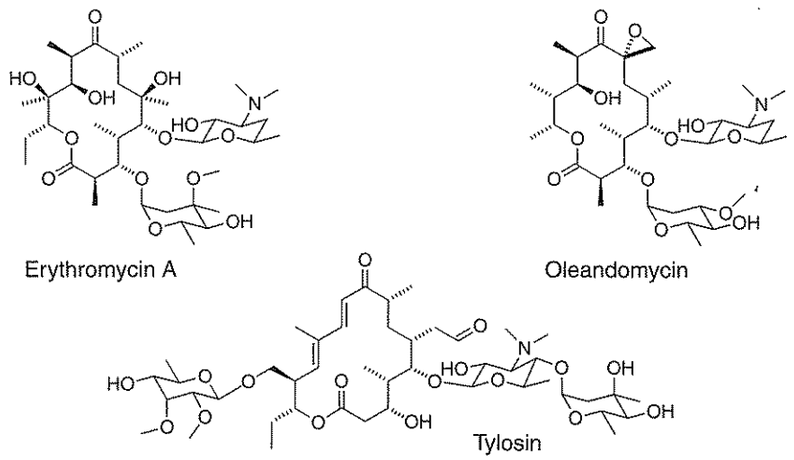
antibiotic genes have been sequenced, one typically finds genes encoding such antibiotic export pumps within the transcriptional clusters, ensuring the coordinate production of the export pumps as antibiotics roll off the enzymatic assembly lines (chapters 12 and 13). In the Pep lantibiotic cluster, *pepA* encodes the precursor to the antibiotic peptide and *pepT* encodes the pump. It may be that many of the later stages of antibiotic assembly, e.g., the protein complex making the lantibiotic proteins (Brotz and Sahl, 2000) (**Fig. 7.4B**), are physically coupled at the membrane to the pumps such that antibiotic synthesis and vectorial efflux from the bacterial cytoplasm are kinetically coupled.

The existence of genes in antibiotic producers that provide intrinsic resistance or autoimmunity also provides a potential reservoir for those genes to be acquired, through a variety of gene transfer mechanisms, by the bacteria that are the intended targets of the antibiotics. Given the resistance gene reservoirs, various transposon and other mobile genetic elements as transfer vehicles, and the selective pressure for bacteria to survive in an antibiotic-rich microenvironment once they have acquired the resistance genes, it is to be expected that the pilfering of such genes would be a common route to acquired resistance. We shall see this exemplified particularly clearly when comparing the autoprotection strategy in the glycopeptide antibiotic producers and the mechanism by which pathogenic enterococci become VRE.

Self-protection in macrolide producers

Streptomycetes produce most of the polyketide-based macrolide antibiotics, including erythromycin, the related 14-membered ring macrolide oleandomycin, and the 16-membered ring homolog tylosin (**Fig. 7.5**; also see Table 11.1). Oleandomycin differs from erythromycin in enzymatic modification of the C_8-CH_3 locus into an epoxide. Three strategies for self-resistance have been described in

Figure 7.5 Structures of the macrolide antibiotics erythromycin A, oleandomycin, and tylosin.

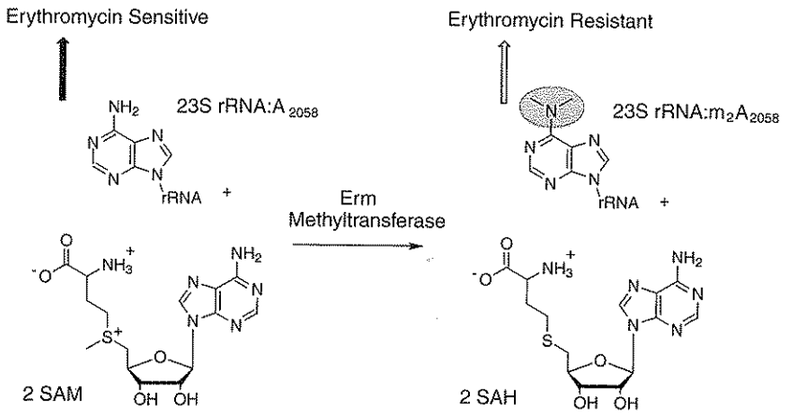


macrolide producers and presage acquired resistance mechanisms in human pathogens. The first is modification, by tandem action of the Erm methyltransferase, of the exocyclic amino group of one adenine residue, A_{2058}, in 23S RNA for mono- or dimethylation. This N-methylation interferes with erythromycin binding to that high-affinity site on the 50S ribosome (**Fig. 7.6**; also see chapter 4). This resistance mechanism is found in erythromycin and tylosin producers (Fierro et al., 1987; Quiros et al., 1998) but not in oleandomycin producers. The second mechanism is the expression of macrolide-exporting transport proteins, powered by ATP hydrolysis and known as ATP-binding cassette (ABC)-type proteins. Confirmation of export pump function comes from overexpression of these ABC proteins and increased resistance to erythromycin, discussed further in chapter 9.

The third self-resistance mechanism, deciphered by Salas and colleagues (Quiros et al., 1998) in the oleandomycin-producing *Streptomyces antibioticus*, involves enzymatic modification at the end of the biosynthetic pathway to keep the macrolide in an inactive form while it is still intracellular. The complementary piece of this elegant self-protection scheme is that once the macrolide is secreted it runs into an extracellular enzyme that converts it into its active form. The transient protecting group is a glucosyl group, encoded by a glucosyltransferase, *oleI*, within the oleandomycin gene cluster. **Figure 7.7** shows that the OleI glucosyltransferase uses UDP-glucose and regiospecifically glucosylates the C_2-OH of the desosamine sugar attached to C_5 of the macrolactone. The resulting macrolide, now with a desosaminyl-2,1-glucose disaccharide at C_5, is inactive as an antibiotic, because the newly introduced glucosyl moiety blocks binding to the 50S ribosome subunit. This causes no harm to the producing *S. antibioticus* cell, even if it accumulates to otherwise toxic levels before being pumped out by the ABC-type pump, the OleB protein.

On the other hand, this tri-sugar-containing macrolide, even when shipped out, is also not toxic to any of the neighboring bacteria. To reactivate the antibiotic properties of this latent glucosyl-oleandomycin, *S. antibioticus* also secretes a glycosidase, the product of the *oleR* gene (Quiros et al., 1998), that now re-

Figure 7.6 Enzymatic mono- and dimethylation of A_{2058} in 23S rRNA in macrolide resistance. SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine.



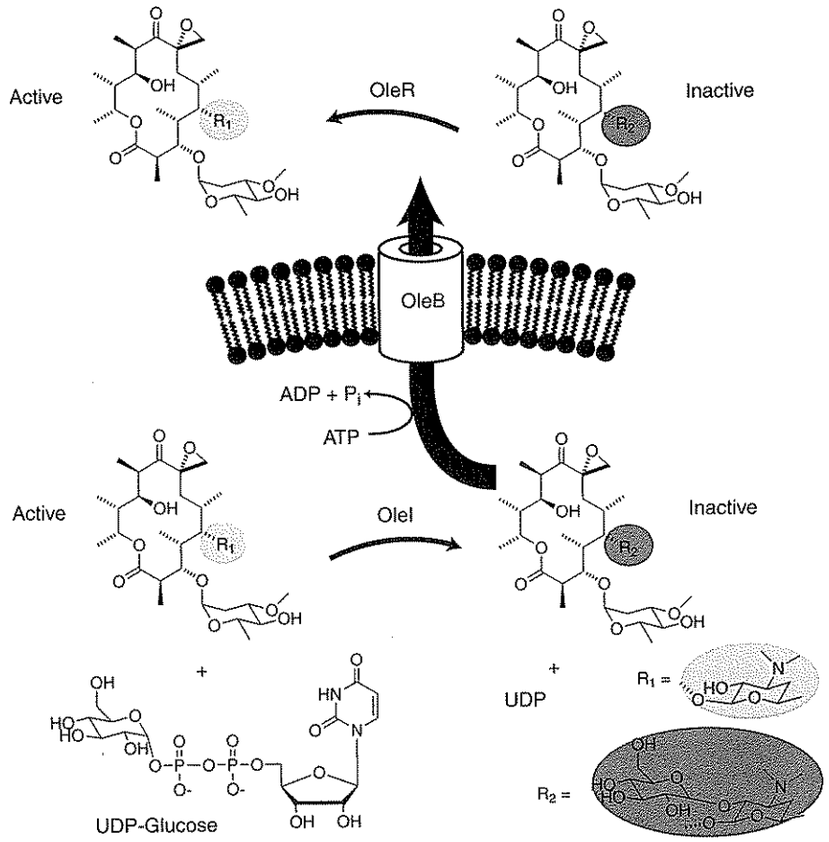


Figure 7.7 Strategy for self-protection by the oleandomycin producer: intracellular glucosylation to an inactive precursor of oleandomycin by OleI, export by the OleB pump, and extracellular reactivation by the glycosidase OleR.

moves the glucose hydrolytically and produces the form of oleandomycin capable of binding to the 50S ribosome (Fig. 7.7). This decoy mechanism does not seem to be followed by the erythromycin-producing *Saccharopolyspora erythraea*, although the EryBI enzyme is homologous to OleR, suggesting that at one time such a glycosylation (inside)/deglycosylation (outside) strategy may have been in play for some phase of the evolution of erythromycin producers, perhaps before export pumps became optimized or the rRNA methylase strategy, found in *S. erythraea* but not *S. antibioticus*, evolved. One last feature of note in *S. antibioticus* self-protection is the observation that the OleB transport/efflux pump will pump out the glucosyl-oleandomycin. It has yet to be determined if OleB has a lessened affinity for oleandomycin itself, once deglycosylated in the extracellular milieu by OleR. A lower affinity for reuptake of oleandomycin would bias the net flux of the pumping system to send glucosyl-oleandomycin out of the cell.

It remains to be seen how general is the cloaking of intracellular antibiotics by glycosylation or other covalent modification. There is a hint that off-pathway

intermediates in the bahlimycin glycopeptide antibiotic biosynthetic pathway are glycosylated in what may be a protective surveillance function (Bischoff et al., 2001). As will be discussed in chapter 14, there is cognate logic in streptomycin producers where intracellular streptomycin is inactive because of phosophorylation which is enzymatically removed after export.

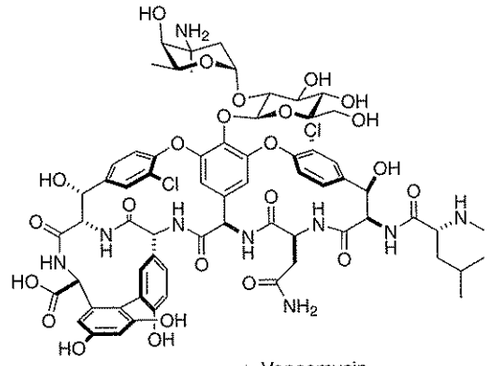
Self-protection in aminocoumarin producers

In the biosynthesis of the gyrase-targeted aminocoumarin antibiotics (chapter 5), it is likely that the coumarin ring is generated relatively early in the biosynthetic pathway (see chapter 14), and several of the intermediates on the way to novobiocin could therefore be potential inhibitors of the target enzyme, DNA gyrase. The producing streptomycetes have been tested for DNA gyrase susceptibility and intrinsic resistance has been observed. These determinants map to the GyrB subunit and are likely to be mutations in the extended ATP binding site which desensitize the site to novobiocin binding (Tsai et al., 1997). This is a case of modification of the target by mutation to retain essential catalytic activity while decreasing binding affinity for the inhibitor. It presages the molecular bases of resistance of pathogens by GyrB mutations after exposure to coumarin antibiotics (Maxwell, 1997).

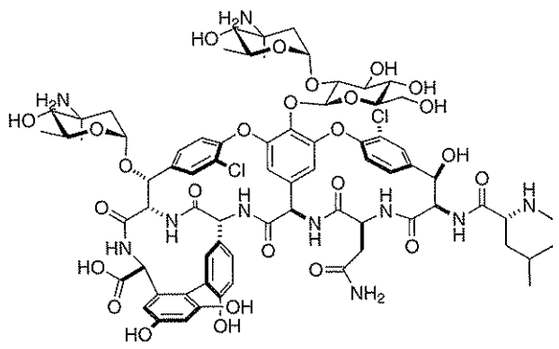
Protection during antibiotic production in vancomycin family producers

The vancomycin group of antibiotics are made by streptomycetes and by actinoplanes. The gene clusters for a vancomycin analog, chloroeremomycin (van Wageningen et al., 1998) (**Fig. 7.8**), and for the variant heptapeptide scaffold of teicoplanin (Sosio et al., 2000) and A47934 (Pootoolal et al., 2002), a nonglycosylated version of teicoplanin from *Streptomyces toyocaensis*, have been sequenced. The details of biosynthetic logic will be covered in chapter 13 and the mechanisms of acquired resistance in VRE in chapter 10, where we note that the VRE phenotypes arise from a reprogramming of the enzymes that make the D-Ala-D-Ala terminus of the peptidoglycan pentapeptide precursor to the D-Ala-D-lactate terminus. This reduces affinity to vancomycin by 1,000-fold (Bugg et al., 1991) and creates the autoimmunity.

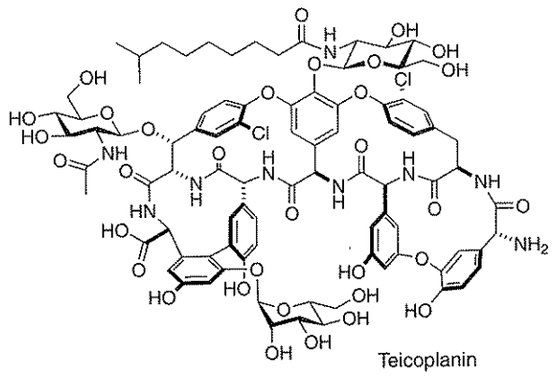
This dipeptide to depsipeptide transition is effected by three enzymes encoded by *vanH*, *vanA*, and *vanX* (see Walsh et al., 1996b). VanH, VanA, and VanX equivalents are found in tandem both in the *S. toyocaensis* producer of A47934 (Marshall et al., 1998) and in the teicoplanin producer, near the teicoplanin biosynthetic cluster (Sosio et al., 2000). The physiology of *S. toyocaensis* has been examined. Cultures are sensitive to A47934 during logarithmic growth when the antibiotic is not being made. During that phase of the life cycle the peptidoglycan chains have D-Ala-D-Ala produced by a normal D-Ala-D-Ala ligase. When the organism enters stationary phase and turns on the genes for antibiotic production it also presumably turns on *vanH*, *vanA*, and *vanX* to make D-Ala-D-lactate, by the A protein, known as ddlM in this organism (Marshall et al.,



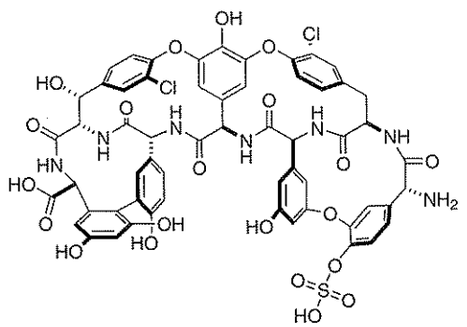
Vancomycin



Chloroeremomycin



Teicoplanin



A47934

Figure 7.8 Glycopeptide antibiotics.

1997), reprogramming the peptidoglycan layer to insensitivity to the antibiotic which is now being made (**Fig. 7.9**). So the switch occurs in a temporally coordinated manner.

Protective strategy in mitomycin producers

Streptomyces lavendulae produces the quinone-containing mitomycin C, which is an antitumor antibiotic by virtue of DNA covalent cross-linking at CpG sequences in both DNA strands after bioreduction (**Fig. 7.10A**) of the benzoquinone portion of the drug by electrons possibly from the respiratory chain of the bacteria. The producing streptomycetes contain a resistance gene, *mcrA*, that encodes a flavoprotein with a flavin adenine dinucleotide cofactor that participates in redox-mediated protection. Mitomycin C is a prodrug in its oxidized form at the completion of its biogenesis. It has to undergo one or two electron

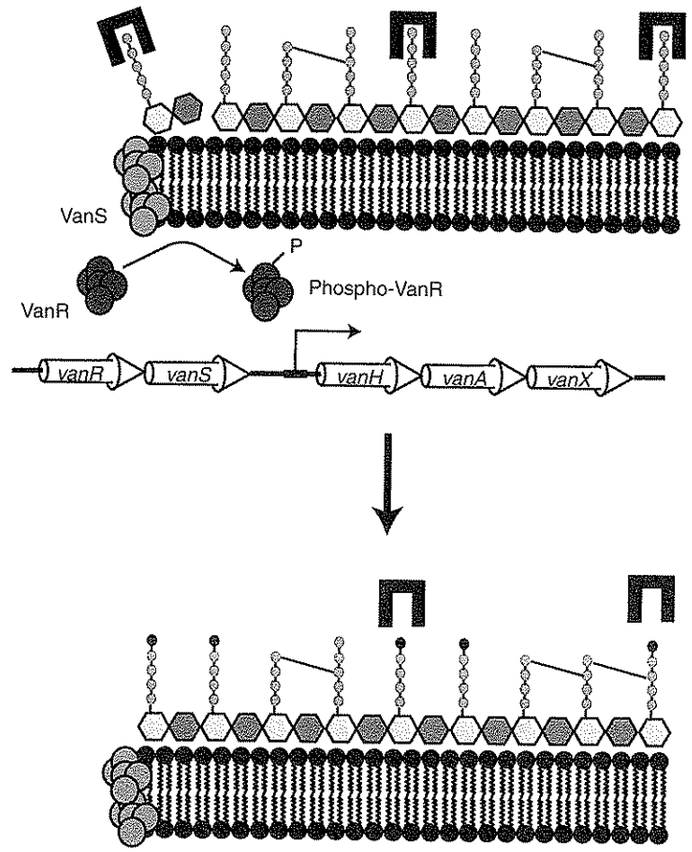


Figure 7.9 Regulation of the *vanH*, *vanA*, and *vanX* genes allows cell wall restructuring to glycopeptide antibiotic insensitivity at the time of antibiotic biosynthesis.

reductions and then it can rearrange by loss of methanol to a quinone methide which now can covalently capture DNA. It is proposed that the redox-active flavin adenine dinucleotide coenzyme of the McrA immunity protein reoxidizes the reduced form of mitomycin in competition with, and thereby blocking the rearrangement to uncover, the DNA-reactive functional group (**Fig. 7.10B**). This is the first example reported of a redox-mediated self-protection mechanism in an antibiotic producer (Sheldon et al., 1997) and emphasizes the amazing biochemical and physiological diversity that antibiotic producers will employ to provide self-immunity to their own chemical weapons.

Natural and acquired resistance in pathogenic bacteria

The examples in the four previous sections typify the kinds of acquired resistance mechanisms that have presumably been accumulated, some from the reservoir

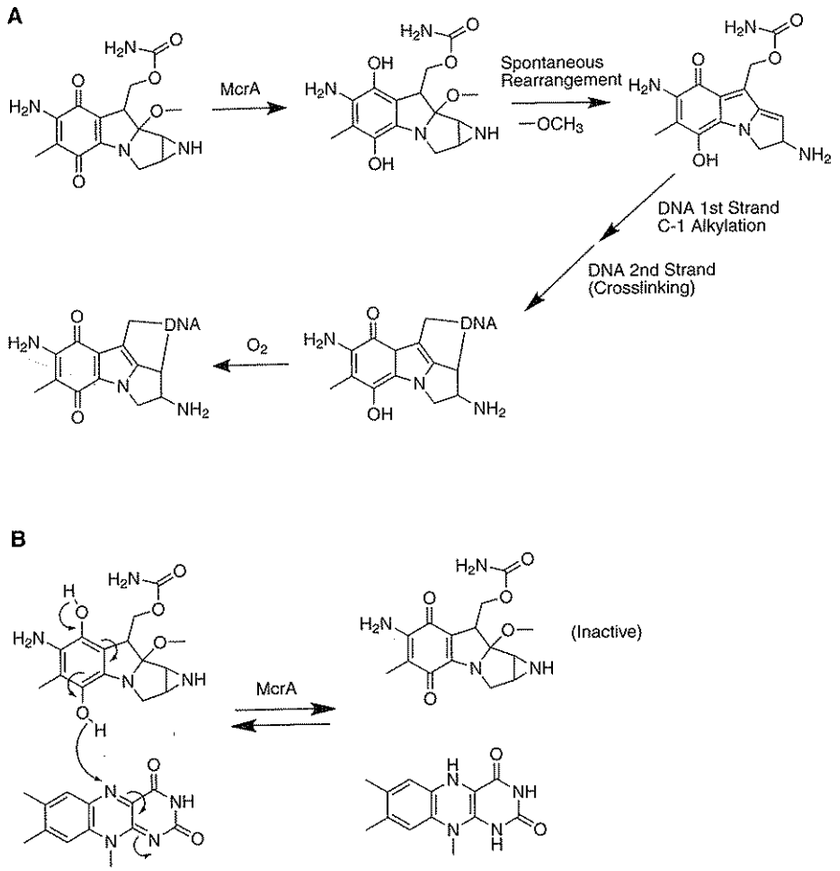


Figure 7.10 Mitomycin: (A) DNA cross-linking by bioreductive alkylation; (B) enzymatic reoxidation of dihydro mitomycin by McrA for self-protection.

of these genes in producer organisms and some from evolution of housekeeping enzymes to new specificities, by soil bacteria in the hundreds of millions of years that they have coevolved with antibiotic-producing neighbors.

The complete genomes of two major human bacterial pathogens, *Pseudomonas aeruginosa* and MRSA, reveal different strategies (Kuroda et al., 2001; Stover et al., 2000) for self-protection.

P. aeruginosa strains can be key culprits in life-threatening bacteremias in burn patients, causative agents in urinary tract infections in patients with urinary catheters, and causative agents in pneumonias in patients on respirators and in chronic lung infections of cystic fibrosis patients. But these pseudomonads are generally much less virulent than *S. aureus* and are often termed opportunistic pathogens because they establish dangerous infections largely in immunocompromised hosts. Two attributes in the first analysis of *P. aeruginosa* PA01's 5,570 open reading frames occasioned note (Stover et al., 2000). First were the large number of two-component regulatory systems—55 sensor kinases, 89 response regulator transcription factors, and 14 sensor/response regulator fusion proteins—allowing for flexibility in response to environmental inputs and integration of many extracellular inputs that may make *P. aeruginosa* a successful pathogen (Rodrigue et al., 2000). Second were the large number of outer membrane porins: 19 members of the OprD family, 34 genes of the TonB family, and 18 in the OprM gene family. Pseudomonads are intrinsically resistant to many or most antibiotics because they keep a low net intracellular concentration. The porin capacity is one index, and a correlated one is the efflux pumps (described in detail in chapter 9), with 10 of the Mex subfamily and 20 of the Bmr subfamily. These pumps can confer protection against foreign compounds, ensuring that the efflux/influx ratio is far on the side of efflux.

The highly virulent MRSA and VRSA (vancomycin-resistant *S. aureus*) strains of *S. aureus* are more virulent human pathogens in disease and have been termed professional pathogens in contrast to the opportunistic ones noted in the preceding paragraph. The genomes of the N315 MRSA strain and the Mu50 VRSA strain (Kuroda et al., 2001) reveal 3 or 4 large gene clusters in pathogenicity islands and 26 to 28 gene clusters on mobile genetic elements. Both types of clusters reflect mechanisms for *S. aureus* to pick up genes from elsewhere and bring them on board for virulence and pathogenicity functions. All told, transposons and insertion sequences make up 7% of these *S. aureus* genomes, while they are almost totally absent from another gram-positive bacterium, *Bacillus subtilis*. Almost 70 novel open reading frames implicated in virulence were found by Kuroda et al. (2001). In terms of specific gene pickups that lead to antibiotic resistance, **Table 7.2** (based on data of Kuroda et al., 2001) notes nine genes that confer antibiotic resistance in the MRSA and VRSA strains, including both *blaZ* and *mecA* for β-lactam resistance (chapters 8 and 10), *ermA* for erythromycin resistance (chapter 10), *ant4′*, *ant9′*, and *aacA-aphD* for aminoglycoside modification (chapter 8), and *tetM* and *qacA* for drug efflux pumps (chapter 9). A pathogenicity island termed SSC_{mec} contains the *bleO, blaZ, mecA, ermA*, and *ant4′* genes and is obviously a genetic determinant that contributes to the prowess of MRSA strains as professional pathogens (Hiramatsu et al., 2001).

Susceptibility of bacteria to antimicrobial drugs has classically been tested with phenotypic assays that evaluate the ability of an antibiotic to inhibit bacterial growth under some specified set of growth conditions. Various formats can be used, including disk diffusion, agar dilution, and broth dilution assays.

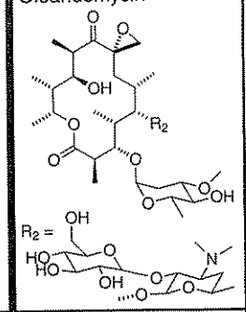
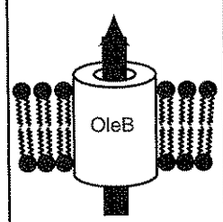
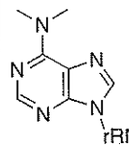
Table 7.2 Antibiotic resistance genes found in MRSA

Protein	Gene	Antibiotic resistance
Bleomycin resistance protein	*bleO*	Bleomycin
PBP2′	*mecA*	β-Lactams
β-Lactamase	*blaZ*	β-Lactams
rRNA methylase	*ermA*	Erythromycin, pristinamycins
O-NucleotidylTransferases	*ant 4′, ant 9′*	Aminoglycosides
Acetylase-phosphotransferase	*aacA-aphD*	Aminoglycosides
TetM efflux protein	*tetM*	Tetracyclines
QacA	*qacA*	Antiseptics

For example, disks impregnated with a colorless cephalosporin substrate, nitrocefem, can be used to test for β-lactamase-producing bacteria. Ring opening of the lactam ring of nitrocefem leads to elimination of an electron-rich nitroaromatic substituent that is yellow, so the assay scores development of color. More recently, methods that directly evaluate antibiotic resistance genotype rather than phenotype susceptibility have come into common use (reviewed by Cockerill, 1999), especially when prevalent mutations causing resistance are known and can be readily screened for genetically. For example, the presence of the *mecA* gene in staphylococcal clinical isolates is readily detected by PCR of a region of *mecA*, followed by gel electrophoresis analysis. The presence of the *mecA* gene is readily detected on ethidium bromide staining of the gel (Cockerill, 1999). Analogously, the acquisition of genes of the TEM β-lactamase family, conferring clinical resistance to ceftazidime, cefotaxime, and aztreonam (see chapters 3 and 8) in *Klebsiella pneumoniae* and *E. coli* isolates, is readily detected by PCR and restriction fragment length polymorphism analysis (Cockerill, 1999). Rifampin resistance in *Mycobacterium tuberculosis* is detected by PCR analysis of mutations in the *rpoB* gene encoding the β subunit of RNA polymerase while fluoroquinolone resistance is detected by PCR analysis and DNA sequencing of fragments of the *gyrA* gene encoding the GyrA subunit of DNA gyrase, accounting for effectively all clinical quinolone resistance (Cockerill, 1999).

In summary, the three methods of self-protection in macrolide antibiotic producers noted in **Fig. 7.11** set the stage for the understanding of the three major strategies for bacterial resistance to antibiotics that we will explore in chapters 8 to 10. The first is exemplified by temporary inactivation of the intracellular form of the antibiotic in oleandomycin producers. The cognate in resistant pathogens can be characterized as enzymatic destruction of the antibiotic, as is the case for β-lactam hydrolysis, aminoglycoside modification, and fosfo-

Figure 7.11 Analogy between producer self-protection and bacterial resistance.

Method of Producer Self-Protection	Temporary Intracellular Inactivation of Antibiotic	Efflux of Produced Antibiotic	Modification of Target in Producer
Example of Macrolide Producer Self-Protection	Glycosylation of Oleandomycin 	Export of Oleandomycin by OleB 	Dimethylation of Adenine in 23S rRNA  23S rRNA:m_2A2085
Analogous Mode of Clinically Observed Bacterial Resistance	Inactivation of Antibiotic	Efflux of Antibiotic	Modification of Target

mycin capture by glutathione (chapter 8). The efflux of intracellular antibiotics practiced by producers, and shown by the action of the OleB pump, presages a general strategy to lower the ambient concentration of antibiotics in the cells of the pathogens. This can be a combination of lowered rates of influx, operating mostly in gram-negative bacteria through the outer membrane permeability barrier, coupled to accelerated rates of efflux. The efflux pump proteins can have narrow specificity, e.g., TetB protein for tetracycline, or have broad selectivity, as with the multidrug efflux pump proteins (chapter 9). The third mechanism is for the pathogen to alter the target protein such that it still retains its physiologic function but now has a lower affinity for antibiotic binding (chapter 10). In some erythromycin producers this strategy is enacted by A_{2058} *N,N*-dimethylation in 23S rRNA to make a resistant 50S ribosome subunit. The alteration of the target in pathogens can be mutations in or replacement of the wild-type protein, as in penicillin-binding proteins, or an alteration in a substrate for a key enzyme, as in the D-Ala-D-lactate reprogramming.