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Plasmid-Determined Resistance to Antimicrobial Drugs and Toxic Metal Ions in Bacteria

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INTRODUCTION

Most clinically significant antibiotic resistance is determined by genes located on extrachromosomal DNA elements called plasmids (31, 108, 154). Different species of bacteria harbor characteristic types of plasmids, some of which can mediate their own transfer by conjugation. Also, resistance genes are often incorporated into discrete genetic units called transposons (204), which have the capacity to transpose from one DNA molecule to another. This has undoubtedly contributed to the rapid dissemination of antibiotic resistance by providing an efficient mechanism for incorporating resistance determinants into new vectors which can transfer to and stably replicate in diverse hosts.

The major objectives of this review are to discuss (i) the biochemical mechanisms of plasmid-specified resistance to drugs and toxic metal ions, (ii) the regulatory mechanisms controlling expression of resistance genes, (iii) comparative studies on resistance determinants, and (iv) the evolutionary origins of resistance genes.

One might ask the questions, why concentrate on plasmid-specified resistance and why consider resistance to drugs and metal ions? As mentioned above, resistance determined by plasmids is most frequently responsible for resistance in clinical situations. Resistance conferred by mutations in chromosomal genes occurs by different biochemical mechanisms and is less commonly encountered in nature. However, important examples of mutational changes have been recognized and these are discussed. The distinction between plasmid-encoded and chromosomally encoded resistance can become blurred because transposons can integrate into the chromosome and have been found in this location in clinical isolates. However, these two types of chromosomally specified resistance can be readily distinguished by biochemical and genetic tests. It is instructive to compare the biochemical mechanisms of resistance to metal ions and drugs since most metal resistance is plasmid determined and has presumably evolved in response to similar selective pressures. Knowledge of metal ion resistance may prove helpful in understanding antibiotic resistance mechanisms.

There are four classic mechanisms of resistance specified by plasmids: (i) inactivation, (ii) impermeability, (iii) bypass, and (iv) altered target site (93). These form the major headings for this review. Recent work allows more precise definitions of some mechanisms and suggests that additional mechanisms might exist. For example, resistance previously described as being due to decreased permeability has been shown to be caused by specific efflux systems. Also, intracellular binding now seems to be a valid mechanism for immobilizing an inhibitor.

The advent of rapid DNA sequencing techniques means that detailed comparisons of resistance genes from different sources can be made to provide insights into their origins and evolution. The notion that R-plasmid-specified antibiotic resistance may have evolved as self-protection mechanisms in antibiotic-producing microorganisms could be strengthened by this approach.

INACTIVATION MECHANISMS

Resistance to β-Lactam Antibiotics

β-Lactam antibiotics inhibit enzymes involved in cell wall biosynthesis and cell division (for review, see reference 317). The proteins to which the drugs bind (penicillin-binding proteins [PBPs]) are integral components of the cytoplasmic membrane of bacteria. Gram-negative bacteria are intrinsically resistant to many β-lactam drugs. This is partly due to the inability of the drugs to diffuse easily through pores in the outer membrane (58, 317) and partly to low levels of chromosomally encoded β-lactamase (81, 284).

Acquired resistance to β-lactam drugs in clinical isolates may be due to β-lactamase activity specified by plasmids or to mutational changes in chromosomal loci. These mutations alter the affinity or amount of PBPs or (in the case of gram-negative organisms) increase the impermeability of the outer membrane. Both types of mutation can be selected in the laboratory (135, 153, 208). Mutations altering PBPs are responsible for penicillin resistance in *Neisseria gonorrhoeae* (100) and *Streptococcus pneumoniae* (145) and for methicillin (155, 158) and cephradine (134) resistance in *Staphylococcus aureus*, and they can be a mechanism for resistance to piperacillin and carbenicillin in *Pseudomonas aeruginosa* (80, 135, 136, 316, 269). In some strains a single-step mutation may be responsible for resistance, whereas in others resistance may be due to several mutations (145, 441). In some isolates of *P. aeruginosa* carbenicillin resistance is due to a combination of reduced sensitivity of PBPs and increased outer membrane impermeability (269, 316).

Another proposed mechanism of resistance to β-lactamase-insensitive cephalosporins is the apparent ability of the periplasmic enzyme to bind to drug and prevent it from reaching its target (384). Also, the phenomenon of tolerance to penicillins in *S. aureus* appears to be due to mutations that reduce autolytic activity (411).

It is conceivable that the resistance mechanisms described above will become more prevalent after the introduction of β-lactamase inhibitors and β-lactamase-insensitive drugs into clinical practice.

Plasmid-determined β-lactamases in gram-negative bacteria. High-level resistance to broad-spectrum β-lactam antibiotics in gram-negative bacteria is usually due to β-lactamase activity specified by plasmids. In addition, most, if not all, gram-negative organisms express low levels of chromosomally encoded β-lactamase which may contribute to intrinsic resistance (313, 376).

The question of β-lactamase nomenclature should be mentioned at this point. β-Lactamases have been classified into three groups (A, B, and C) on the basis of evolutionary and mechanistic considerations (8). Class A includes the *S. aureus* penicillinases and the TEM β-lactamase specified by plasmids in gram-negative bacteria. The chromosomally encoded cephalosporinases of gram-negative bacteria are class C enzymes. Another classification system based on biochemical criteria places β-lactamases into five groups (I to V), with the chromosomal (class C) cephalosporinases in group I and the plasmid-specified penicillinases in groups III and V (313). Some authors have preferred to name plasmid-specified enzymes of gram-negative bacteria according to the bacterial host in which the enzyme was first isolated or after the β-lactam drug which they best hydrolyze. In the absence of an agreed convention, this scheme is used here for ease of reference to published work.

A number of plasmid-specified β-lactamases in gram-negative bacteria have been distinguished by isoelectric focusing and inhibitor studies and by substrate specificity (Table 1). All are expressed constitutively and are located in the periplasmic space. Most are predominantly penicillinases. These may have low activity against cephalosporins such as cephaloridine and cephalothin. Other drugs such as cephalexin and cefuroxime are hydrolyzed poorly, if at all, by most enzymes (Table 1).

The TEM enzyme was the first R-plasmid-specified β-lactamase to be recognized in gram-negative bacteria (90). The enzyme was named after the RTEM plasmid. There are two subtypes called TEM-1 and TEM-2, which can be distinguished by isoelectric focusing. They differ in sequence by a single amino acid (9). These

TABLE 1. Properties of β-lactamases of gram-positive and gram-negative bacteria[a]

Type of β-lactamase	Relative rates of hydrolysis[b]						Inhibition by[c]:			pI	Mol wt (10^3)	Reference(s)
	Pen	Amp	Carb	Oxa	Meth	Cep	Clox	pCMB	NaCl			
TEM-1	100	106	10	5	0	76	S	R	R	5.4	28.5[d]	90 160
TEM-2	100	107	10	5	0	74	S	R	R	5.6	28.5[d]	
SHV-1	100	212	8	0	0	56	S	S/R	R	7.6	17.0	251
HMS-1	100	253	14	0	0	183	S	S	R	5.2	21.0	251
ROB	100	186	25	6		24				8.1		323
OXA-1	100	382	30	197	332	30	R	PS	S	7.4	23.3	83, 160
OXA-2	100	179	15	646	23	37	R	R	S	7.45/7.7	44.6	83, 160
OXA-3	100	178	10	336	29	44	R	R	S	7.1	41.2	83, 160
OXA-4(PSE-2)	100	267	121	317	803	32	R	S	S	6.1	12.4	248
CARB-1(PSE-4)	100	88	150	8	16	40		R		5.3	32.0	162
CARB-2(PSE-1)	100	90	97	0	0	18	R	S		5.7	28.5	248
CARB-4(PSE-3)	100	101	253			10		R		6.9	12.0	326
CEP-1	100	5		0		325		R	R	8.1	31.8[d]	27
CEP-2	100	low	48			108[e]	R	R		8.1	36.2	231
S. aureus A, B	100	185		4.5	1.5	10					28.8[d]	102, 307
S. aureus C	100			1	0.6							102, 307

[a] Abbreviations: Pen, Benzyl penicillin; Amp, ampicillin; Carb, carbenicillin; Oxa, oxacillin; Meth, methicillin; Clox, cloxacillin; Cep, cephaloridine; pCMB, *p*-chloromercuribenzoate.

[b] The rates of hydrolysis are expressed as a function of the benzyl penicillin rate. The data were selected from Matthew (248), Richmond (307), and Dyke (102) primarily to highlight comparatively the important features of the enzymes.

[c] S, Sensitive; R, resistant; PS, partially sensitive.

[d] Molecular weight deduced from DNA sequence of the gene (189, 371) or amino acid sequence or both of the polypeptide (7, 9). The molecular weight given for CEP-1 is that of the *ampC* β-lactamase of *E. coli*.

[e] Also hydrolyzes cephalothin, cefamandole, and cefazolin at rates similar to that of cephaloridine.

enzymes are widely distributed in nature in terms of geographical distribution, association with plasmids of different incompatibility groups, and occurrence in different bacterial species (249). Undoubtedly, this widespread occurrence is the result of the TEM β-lactamase gene being carried by transposons (161, 209).

The SHV-1 (sulfhydryl variable) β-lactamase has a substrate profile similar to that of TEM enzymes but differs by hydrolyzing ampicillin at a greater rate (249, 251). It has the distinctive property of variable inhibition by *p*-chloromercuribenzoate depending on the substrate. Thus, hydrolysis of benzyl penicillin is not affected by *p*-chloromercuribenzoate, whereas the reaction with cephaloridine is inhibited.

β-Lactamase HMS-1 is very similar to SHV-1 in substrate profile, with the exception that it hydrolyzes cephaloridine at a threefold greater rate. Also, it is uniformly sensitive to *p*-chloromercuribenzoate. It is a rare enzyme, having been found in a single strain of *Proteus mirabilis* (249, 251).

Another enzyme (ROB) with a broad TEM-like substrate specificity has been isolated from *Haemophilus influenzae* type b (323). It differs from TEM in having a higher pI and a faster rate of ampicillin hydrolysis.

The OXA group of β-lactamases has the distinctive property of hydrolyzing isoxazoyl penicillins (including oxacillin) and methicillin. Three different OXA enzymes were originally described (83). In addition, the enzyme classified as PSE-2 because it was discovered in *P. aeruginosa* (see below) has been renamed OXA-4 (A. M. Philippon, G. Paul, and G. A. Jacoby, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 21st, Chicago, Ill., abstr. no. 680, 1981). OXA-1 and OXA-4 hydrolyze methicillin at much greater rates than do OXA-2 and OXA-3. The OXA-1, -2, and -3 enzymes are specified by plasmids of three, four, and two different incompatibility groups, respectively (249). In addition, OXA-1 and OXA-3 have been encountered in *P. aeruginosa* (185; A. Philippon, G. Paul, and G. A. Jacoby, 13th Int. Conf. Microbiol., abstr. no. P42:1, 1982). Also, PSE-2/OXA-4 has been found in several species of enteric bacteria (D. M. Livermore and J. D. Williams, personal communication). The OXA-1 determinant has been shown to be transposable (435).

Several plasmid-specified β-lactamases hydrolyze carbenicillin at a greater rate than does penicillin. They were originally discovered in *P. aeruginosa* and are called pseudomonas-specific

enzymes (PSE; 185). These enzymes have also been named after the drug which they best hydrolyze (211; A. Philippon and M. Matthew, personal communications). Thus, PSE-2 is called OXA-4 because it attacks isoxazoyl penicillins best (see above). The PSE-4/CARB-1 enzyme is indistinguishable from the classic chromosomal Dalgleish enzyme (126, 162). It is commonly specified by a transposon located in the *P. aeruginosa* chromosome (349). The type strain for plasmid-specified PSE-4/CARB-1 (PU21, pMG19) actually carries the β-lactamase gene on a similar chromosomal transposon (G. R. Jacoby, L. R. Knobel, and L. Sutton, 13th Int. Conf. Microbiol., abstr. no. P21:9, 1982). Indeed, it is possible that the original Dalgleish strain carries its PSE-4/CARB-1 determinant on such an element.

The PSE-1/CARB-2 enzyme was originally a determinant of the narrow-host-range IncP2 plasmids of *P. aeruginosa* (252), but has recently been reported in enteric bacteria and is linked to transposons (196, 259). CARB-4/PSE-3 is rare, being determined by a single plasmid in *P. aeruginosa* (249, 326). Another carbenicillin-hydrolyzing enzyme, CARB-3 (211), does not seem to be plasmid specified and is not listed in Table 1.

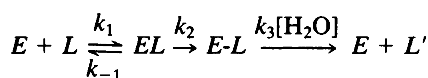
There have been two reports of plasmid-specified β-lactamases which predominantly hydrolyze cephalosporins. The CEP-1 determinant was transferred from *Proteus mirabilis* into *Escherichia coli*, where it specified a β-lactamase with properties indistinguishable from those of the *E. coli* chromosomal *ampC* enzyme (27). It has not been demonstrated unequivocally that the CEP-1 structural gene resides on the plasmid (27; M. Matthew, personal communication). It is possible that the plasmid encodes a regulatory function which stimulates expression of *ampC*. A true plasmid-encoded cephalosporin-hydrolyzing enzyme was recently discovered in a strain of *Achromobacter* sp. (230, 231).

In addition to the enzymes described above and in Table 1, five new β-lactamases have recently been found in *E. coli* and *P. aeruginosa*. One has broad TEM-like substrate specificity, whereas the others are OXA-like but have pI values different from those of OXA-1 to -4 (A. A. Medeiros and G. A. Jacoby, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 716, 1982).

The relationships between the plasmid-specified β-lactamases have yet to be established. Immunological studies have provided some information. The CARB/PSE and OXA enzymes did not cross-react with anti-TEM serum (377). There have been conflicting reports of cross-reaction between TEM-1 and SHV-1 (377; G. Paul, A. Philippon, M. Bartelemy, R. Labia, and P. Nevet, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 21st, abstr. no. 681, 1981). The evolutionary relationships between β-lactamases could be profitably studied by DNA hybridization and sequencing studies.

Plasmid-specified β-lactamases in gram-positive bacteria. Penicillin resistance in *S. aureus* is determined by β-lactamases which are usually plasmid encoded. There are four minor variants of staphylococcal penicillinase (types A to D), three of which (types A, B, and C) have been studied in some detail (307). The enzymes are predominantly penicillinases, hydrolyzing benzyl penicillin and ampicillin at high rates. They hydrolyze methicillin, oxacillin, and cloxacillin poorly. With the exception of the type D enzyme (320), they are expressed at high levels only after induction, with most of the activity being extracellular. It is assumed that the four penicillinases are closely related, with a small number of differences in amino acid residues being sufficient to cause the small but significant differences in hydrolysis rates (307).

Mechanism of hydrolysis of β-lactams. Recently the serine-70 residue has been shown to be the active-site nucleophile of the class A *Bacillus cereus* and TEM β-lactamases (67, 111, 112). It is now clear that the class C β-lactamases are also serine enzymes (203), although they are otherwise unrelated to class A enzymes (189). The interaction between β-lactam drugs and β-lactamases is kinetically complex and may be represented, in the simplest case, as



$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL \xrightarrow{k_2} E\text{-}L \xrightarrow{k_3[H_2O]} E + L'$$

where EL is a noncovalent complex between the enzyme (E) and the β-lactam (L) which may either dissociate (k_{-1}) or break down (k_2) to form a covalent intermediate, the acyl enzyme E-L. The acyl enzyme may hydrolyze (k_3) to yield free enzyme and the acid derivative of the β-lactam (L') (Fig. 1). It appears that the rate of hydrolysis of the acyl enzyme determines whether compounds are degraded efficiently or behave as β-lactamase inhibitors (113). The acyl enzyme may also undergo β-elimination to yield enzyme-bound chromophores (113).

Factors affecting expression of β-lactam antibiotic resistance in gram-positive and gram-negative bacteria. The gram-positive bacterium *S. aureus* secretes copious amounts of β-lactamase into the surrounding medium. The enzyme must inactivate the drug before it penetrates the cell wall and reaches the cytoplasmic membrane-

located PBP targets. There is a pronounced "inoculum effect" on the level of resistance that can be attained in *S. aureus*. A single cell is virtually defenseless against even low concentrations of the drug (102). However, the resistance level increases by as much as 10,000-fold when larger inocula are used (102).

The kinetic properties of the β-lactamase also influence the level of resistance (150). For example, the *S. aureus* β-lactamase has a very low affinity for methicillin (278). In contrast, the affinity for cephalothin is very high but the rate of hydrolysis is low (149). In both cases the rate of hydrolysis is so slow that the drug is essentially stable and can be used at low concentrations to kill penicillin-resistant staphylococci. The amount of enzyme synthesized will also be an important determinant of resistance.

Plasmid-specified β-lactamases of gram-negative bacteria are expressed constitutively and are located in the periplasmic space between the

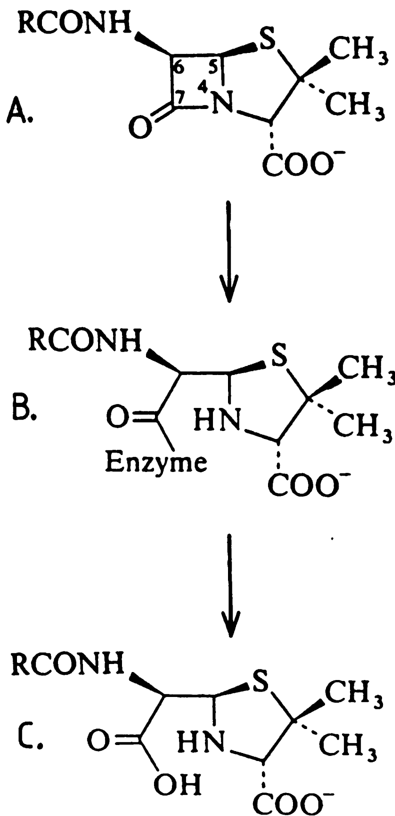


FIG. 1. Inactivation of penicillin antibiotics by β-lactamase. The penicillin molecule (A) forms an acyl enzyme intermediate (B) with a serine residue in the active center of the β-lactamase. The hydrolyzed penicilloic acid derivative (C) is released.

cytoplasmic and outer membranes. Because of this strategic location the cells are protected by smaller amounts of an enzyme which generally has a low affinity for its substrate compared with the gram-positive enzymes (150, 310). There is no marked inoculum effect if the β-lactamase can cope with the drug as it passes into the periplasm, and single cells will be able to grow in quite high concentrations of drug. In contrast, if the enzyme cannot inactivate the drug quickly enough, an inoculum effect may occur. Some of the cells in a large population will be killed by the drug and will release their β-lactamase into the medium. This will hydrolyze the drug and allow some of the surviving cells to grow.

The level of resistance to β-lactam antibiotics in gram-negative bacteria is determined by the interplay of three factors: (i) the amount of enzyme, (ii) kinetic properties of the enzyme (i.e., rate of hydrolysis), and (iii) the ability of the drug to penetrate the outer membrane.

(i) Amount of enzyme. In *E. coli* there is a direct relationship among the number of copies of the TEM β-lactamase gene, the amount of enzyme synthesized, and the resistance level expressed (a gene-dosage effect) (399). Also, TEM β-lactamase genes located on plasmids of different incompatibility groups which are indigenous to one or a few bacterial species often express different levels of enzyme when introduced into the same strain of *E. coli*. This may be due to different rates of transcription of the gene (436). Perhaps mutations have occurred in TEM promoters to adapt them to transcription in different species of gram-negative bacteria, resulting in a variation in transcription by *E. coli* RNA polymerase. Poor transcription may also explain the observation that plasmid RP1 expresses 1/50 the level of β-lactamase in *Proteus mirabilis* compared with *E. coli* (313).

The level of β-lactamase will also be dependent on factors that influence plasmid copy number and thus the number of gene copies per cell. The copy number of plasmid R1 is higher at slower growth rates, with a corresponding gene-dose effect for TEM β-lactamase (105). Differences in plasmid copy number may also explain the increased synthesis of β-lactamase by anaerobically grown *E. coli* (304).

(ii) Ability of the drug to cross the outer membrane. The intrinsic resistance of plasmid-free cells is partly determined by the barrier to drug penetration posed by the outer membrane (58, 71, 310, 311). The ability of β-lactam drugs to diffuse through outer membrane pores is dependent on their charge and hydrophilic properties (58). Also, the expression of resistance in β-lactamase-producing cells is in part controlled by this surface layer. For example, *E. coli* cells

synthesizing TEM β-lactamase are quite sensitive to cephaloridine despite the fact that the purified enzyme hydrolyzes the drug at almost the same rate as ampicillin (Table 1) (313), to which high-level resistance is expressed. Cephaloridine is thought to penetrate the outer membrane very rapidly and swamps the β-lactamase (313). Different rates of drug penetration are also responsible for the cryptic nature of β-lactamase activity towards some drugs in gram-negative organisms. The specific activities of β-lactamases are often higher in broken cell extracts than in whole cells (313). Also, the outer membranes of different gram-negative bacteria vary in their ability to exclude β-lactams. Extreme examples are the highly impervious *P. aeruginosa* (311) and the relatively porous *H. influenzae* membranes.

(iii) Kinetic properties of β-lactamase. Just as for staphylococcal β-lactamases discussed above, the affinity of the enzyme for substrate and the rate of reaction are important determinants of resistance (150).

Do some plasmids determine a barrier to the penetration of β-lactam antibiotics? The suggestion that some β-lactamase-specifying plasmids also determine a barrier that hinders the diffusion of drugs across the outer layers of the cell was proposed to explain the expression of high-level carbenicillin resistance in strains with low or undetectable enzyme activity (82, 437). Recently it was shown that the resistance expressed by plasmids with defective β-lactamases was entirely attributable to residual β-lactamase activity of the mutant proteins (76, 77).

Regulation of β-lactamase expression. *(i) Gram-negative bacteria.* All plasmid-specified β-lactamases of gram-negative bacteria are expressed constitutively. Factors that can indirectly affect the level of β-lactamase expression and hence the resistance level of the cell are plasmid copy number, which may be higher at slower growth rates (105), and anaerobic growth (304).

In contrast, the chromosomally encoded β-lactamase of *P. aeruginosa* is expressed inducibly (81, 284, 313), although the regulatory mechanism involved is not understood. The transcription of the *E. coli* chromosomal *ampC* gene is subject to growth rate-dependent regulation by an attenuation mechanism (190).

(ii) Gram-positive bacteria. The type A, B, and C penicillinases specified by *S. aureus* plasmids are expressed inducibly (102, 307, 308), whereas the type D enzyme is constitutive (320). Inducible penicillinase synthesis is controlled negatively by the product of the *penI* gene (308). *Trans*-recessive *penI* mutations caused constitutive expression of penicillinase (308). Also, penicillinase synthesis can be derepressed by incorporation of the amino acid analog 5-methyltryptophan into repressor protein (180). The operator binding function of the repressor is probably inactivated.

Mutants which expressed low basal levels of wild-type penicillinase either partly or noninducibly formed an active repressor in diploid cells carrying a compatible *penI* mutant plasmid (309). This led to the suggestion of a second plasmid-located regulatory gene. However, a more acceptable explanation is that the noninducible phenotype is caused by a $penI^s$ (super-repressor) mutation. In diploid cells carrying the putative $penI^s$ mutant along with a $penI^-$ element, mixing of mutant repressor subunits could occur to form an active molecule by a form of intragenic complementation. Also, the putative $penI^s$ mutants were inducible by 5-methyltryptophan, a finding that is difficult to reconcile with a positive regulator model (183). Another class of mutant that expressed very low constitutive levels of penicillinase has two mutations, a $penI^s$ lesion along with a structural gene mutation (183). The simplest model for penicillinase regulation involves a repressor protein binding to an operator site to regulate transcription of the structural gene. The putative operator site has been identified as a 22-base pair (bp) inverted repeat sequence which overlaps the structural gene promoter (254).

Another factor in the regulation of penicillinase in *S. aureus* is a chromosomal locus which appears to affect the level of penicillinase synthesis (180, 181). Mutations in this gene caused a *meso*-inducible phenotype (180, 181). It is possible that the host specifies an antirepressor which complexes the plasmid repressor in the presence of inducer (181).

Resistance to Chloramphenicol

Chloramphenicol (Cm) diffuses passively into the cytoplasm of bacterial cells, where it interacts with the 50S ribosome subunit to inhibit the peptidyltransferase step in translation (130). Resistance to Cm is usually due to inactivation of the drug by acetylation (335, 336, 374). The enzyme responsible is Cm acetyltransferase (CAT), which is usually plasmid encoded in clinical isolates. The properties of CAT have recently been comprehensively reviewed (337). It should be noted that plasmid-determined Cm resistance in gram-negative bacteria is occasionally caused by another mechanism which does not involve inactivation (99, 128, 271).

CATs in gram-negative bacteria. CATs have been found in many different bacterial genera (337). The enzymes are tetramers of identical

subunits with apparent molecular weights in the range of 23,000 to 25,000. The plasmid-encoded enzymes in gram-negative bacteria are expressed constitutively, whereas those of gram-positive organisms are inducible. The CAT enzyme variants have been classified on the basis of electrophoretic mobility, kinetics, inhibitor susceptibility, and reactivity to antisera (114, 337).

There are three types of plasmid-encoded CATs in enteric bacteria (129). The ubiquitous type I enzyme has been studied in most detail. Its primary amino acid sequence has been compared with the DNA sequence of the CAT genes (6, 244, 342). The type I CAT has the unusual property of binding strongly to fusidic acid (406) and rosaniline dyes (302). The partial amino acid sequences of the type II and III enzymes are also known (275, 440). The chromosomal enzyme of *Proteus mirabilis* is related to the type I variant (440). CAT has also been found in nonenteric species of gram-negative bacteria. The plasmid-specified enzyme of *H. influenzae* is related to type II CAT(314), as is the enzyme of *Bacteroides fragilis* (30, 339), whereas the *Bacteroides ochraceus* enzyme is similar to type I (337). Chromosomally determined enzymes have also been described in *Agrobacterium* sp. (an inducible enzyme; 143, 440), *Flavobacterium* sp. (440), and *Myxococcus* sp. (440).

CATs in gram-positive bacteria. Five CAT variants have been observed in staphylococci. These include the prototype A to D enzymes (93, 324, 336–338) and the newly described CAT of pC194 (174). The amino acid sequence of the pC194 CAT was inferred from the DNA sequence (174). Amino-terminal sequences of the A, B, and D variants and a more extended partial sequence of the type C enzyme have also been determined (115).

The *Streptococcus agalactiae* (440), *S. faecalis* (74), and *S. pneumoniae* (86) CATs are related to staphylococcal enzymes. The *S. pneumoniae* determinant is chromosomal, whereas the others are plasmid located. *Clostridium perfringens* (440), *Bacillus pumilus* (201), and *Streptomyces* sp. (341) also specify CAT activity.

Mechanism of Cm inactivation. Early studies established that Cm inactivation resulted from the formation of 3-acetoxy-Cm, followed by the slower appearance of 1,3-diacetoxy-Cm (Fig. 2) (335, 374). The 3-acetoxy group is transferred non-enzymatically by an intramolecular rearrangement to yield 1-acetoxy-Cm, which in turn is acetylated to give the diacetoxy form (Fig. 2). All of the acetoxy derivatives of Cm are inactive as antibiotics because they cannot bind to the ribosome (343). Thus, resistant cells need only to acetylate Cm once to inactivate it, but are obliged to divert a second molecule of acetyl coenzyme A (CoA) into another acetylation reaction. This may not present a problem to wild-type cells growing in low concentrations of drug. However, the lack of unlimited supplies of acetyl CoA is likely to be responsible for the nonlinear relationship between the number of enzyme molecules (generated by amplifying the *cat* gene) and the Cm resistance level (277).

Catalytic properties of CAT. The substrate specificity of the type I and C CAT variants and peptidyltransferase (the ribosomal target for Cm) have been compared (337). All share the absolute specificity for the D,threo isomer of Cm and a requirement for a substitution of the C-2 amino group and for absence of substitution of the C-1 and C-3 protons. CAT is more tolerant than peptidyltransferase of C-2 amino group substitutions or *para*-substitutions of the phenyl group. Fluoro substitutions of the 3-hydroxyl group of Cm results in a drug with good antimicrobial activity which cannot be inactivated by CAT (270). Indeed, fluoro-Cm is a competitive inhibitor of CAT, forming a nonproductive complex with the enzyme and the acetyl donor.

The critical first acetylation step couples the breaking of a high-energy thiol ester bond with formation of an *O*-acyl derivative of Cm. Circumstantial evidence originally suggested a direct role for a thiol group in the active center of gram-negative CAT variants, but not for those of gram-positive bacteria (129, 439, 440). The sensitivity to thiol inhibitors of the enteric CAT enzymes is now thought to be due to a cysteine residue located near the active site, which does not participate directly in the catalytic reaction (337). Thus, kinetic studies of the type I and C enzymes favor the formation of a ternary complex whereby CAT binds both acetyl CoA and Cm together at the active site rather than a sequential mechanism involving an acyl enzyme intermediate with an active-site cysteine. The three enteric CAT variants have reactive cysteine residues at an equivalent position, whereas the staphylococcal enzymes do not and are thus resistant to thiol-directed inhibitors (276, 277).

A detailed model of the active center of CAT has been proposed and the catalytic mechanism has been discussed (337). Of central importance is a highly reactive histidine residue (His-193 of type I CAT) whose reactivity to alkylating and modifying reagents is protected by Cm. This histidine is present at an equivalent position in all CAT variants studied (337).

Type I CAT confers resistance to fusidic acid. Gram-negative bacteria are intrinsically resistant to fusidic acid because this steroid antibiotic cannot penetrate the outer membrane. Fusidic acid resistance specified by R plasmids of enter-

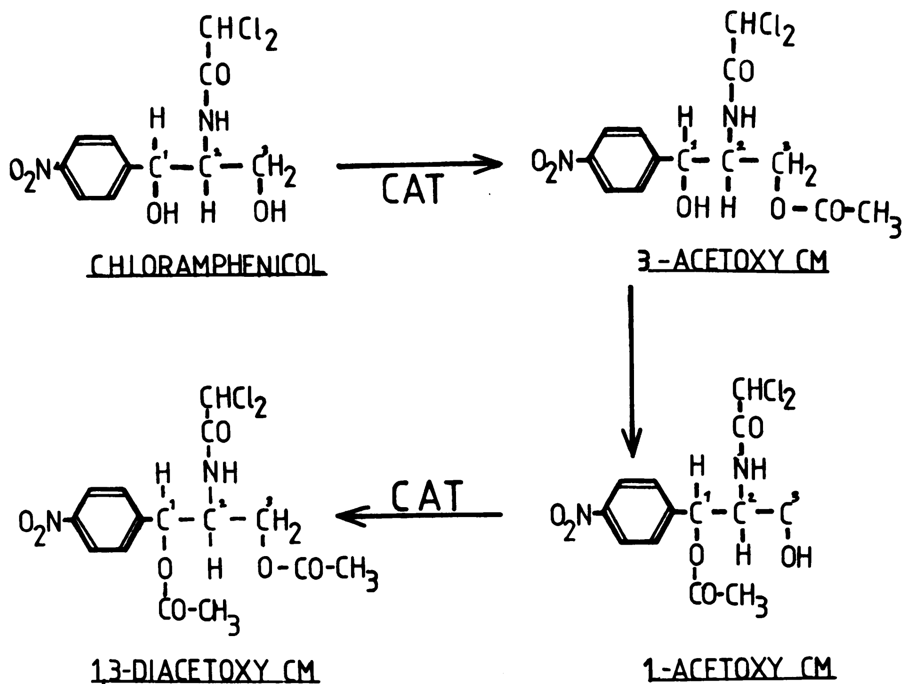


FIG. 2. Inactivation of Cm by CAT.

ic bacteria can only be detected in strains with alterations in outer membrane structure which can be correlated with increased sensitivity to fusidic acid. Many R plasmids have been shown to promote resistance to fusidic acid in such strains (89). Genetic analysis of the r-determinant region of R100 showed that *cat* and *fus* were closely linked (219, 262, 263) but appeared to be separable (96). Recently, however, *cat* and *fus* have been shown to be the same gene by the isolation of amber mutations affecting both *cat* and *fus* simultaneously (406). DNA sequence analysis showed that one amber mutation (at Gln-38) preceded the only possible internal translational start (at Met-77), thus eliminating the possibility that the *fus* product could be translated from an initiation codon internal to the *cat* message.

Resistance to fusidic acid is specified only by type I CAT. The enzyme protein must mediate resistance to the antibiotic despite the lack of structural similarity between fusidic acid and Cm and the fact that fusidic acid is not inactivated or modified in any way (337). Thus, CAT binds fusidic acid very strongly to its active center and fusidic acid acts as a competitive inhibitor of Cm acetylation (337). This may be related to the finding that rosaniline dyes such as crystal violet and carbol fuchsin also bind strongly to CAT and are competitive inhibitors of acetylation (302). Type I CAT seems to have a binding "pocket" or domain which readily accommodates hydrophobic and, in particular, aromatic groups of compounds which are not related structurally.

The high concentrations of CAT protein in the cytoplasm combined with the strong affinity of fusidic acid for the protein and its slow diffusion into the gram-negative cell makes a "sponge" or "buffer" mechanism seem plausible, whereby an inhibitor is rapidly sequestered by a binding protein before it can reach its target. A similar mechanism has been postulated for CadB-mediated cadmium resistance (295).

Regulation of CAT synthesis in *E. coli*. It has been known for some time that type I CAT synthesis in *E. coli* is subject to catabolite repression. Constitutive synthesis of the enzyme occurred at a higher rate in glycerol-grown cultures than in those grown in glucose (156). Mutants lacking adenylate cyclase or catabolite activator protein (CAP) failed to support high-level CAT synthesis, and cells lacking CAP could not be stimulated to do so by the addition of cyclic AMP (94). Furthermore, in vitro coupled transcription-translation of the *cat* gene

demonstrated the requirement for both cyclic AMP and CAP for maximum CAT synthesis (94).

DNA sequence analysis of the type I CAT gene and DNase protection experiments identified two CAP sites in the promoter-proximal region of the gene (227). One site is located 130 bp upstream from the start point of transcription but is not required for the stimulation of transcription. The CAP site which overlaps the promoter is involved in regulating the transcription of *cat*. The cyclic AMP-CAP complex may interact with RNA polymerase and also change the structure of the promoter.

Regulation of CAT synthesis in *S. aureus*. CATs are encoded by small multicopy plasmids in *S. aureus*. CAT is expressed at a high level only after induction with Cm. Such induction experiments are normally complicated by the fact that the inducer is inactivated by the newly synthesized CAT (acetoxy-Cm cannot act as an inducer) and also because the inducer is an inhibitor of the protein synthesis required for induction to occur (340, 425). These problems can be circumvented by using 3-deoxy-Cm, which is a gratuitous inducer (425). Apart from the absence of a requirement for a 3-OH group, the structural requirements for the inducer parallel those of Cm as an antibiotic and CAT substrate.

The mechanism of regulation of CAT expression in *S. aureus* is poorly understood. The failure of repeated attempts to generate Cm^r constitutive mutants (337) would argue against a simple negative (repressor-controlled) regulatory mechanism. Also, it is known that induction fails to occur in the presence of inhibitors of transcription, an observation which diminishes the likelihood of a translational attenuation mechanism as has been proposed for the induction of staphylococcal macrolide-lincosamide-streptogramin B (MLS) resistance (171, 345).

Cloning and DNA sequencing studies have provided some insight into the regulatory process. Staphylococcal CAT determinants express Cm^r inducibly when cloned in an *E. coli* vector plasmid (174, 424), although the 5-fold level of CAT induction was poor compared with the 100-fold induction seen in the native host. A small fragment of pC194, where the only open translational reading frame was for CAT protein, still expressed inducible Cm^r in *E. coli*. This suggested that no plasmid-encoded regulatory protein (apart from CAT) could be involved in *S. aureus* CAT induction in *E. coli*. This and the presence of a 37-bp inverted repeat sequence between the likely start of transcription and the translational initiation codon for CAT led to the suggestion that *cat* gene transcription is controlled autogenously by the CAT protein. Two simple autogenous regulation models are possible: (i) negative regulation, where CAT protein acts as a repressor of *cat* gene transcription; and (ii) positive regulation, where CAT acts as an inducer in the presence of Cm. Either model would need substantiation by showing that CAT protein has in vitro DNA binding activity and can alter the rate of labeled CAT synthesis in a coupled in vitro system. So far, attempts to demonstrate in vitro regulation by pure CAT protein in an *E. coli*-coupled system have failed (337). Evidence that CAT is a negative autoregulator could come from the isolation of Cm^s point mutants in which the altered CAT protein was synthesized constitutively.

Resistance to Aminoglycoside-Aminocyclitol Antibiotics

The inhibitory effect of aminoglycosides on sensitive bacterial cells is due to the binding of the drug to ribosomes, thus interfering with translation (130). Defining the lethal event has been confused by the pleiotropic effects that occur after sensitive cells are exposed to the drug and by controversy about effects of aminoglycosides on translation (for reviews, see references 130, 151, 152).

Mutations causing resistance to aminoglycosides either reduce drug binding to the ribosome or impair transport across the cytoplasmic membrane. Single-step mutations in the *rpsL* gene of *E. coli* K-12 cause high-level resistance to streptomycin by altering the structure of ribosomal protein S12 and preventing the drug from binding to its target (130). Ribosomal resistance to streptomycin is found in some clinical isolates of *N. gonorrhoeae* (241), enterococci (442), *S. aureus* (213), and *P. aeruginosa* (396). Mutations which cause decreased accumulation of aminoglycosides due to impaired transport across the membrane, resulting from a defect in membrane energization, can be selected in the laboratory (1, 38, 267) and also occur in clinical isolates (34, 36). In addition, obligate and facultative anaerobes growing anaerobically are intrinsically resistant to aminoglycosides because they cannot accumulate the drug (35, 44).

High-level ribosomal resistance to deoxystreptamine-containing aminoglycosides (see below) cannot be selected in a single step (130), probably because these drugs have several binding sites on ribosomes. Low-level resistance due to changes in ribosomal proteins have been reported (40, 388, 432). Sometimes resistant strains have more than one mutation, each one contributing a small increment in resistance (1,

40, 388). Thus, gentamicin resistance can be due to a mutation in *rplF* affecting ribosomal protein L6 (40), which alters drug binding to ribosomes, in combination with an *unc* mutation which impairs transport across the membrane (1). High-level resistance to the aminocyclitol spectinomycin can be selected (*rpsE*; 106, 428), whereas hygromycin B resistance is caused by impaired uptake (1). Resistance to kasugamycin may be by either mechanism (106).

Most clinically significant resistance to aminoglycosides is caused by R-plasmid-specified phosphotransferase, acetyltransferase, and adenyltransferase enzymes (78, 306, 333). The modified antibiotics no longer bind to ribosomes and cannot inhibit protein synthesis (433). However, unlike other drug resistance mechanisms involving enzymatic inactivation, only a small proportion of the drug in the medium is actually destroyed (93). Furthermore, less drug is accumulated in resistant cells (97, 169).

Transport of aminoglycosides in sensitive and resistant bacteria. To reach the ribosomes, aminoglycoside antibiotics must penetrate the cell surface layers. Uptake of aminoglycosides in sensitive strains of both gram-positive and gram-negative bacteria occurs in three stages (36–38, 97, 168).

The first phase of uptake is rapid energy-independent (EIP) binding to the cell surface. In gram-negative bacteria this includes passive diffusion through outer membrane pores. Positively charged aminoglycosides can readily pass through these pores despite being larger than the size exclusion limit for uncharged molecules (272).

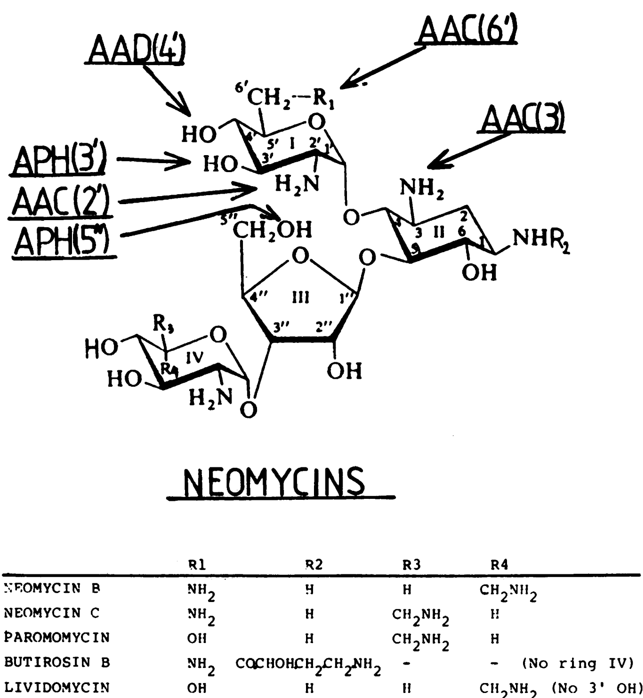
The second stage of uptake is the slow energy-dependent phase I (EDPI), which represents initial passage of the drug across the cytoplasmic membrane. It requires the membrane to be energized sufficiently and uses the $\Delta\Psi$ (electric potential) component of proton motive force (85, 246, 267). Indeed, there is a direct relationship between the magnitude of $\Delta\Psi$ and the rate of uptake and killing by aminoglycosides (85, 246). The cells remain viable during EDPI and polypeptide synthesis continues (1, 267), although misreading may occur. The duration of EDPI depends on the concentration of drug in the medium and the susceptibility of the ribosomes (1). The nature of the carrier in the membrane is not known; different authors have proposed respiratory quinones (33), cytochromes (44), and the combined use of several different carbohydrate transport systems (85).

The third phase of aminoglycoside transport is energy-dependent phase II (EDPII). It is initiated when sufficient drug is present in the cytoplasm to bind to all of the ribosome particles. It signals the onset of lethality and the cessation of protein synthesis. Its initiation requires that susceptible ribosomes in the cell be actively engaged in protein synthesis (1, 177). The suggestion that EDPII results from induction of a cognate polyamine transport system (168) is now discounted (1, 44, 85). Indeed, EDPII may not actually be due to energy-dependent transport per se, but could result from an increase in "runoff" ribosomes from polysomes to provide unimpeded binding sites (177) or from a pleiotropic alteration to membrane permeability (1).

Cells resistant to aminoglycosides because of R-plasmid-specified modifying enzymes exhibit only the EIP and EDPI phases of transport (97, 169). This is because any drug that manages to penetrate the cytoplasm during EDPI is inactivated and fails to induce EDPII because it cannot bind to ribosomes and inhibit protein synthesis (433). However, if the rate of drug transport during EDPI exceeds the rate of drug inactivation, then significant amounts of unmodified drug will penetrate the cytoplasm and bind to ribosomes. Thus, some strains which are phenotypically susceptible to certain drugs exhibit aminoglycoside-modifying activity when assayed in vitro (25, 144, 403). Also, competitive inhibitors of enzyme activity such as the aminoglycoside adenyltransferase inhibitor 7-hydroxytropolone may decrease the rate of inactivation sufficiently to cause a significant decrease in resistance level (4).

Location of aminoglycoside-modifying enzymes in the bacterial cell. There has been some confusion about the cellular location of aminoglycoside-modifying enzymes. Originally it was suggested that these enzymes were located in the periplasmic space of gram-negative bacteria because significant amounts were released by osmotic shock (91, 137, 138, 422). However, it is difficult to imagine how enzymes that require the high-energy cofactors ATP or acetyl CoA could be active in the periplasm. Recent experiments with an aminoglycoside 3′-phosphotransferase in *E. coli* showed that the enzyme is located in the cytoplasm (290). It would be attractive to think that modifying enzymes were attached to the cytoplasmic membrane where they would be strategically placed to inactivate incoming drug molecules before they could reach the ribosomes. However, there is no direct evidence for these enzymes being integral membrane proteins since purified membrane fractions from resistant bacteria have little activity. Any membrane attachment of enzymes must be loose and easily disrupted.

It is now considered that osmotic shock treatment may result in release of some cytoplasmic enzymes (290). To establish that an enzyme is



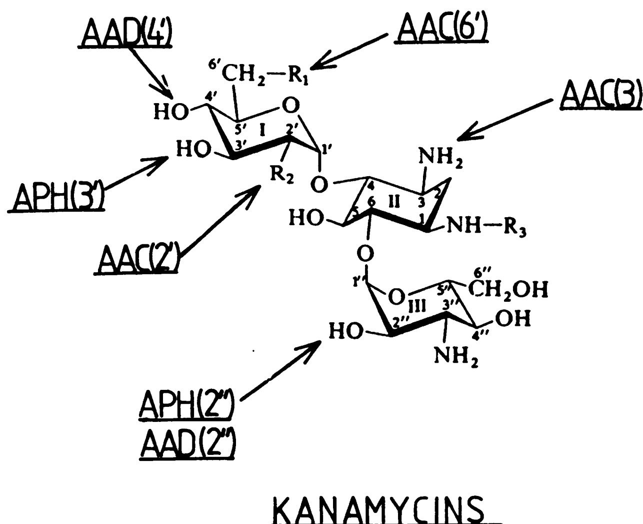
	R1	R2	R3	R4
NEOMYCIN B	NH_2	H	H	CH_2NH_2
NEOMYCIN C	NH_2	H	CH_2NH_2	H
PAROMOMYCIN	OH	H	CH_2NH_2	H
BUTIROSIN B	NH_2	$COCHOHCH_2CH_2NH_2$	-	- (No ring IV)
LIVIDOMYCIN	OH	H	H	CH_2NH_2 (No 3' OH)

FIG. 3. Structure and modification of neomycin antibiotics. The structure of the neomycin family of antibiotics is shown. The structure of variants can be deduced from the table under the structure. The sites of phosphorylation (APH), acetylation (AAC), and adenylylation (AAD) are indicated by arrows.

periplasmic, it is important to test for release from lysozyme-EDTA-generated spheroplasts and from periplasmic-leaky mutants as well as for release by osmotic shock.

Classification of aminoglycoside-aminocyclitol antibiotics and their modifying enzymes. Aminoglycoside-aminocyclitol antibiotics are classified into two major groups, those containing streptidine (e.g., streptomycin) and those containing 2-deoxystreptamine. The 2-deoxystreptamine antibiotics are subdivided on the basis of substitutions to the 2-deoxystreptamine ring: (i) 4,5 substitutions as in neomycin, paromomycin, butirosin, and lividomycin (Fig. 3); (ii) 4,6 substitutions as in kanamycins, gentamicins, tobramycin, and amikacin (Fig. 4 and 5). Other related drugs which do not fit in these groups are the aminocyclitols spectinomycin and hygromycin B, as well as kasugamycin. There are three types of aminoglycoside-modifying enzymes: aminoglycoside-*O*-phosphotransferase (APH), aminoglycoside-*N*-acetyltransferase (AAC), and aminoglycoside-*O*-nucleotidyltransferase (AAD). The reactions that they catalyze are shown in Fig. 6. The phosphorylation and nucleotidylation reactions use ATP as a cofactor, whereas acetyltransferases use acetyl CoA. The site of modification of the drug is indicated by the number in parentheses. Thus, AAC(6') acetylates the 6'-amino group on aminohexose I of susceptible drugs. The modification sites are summarized in Fig. 3, 4, 5, and 7, and in Tables 2, 3, and 4. It may be possible to infer the type of enzyme present in cells from the pattern of resistance to growth inhibition by a standard set of antibiotics (266). This could be a convenient method for screening strains but is no substitute for biochemical analysis, particularly because some strains express more than one aminoglycoside-modifying activity.

Some enzymes have been divided into subtypes, primarily on the basis of differences in substrate profile (Tables 2, 3, and 4). However, this has not always been done consistently, and there seems to have been little attempt to compare directly enzymes isolated in different laboratories. It should be emphasized that being assigned to the same subgroup does not necessarily imply evolutionary relatedness. In most cases immunological cross-reaction and DNA hybridization data are lacking. Also, it is possible that some of the subgroups listed in Tables 2,



	R1	R2	R3
KANAMYCIN A	NH_2	OH	H
KANAMYCIN B	NH_2	NH_2	H
KANAMYCIN C	OH	NH_2	H
AMIKACIN	NH_2	OH	$COCHOHCH_2CH_2NH_2$
TOBRAMYCIN	NH_2	NH_2	H (3' OH MISSING)

FIG. 4. Structure and modification of kanamycin antibiotics. The structure of the kanamycin family of antibiotics is shown. The structure of variants can be deduced from the table under the structure. The sites of phosphorylation (APH), acetylation (AAC), and adenylylation (AAD) are indicated by arrows.

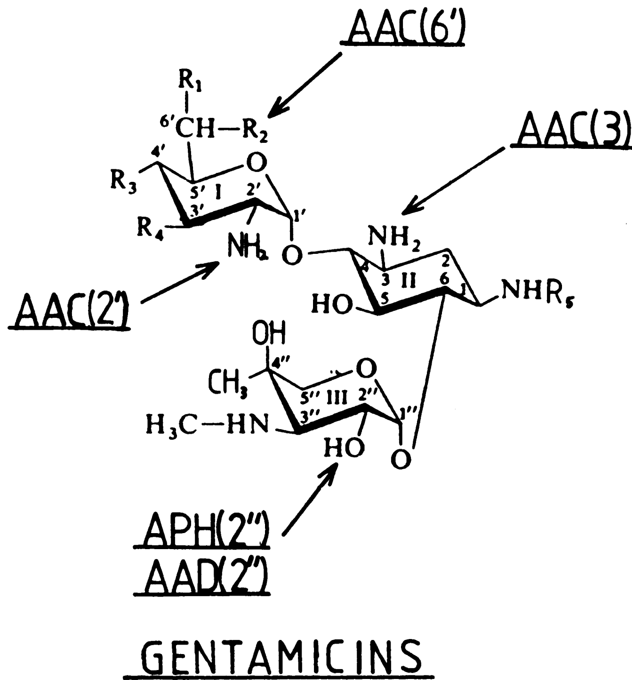
3, and 4 [e.g., some AAC(6') enzymes] are not sufficiently different in substrate profile to warrant separate classification. Other possible sources of confusion are the presence of more than one enzyme in the same strain and the facts that the same enzyme may not confer the same phenotype in different hosts and modification of the drug in vitro does not imply that the enzyme will confer resistance in vivo. Perhaps enzymes should only be reported and classified after kinetic tests have been done with purified proteins.

Resistance to Mercuric Ions and Organomercurials

Sensitivity and resistance to mercurial compounds. Mercuric ions are toxic to bacteria because they bind avidly to sulfhydryl groups and inhibit macromolecule synthesis and enzyme action. Many enzymes have critical thiol groups and are sensitive to Hg^{2+} in vitro. Transcription and translation are particularly sensitive. This may be due to inhibition of precursor synthesis or Hg^{2+} binding to polynucleotides (84). Also, RNase may be activated by Hg^{2+} (22).

Resistance to mercurials (Hg^r) is a common plasmid-determined property of both gram-positive and gram-negative bacteria (64, 331, 369, 417, 418). This may have been caused by the use (until recently) of mercurials such as phenylmercury and thimerosal as hospital disinfectants (148) or by industrial and urban pollution. A decreasing incidence of mercurial resistance in hospital strains has coincided with the discontinuation of mercurial disinfectant usage (300). Also, Hg^r is frequently specified by drug resistance plasmids (331) and is also common in soil pseudomonads and bacilli. It has recently been found in *Thiobacillus ferrooxidans* (286).

Bacterial resistance to Hg^{2+} is determined by enzymatic reduction of the ion to Hg^0, which is much less toxic (368, 369). Also, Hg^0 is virtually insoluble in water and evaporates because of its high vapor pressure. The enzyme that catalyzes the reduction of Hg^{2+} is the intracellular, cytoplasmic, FAD-containing mercuric reductase



		R1	R2	R3	R4	R5
GENTAMICIN	C1a	H	NH_2	H	H	H
GENTAMICIN	C1	CH_3	$NHCH_3$	H	H	H
GENTAMICIN	C2	CH_3	NH_2	H	H	H
SISOMICIN		H	NH_2	-	H	H
NETILMICIN		H	NH_2	-	H	C_2H_5

FIG. 5. Structure and modification of gentamicin antibiotics. The structure of the gentamicin family of antibiotics is shown. The structure of variants can be deduced from the table under the structure. The sites of phosphorylation (APH), acetylation (AAC), and adenylylation (AAD) are indicated by arrows.

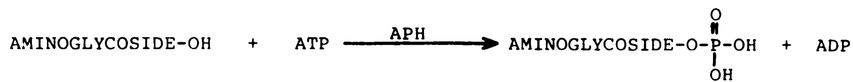


FIG. 6. Biochemical mechanisms of aminoglycoside modification. The mechanisms of modification of aminoglycoside antibiotics by phosphotransferases (APH), adenyltransferases (AAD), and acetyltransferases (AAC) are summarized.

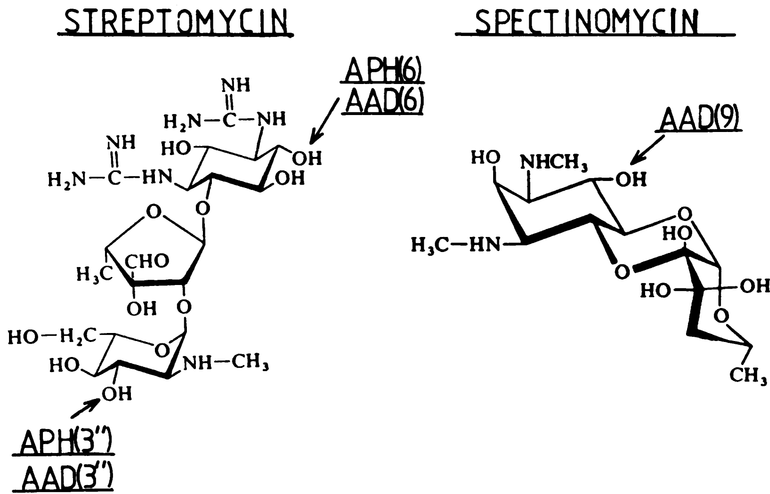


FIG. 7. Structure and modification of streptomycin and spectinomycin. The sites of modification of streptomycin and spectinomycin by adenyltransferases (AAD) and phosphotransferases (APH) are indicated by arrows.

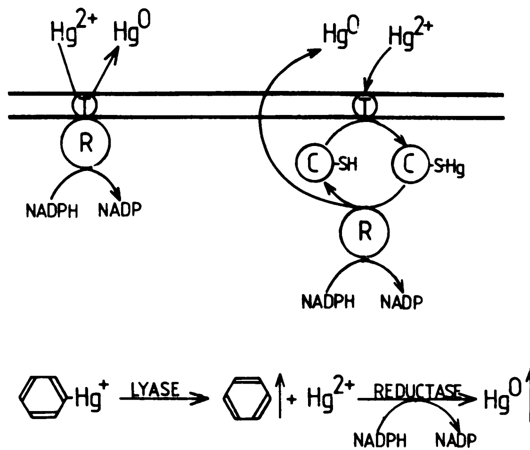


FIG. 8. Mechanism of resistance to mercuric ions. The upper part shows in diagrammatic form two possible mechanisms for detoxification of mercuric ions. The parallel lines represent the cytoplasmic membrane. The diagram on the left indicates that the reductase protein (R) interacts directly with the membrane-bound transport protein (T). On the right it is suggested that another *mer* operon-specified protein (possibly the *merC* product; C) is required to bind the incoming Hg in the form of an adduct and transport it to cytoplasmic reductase (R) molecules. The lower diagram shows the reactions involved in detoxifying the organomercurial phenyl mercury. The C—Hg bond of phenyl mercury is cleaved by organomercurial lyase to form benzene and Hg^{2+}. The Hg^{2+} is subsequently converted to Hg^0 by reductase.

(330, 369). The resistance mechanism also involves a plasmid-specified Hg-specific transport system (274). It seems to be required to direct Hg^{2+} through the cytoplasmic membrane, where it would otherwise encounter sensitive enzymes. It is attractive to think that the reductase and transport functions might interact physically. Indeed, some reductase protein appears to be membrane associated in *E. coli* minicells (187), although this was not found in experiments with whole-cell envelopes (330, 368). The mechanism of resistance is presented diagrammatically in Fig. 8.

Strains which detoxify organomercurials specify a second cytoplasmic enzyme, organomercurial lyase (330, 383). This cleaves C—Hg bonds to release Hg^{2+}, which is in turn volatilized by the reductase (Fig. 8).

Spectrum of resistance to organomercurials in gram-positive and gram-negative bacteria. Two different mercurial resistance phenotypes have been found in gram-negative bacteria (331, 418). Narrow-spectrum resistance is determined by strains that express reductase alone, whereas those strains that specify both reductase and lyase have the broad-spectrum resistance phenotype. However, the phenotypes of enteric bacteria and *P. aeruginosa* differ even when the bacteria harbor the same plasmid (64, 418). An additional complication is that resistance to some organomercurials does not involve enzymatic degradation. All staphylococci resistant to

TABLE 2. Aminoglycoside-modifying enzymes: acetyltransferases

Enzyme	Abbreviation	Site of modification	Where found	Substrate(s)	Comments	Reference(s)
3-*N*-Acetyltransferase	AAC(3)I	3-Amino group on deoxystreptamine	*P. aeruginosa* Enterobacteria	Gentamicin C, sisomicin	Tobramycin is a poor substrate Resistance not conferred	39, 422
	AAC(3)II		Enterobacteria		Different pI from AAC(3)I but otherwise very similar	226
	AAC(3)III		*P. aeruginosa*	Gentamicin C, sisomicin, kanamycin A, B, neomycin, paromomycin, tobramycin	Resistance not conferred to neomycin; wider substrate range than AAC(3)I	25
	AAC(3)IV		Enterobacteria	As above, but also the structurally unrelated apramycin	Broadest substrate range of AAC(3) enzymes	92
2′-*N*-Acetyltransferase	AAC(2′)	2′-Amino group of aminohexose	*Providencia* Sp. *Proteus* Sp.	Kanamycin C, gentamicins, sisomicin, netilmycin, tobramycin		53
6′-*N*-Acetyltransferase	AAC(6′)I[a]	6′-Amino group of aminohexose I	Enterobacteria (R5)	Neomycin, kanamycin A, B, gentamicin Cla, sisomicin, butirosin	In *E. coli*, resistance is not conferred to neomycin, tobramycin, or sisomicin	19, 93
			P. aeruginosa GN315	As above, but also amikacin	Similar substrate profile to R5 enzyme, but resistance spectrum is broader	144
	AAC(6′)II[a]		*Moraxella* sp.	As for AAC(6′)I	Biochemical differences from AAC(6′)I	223
			Enterobacteria	Similar to AAC(6′)I	May not warrant separate classification to AAC(6′)I	268
	AAC(6′)III[a]		*P. aeruginosa* 3796	Neomycin B, kanamycin A, B, gentamicin Cla, sisomicin, tobramycin	Narrower substrate range compared with AAC(6′)I; amikacin and butirosin not modified signficantly	144
	AAC(6′)IV[a]		Staphylococci	Kanamycin, A, B, gentamicin Cla	Neomycin and amikacin poor substrates	101, 225

[a] The enzymes classified as AAC(6′) by Mitsuhashi's group (199, 199a) do not correspond to the scheme used here. It is likely that more than four types of AAC(6′) exist.

TABLE 3. Aminoglycoside-modifying enzymes: nucleotidyltransferases

Enzyme	Abbreviation	Site of modification	Where found	Substrate(s)	Comments	Reference(s)
Enzymes modifying 2-deoxystreptamine antibiotics						
2″-*O*-Nucleotidyltransferase	AAD(2″)I	2″-Hydroxyl on aminohexose III of deoxystreptidine	Enterobacteria *P. aeruginosa*	Kanamycins, gentamicins, sisomicin, tobramycin		20
	AAD(2″)II		Enterobacteria	As above and amikacin	Activity towards gentamicin C1a and C2 reduced.	70
4′-*O*-Nucleotidyltransferase	AAD(4′)	4′-Hydroxyl on aminohexose of deoxystreptamine	Staphylococci Streptococci	Neomycins, kanamycins, butirosin, paromomycin, tobramycin (lividomycin amikacin)	No activity towards gentamicins	179 325
Enzymes modifying streptomycin and spectinomycin						
3″(9)-*O*-Nucleotidyltransferase	AAD(3″)/ AAD(9)	3″-Hydroxyl group on aminohexose III of streptomycin or 9-hydroxyl of actinamine ring of spectinomycin	Enterobacteria *P. aeruginosa*	Streptomycin Spectinomycin		287
6-*O*-Nucleotidyltransferase	AAD(6)	6-Hydroxyl of streptidine ring of streptomycin	Staphylococci, *S. faecalis*	Streptomycin		373 74
9-*O*-Nucleotidyltransferase	AAD(9)	9-Hydroxyl of actinamine ring of spectinomycin	Staphylococci	Spectinomycin	Not studied in detail	93

TABLE 4. Aminoglycoside-modifying enzymes: phosphotransferases

Enzyme	Abbreviation	Site of modification	Where found	Substrate(s)	Comments	Reference(s)
Enzymes modifying streptomycin						
3″-*O*-Phosphotransferase	APH(3″)	3″-Hydroxyl group aminohexose III of streptomycin	Enterobacteria *P. aeruginosa*	Streptomycin	*E. coli* JR35 enzyme differs in substrate profile and cofactors used from the *P. aeruginosa* H9 enzyme	198 206 287
6-*O*-Phosphotransferase	APH(6)	6-Hydroxyl group of streptidine ring	*P. aeruginosa* GN573	Streptomycin		202
Enzymes modifying 2-deoxystreptamine aminoglycosides						
3′-*O*-Phosphotransferase	APH(3′)I	3′-Hydroxyl group of aminohexose I, 5″-hydroxyl group of lividomycin	Enterobacteria (Tn*6*, Tn*601*)	Kanamycins, neomycin, paromomycin, lividomycin	Modifies lividomycin at 5″-OH position, not butirosin	23 400
			P. aeruginosa		The 3 enzymes studied differ in molecular weights, etc.	247
			Staphylococci			206
	APH(3′)II	3′-Hydroxyl group of aminohexose I	Enterobacteria (Tn*5*)	Kanamycins, neomycin, paromomycin, butirosin	Modifies butirosin, not lividomycin	20
			P. aeruginosa			247
	APH(3′)III	3′-Hydroxyl group of aminohexose I; 5″-hydroxyl group of lividomycin	Staphylococci	Kanamycin, neomycin, paromomycin, butirosin, lividomycin, amikacin	Amikacin is phosphosylated but resistance is not conferred Both lividomycin and butirosin inactivated	72
			P. aeruginosa	Kanamycin, neomycin, paromomycin, butiroson, lividomycin, amikacin		400
			P. aeruginosa GN573	Also ribostamycin	Probably APH(3′)III; Ribostamycin phosphsylated at 5′-OH	
			S. faecalis			74
	APH(3′)IV		Staphylococci	As for APH(3″)III	Possibly same enzyme as APH(3′)III	200

2″-*O*-Phosphotransferase	APH(2″)	2″-Hydroxyl group of aminohexose III	Staphylococci	Gentamicins, sisomycin, amikacin, tobramycin, kanamycin	Invariably present in strains producing AAC(6′)IV; 4,5-substituted derivatives (neomycins) are not substrates	101, 225
Enzyme modifying hygromycin B						
4-*O*-Phosphotransferase	APH(4)	4-Hydroxyl group of hyosamine	*E. coli*	Hygromycin and closely related drugs	Expressed in same transcription unit as AAC(3)IV by plasmid JR225	Rao, personal communication

mercurials express a broad-spectrum phenotype which differs from that of gram-negative organisms. These phenotypes are summarized below.

(*i*) *Narrow-spectrum resistance in enteric bacteria.* Enteric bacterial strains volatilize Hg^{2+} but also confer resistance to merbromin and fluorescein mercury acetate without hydrolysis or reduction of mercury (331, 418). A permeability barrier has been postulated (369), but the gene responsible in the *mer* operon has not been identified.

(*ii*) *Broad-spectrum resistance in enteric bacteria.* Broad-spectrum resistance is restricted to a small number of plasmids from incompatibility groups A to C, L, and H2. The narrow-spectrum phenotype is extended to include phenylmercury and thimerosal (331, 418). Although Hg^0 is formed by volatilization from thimerosal, *p*-hydroxymercuribenzoate (pHMB), methyl mercury, and ethyl mercury, resistance is only conferred to thimerosal. This is attributed to low-level lyase activity and the greater toxicity of ethyl and methyl mercury. The IncA–C determinants specify very low lyase activities and are also sensitive to thimerosal.

(*iii*) *Narrow-spectrum resistance in P. aeruginosa.* Narrow-spectrum resistance in *P. aeruginosa* differs from that in *E. coli* in that resistance is conferred to pHMB without volatilization of Hg^0 (64). A host factor is probably responsible because broad-host-range Hg^r plasmids do not confer resistance to pHMB in *E. coli* (64, 418).

(*iv*) *Broad-spectrum resistance in P. aeruginosa.* As with the narrow-spectrum *P. aeruginosa* determinants described above, resistance to pHMB occurs without hydrolysis or volatilization, whereas *E. coli* plasmids slowly volatilize Hg^0 from pHMB yet are sensitive to the compound (418). These plasmids specify resistance (with volatilization) to phenyl, methyl, and ethyl mercury (418).

(*v*) *Resistance in S. aureus.* All *S. aureus mer* plasmids determine broad-spectrum resistance. Two phenotypic groups have been described. The first differs from the gram-negative broad-spectrum phenotype by not involving resistance to merbromin. Resistance to pHMB and fluorescein mercury acetate is conferred without volatilization (417). Furthermore, these strains are sensitive to thimerosal, although slow hydrolysis and volatilization were detected. The second class of determinants expresses resistance to thimerosal, merbromin, and methyl mercury. Thimerosal resistance was attributed to this compound being a better inducer of the *mer* operon as well as to the lyase having greater hydrolytic activity (300). Also, that methylmercury was hydrolyzed suggests that a broader-spectrum lyase was involved.

Mercuric reductase and mechanism of reduction of mercuric ions. Mercuric reductase is a soluble cytoplasmic protein (330, 369). The enzymes specified by plasmid R100 and transposon Tn*501* in *E. coli* are composed of subunits of about 63,000 molecular weight. Each subunit has a bound FAD moiety. The subunit structure of the enzyme from different sources seems to vary remarkably. The soil *Pseudomonas* sp. strain K62 enzyme is apparently a monomer of 67,000 daltons (127). Two distinct types of reductase enzyme encoded by plasmids in gram-negative bacteria have been recognized by immunological studies and by differences in charge and molecular weight (121, 330). The prototype class I enzyme specified by Tn*501* is a dimer of 125,000 daltons, whereas the type II (R100) enzyme appears to be a trimer of 180,000 daltons. Despite these differences in subunit structure, the type I and II enzymes are closely related to each other in terms of amino acid composition, immunological cross-reaction (T. Kinscherf and S. Silver, personal communication), and DNA sequence homology between the *mer* genes (S. Silver and N. L. Brown, personal communications).

The reductase encoded by Tn*501* has been studied in greatest detail at the biochemical level (121). It has a redox-active cysteine at the active site which acts as an electron acceptor. Hg^{2+} is presented to the enzyme as a dithiol adduct RS-Hg-SR (121). The possible biochemical mechanisms involved in the reduction of mercuric ions have been discussed before (121).

Detoxification of organomercurial compounds. The relationship between hydrolysis of organomercurials and resistance phenotypes in different organisms has been discussed above. Broad-spectrum organomercurial-resistant bacteria contain the enzyme organomercurial lyase (330, 383), which cleaves the C—Hg bonds of phenyl, ethyl, and methyl mercury and in some cases thiomerosol (Fig. 8). Two distinct lyases were separated from the soil *Pseudomonas* sp. strain K62 (383). There is also a possibility that R831 specifies two different, but so far inseparable, activities in *E. coli* (330). Genetic studies are required to determine whether one or two structural genes are responsible for the two activities.

Evidence for an Hg^{2+}-specific transport system and its role in resistance. The finding that mutations which inactivated mercuric reductase caused hypersensitivity to Hg^{2+} was the key to revealing the Hg^{2+}-specific transport system (119, 274). Hypersensitive strains are sevenfold more sensitive to Hg^{2+} than plasmid-free cells and bind ^{203}Hg more rapidly (274).

The Hg^{2+} transport system has been difficult to characterize. It does not involve titratable thiol groups (274). It has been suggested that enhanced uptake of Hg^{2+} is due to facilitated diffusion, with Hg^{2+} passing more rapidly into cells and saturating the available binding sites more quickly (367).

The gene which specifies the transport function (*merT*) is the closest structural gene to the *mer* operon promoter (Fig. 9). Attempts have been made to assign one of the polypeptides expressed in *E. coli* minicells to *merT*. Thus, the 15K and 14K Hg^{2+}-inducible polypeptides bound to the cytoplasmic membrane fraction could be candidates for the *merT* protein. The Tn*501* DNA sequence suggests that the *merT* product is a 12.5K highly hydrophobic protein with two cysteine residues (N. L. Brown, R. D. Pridmore, and D. C. Fritzinger, personal communication). To assign polypeptides unequivocally to genes, it will be necessary to examine

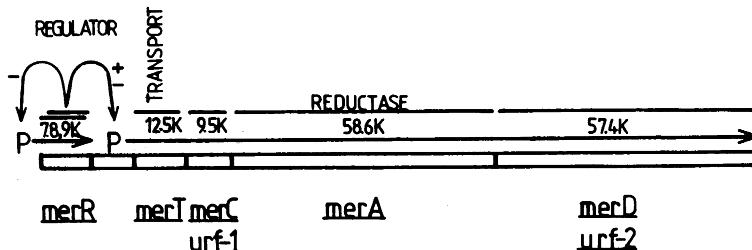


FIG. 9. Structure of the *mer* operon. Data obtained from genetic analysis of the R100 *mer* region and from DNA sequence analysis of the closely related Tn*501* element are summarized. The putative *mer* genes *merC* and *merD* correspond to the open translational reading frames *urf-1* and *urf-2* revealed in the Tn*501* sequence. The direction of transcription of the *merR* and the *merTCA* genes from promoters (P) are shown by arrows. Above these lines are the sizes of putative *mer* polypeptides deduced from the Tn*501* DNA sequence. The regulatory protein(s) specified by the *merR* region acts as both a positive and a negative regulator of the *merTCA* operon and also autogenously regulates *merR* expression.

polypeptides expressed in in vivo systems by wild-type and mapped mutations.

At first glance it might seem strange that a resistance mechanism should involve a process that specifically transports a toxic ion into the cell. However, it can be argued that this is necessary to prevent the highly toxic Hg^{2+} from binding to sensitive sulfhydryl groups as it passes through the membrane. Thus, a strain harboring a *merT* mutant plasmid which still expresses a near-wild-type level of reductase is phenotypically sensitive to Hg^{2+}, despite the fact that it can rapidly volatilize the mercury from the medium (N. Ní Bhriain and T. J. Foster, unpublished data).

It is reasonable to think that reductase interacts with the membrane-bound transport protein (Fig. 8). This might be necessary to prevent Hg^{2+} from binding to sensitive cytoplasmic enzymes once it has been transported through the membrane. However, there is no evidence for reductase being associated with the membrane fraction of lysed cells, although it was suggested that the 66K processed form of reductase might be loosely associated with the minicell membrane (187), possibly by loose ionic binding. Alternatively, another *mer* operon protein could be involved in binding Hg in the cytoplasm and carrying it as an adduct to the reductase. The protein which could be specified by *urf-1* Tn*501* (which is equivalent to *merC* in R100; see below and Fig. 9) has two cysteine residues (Brown et al., personal communication) and could be a candidate for such a role.

Expression of resistance by multicopy plasmids. Comparison of the volatilizing activity of *E. coli* strains carrying different copy number *mer* plasmids revealed a cryptic gene-dosage effect for reductase (273). Strains carrying the R100 *mer* genes cloned in the ColE1::Tn*A* vector RSF2124 (16 copies per chromosome) expressed an eightfold greater reductase activity in cell extracts than did R100 (one to two copies per chromosome), although there was no increase in resistance level or volatilizing activity of intact cells. It was suggested that crypticity was due to a limitation in Hg^{2+} transport sites in strains carrying multicopy *mer* plasmids. The rate of Hg^{2+} transport would be expected to be the same irrespective of the number of copies of the *merT* gene and the amount of reductase within the cell.

Our recent experiments have indicated that there is a gene-dosage effect for the transport function in *E. coli* (Ní Bhriain and Foster, unpublished data). The R100 *mer* genes were cloned into pBR322, a plasmid with a higher copy number than that of ColE1. Reductase-deficient *merA* mutants determined greater Hg^{2+} hypersensitivity than did *merA* mutations on ColE1 or R100. The pBR322 *mer*$^+$ plasmid also specified a higher level of reductase than the other plasmids. However, it did not determine a normal wild-type Hg^r phenotype. The incomplete resistance to Hg^{2+} could be explained by overproduction of the transport function to such an extent that the cells cannot cope with the flood of incoming Hg^{2+}, despite the simultaneous induction of large amounts of reductase. A more extreme manifestation of this imbalance was observed in cells carrying the single-copy wild-type *mer*$^+$ plasmid R100 along with a multicopy *merA* mutant plasmid. These cells were more sensitive to Hg^{2+} than multicopy *mer*$^+$ plasmid-carrying cells or plasmid-free cells, despite the production after induction of substantial reductase activity by the R100 *merA*$^+$ gene (Ní Bhriain and Foster, unpublished data).

Genetic analysis of the *mer* region. One genetic study of *mer* analyzed transposon Tn*A* insertions in plasmid R100 (119). Three genes were recognized and mapped: the regulatory gene (*merR*), the transport function gene (*merT*), and the reductase gene (*merA*). An operon structure was proposed to explain the coordinate induction of *merT* and *merA*, and the approximate physical locations of the genes on the plasmid were deduced (119). We have been engaged in a more detailed genetic analysis of *mer* and have confirmed the main features of the model (Ní Bhriain and Foster, unpublished data; Fig. 9). This can now be interpreted in relation to the DNA sequence of the *mer* region of Tn*501*, with the slight caveat that the two systems specify different types of reductase and probably differ somewhat at the DNA sequence level.

The physical coordinates of the *mer* genes of R100 were deduced by mapping a number of different transposon Tn*5* insertions. This approach allows the minimum and maximum sizes of the gene products to be estimated. The regulatory gene *merR* is located between the IS*1* sequence derived from the r-determinant region of R100 (Fig. 9) and the *merT-merA* genes. The *merR* region of Tn*501* has two partially overlapping protein-coding sequences which could specify polypeptides of 9K and 7.5K. It is not known whether the *merR* region of R100 has the same organization or whether the two proteins are expressed in vivo and are required for *merR* function.

Transposon insertions in *merR* and *merT* both cause a sensitive phenotype and low constitutive expression of reductase. They can be distinguished by complementation tests. Located between *merT* and *merA* is a stretch of DNA where Tn*5* insertions cause a hypersensitive phenotype but allow synthesis of low constitutive reduc-

tase. This region could correspond to a new *mer* gene which we have called *merC* and an open reading frame (*urf-1*) of Tn*501*. The protein product of *urf-1* would be 9.5K (Brown et al., personal communication). However, this region has not been assigned a minicell-specified protein or a function in resistance.

Unexpectedly, several Tn*5* insertions in the multicopy *mer* plasmid which caused a sensitive phenotype mapped promoter-distal to the hypersensitivity-conferring *merA* mutants (Foster and Ní Bhriain, unpublished data). They expressed normal levels of a reductase protein which was indistinguishable from wild type in terms of its stability, catalytic properties, and molecular weight (F. D. Porter and S. Silver, personal communication). It seems unlikely that the mutations have affected the carboxy terminus of the reductase. Interestingly, Tn*501* has an open translational reading frame (*urf-2*) in the corresponding location, which could specify a polypeptide of 57.5K. We have assigned the Tn*5* mutations to a new gene (*merD*). However, there must be some doubt about *merD* because when the copy number of the *merD*::Tn*5* insertions was reduced a wild-type Hg^r phenotype was expressed. Also, we have shown that *merD* is not responsible for merbromin resistance (see above). No polypeptide has so far been assigned to *merD* or *urf-2*. However, the *urf-2* region of Tn*501* is probably transcribed because induction of the *mer* operon stimulates expression of the adjacent transposase and resolvase genes, presumably by transcription from the *mer* operon promoter (203).

Regulation of expression of *mer* genes. Mercury resistance is expressed only after exposure to subtoxic levels of Hg^{2+} (369). This correlates with induction of volatilizing activity (330, 369) and Hg^{2+} hyperbinding by hypersensitive mutants (274). Possibly the *mer* genes are regulated both positively and negatively in a similar fashion to *araC*-mediated control of the arabinose operon (283). Circumstantial evidence for this type of regulation was the difficulty in isolating constitutive mutants and the fact that mutations causing temperature-sensitive induction of reductase have been obtained (369). In addition, insertions in *merR* caused a sensitive phenotype and allowed expression of low constitutive levels of reductase. These mutations could be complemented to give a wild-type inducible phenotype (119). Reductase was fully repressed in uninduced diploid *merR* $merA^+/merR^+$ *merA* cells and could be induced by Hg^{2+}, showing that *merR* encodes a *trans*-acting regulatory element which acts as both a repressor and an inducer of the *mer* operon (Ní Bhriain and Foster, unpublished data).

The *araC* protein negatively regulates its own transcription as well as controls the *araBAD* operon (283). We have investigated the regulation of *merR*, using *merR-lac* transcriptional fusions (Foster and Ní Bhriain, unpublished data). The constitutive expression of β-galactosidase by *merR-lac* fusions was repressed eightfold by $merR^+$ plasmids and was not elevated by induction. Thus, *merR* protein negatively regulates the transcription of the *merR* gene.

DNA sequence analysis of Tn*501* suggests that the structural organization of *mer* differs from that of *ara*. Thus, *merR* and *merTCA* are transcribed in the same direction from well-separated promoters (Brown et al., personal communication; Fig. 9), whereas *araC* and *araBAD* are transcribed divergently from overlapping promoters located in a common regulatory region (283).

Expression of reductase also appears to be sensitive to catabolite repression in *E. coli*. Thus, reductase induced in glucose-grown cells is twofold lower than in glycerol-grown cells. Reductase expression was also lower in *cya* and *crp* mutants (367).

BYPASS MECHANISMS

Sulfonamide Resistance

Inhibitory effect of sulfonamides. Sulfonamides diffuse passively into bacterial cells and inhibit growth by interfering with the biosynthesis of folic acid (32). The sensitive step is the condensation of *p*-aminobenzoic acid (of which sulfonamides are structural analogs) with dihydropteridine to form dihydropteroic acid (Fig. 10). The inhibitory action of the drug can be explained both by competitive inhibition of dihydropteroate synthase (DHPS) and by draining the supply of pteridine precursor into a sulfonamide analog of dihydropteroate which is excreted into the medium (Fig. 10). Insufficient pterin-sulfonamide remains in the cell to inhibit dihydrofolate synthase, although it inhibits this enzyme in vitro. In addition, the pteridine precursor does not accumulate in inhibited cells; it is consumed at the same rate in the presence or absence of the drug (319). Thus, reversal of sulfonamide action by precursor accumulation could not occur.

Plasmid-specified Su^r DHPS. Early reports that plasmid-specified sulfonamide resistance (Su^r) was due to impermeability have not been substantiated. Gram-negative bacteria carrying Su^r plasmids specify DHPS activity which can function normally in the presence of high concentrations of drug (351, 352, 375, 426). These

resistant strains thus have two different DHPS enzymes which can be separated by gel filtration (375). A convenient, but not infallible, method for screening for plasmid-encoded DHPS activity is to test for suppression of a mutation in the chromosomal DHPS gene which directs synthesis of a heat-labile enzyme (351, 375).

Two different types of plasmid-encoded Su^r DHPS have been reported (375; G. Swedberg, Ph.D. thesis, University of Uppsala, Uppsala, Sweden, 1982). These enzymes differ markedly in heat stability and are encoded by DNA sequences that have no measurable homology as assessed by Southern blot hybridization and restriction mapping. They are slightly smaller than the chromosomal DHPS, having a molecular weight of around 40,000 (375). The type I Su^r DHPS specified by IncFII plasmids such as R1 and R6 and the IncW plasmid R388 is extremely thermolabile (352, 375), hence the difficulty in the above-mentioned *ts* mutation suppression test. The earlier report of failure to detect Su^r DHPS activity in R388-carrying cells is now attributed to the synthesis of small amounts of a very labile enzyme (Swedberg, Ph.D. thesis). Resistant DHPS activity was detected in cells carrying the R388 Su^r determinant cloned in pBR322, which resulted in amplification of the gene and a gene-dosage effect for the enzyme.

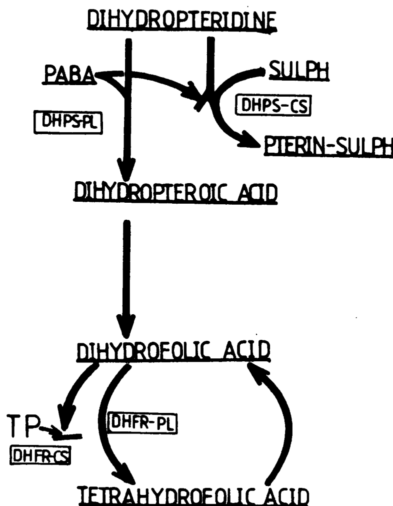


FIG. 10. Inhibition of the folic acid biosynthetic pathway by Su and Tp. The folic acid biosynthetic pathway is shown in outline along with the effects of Su and Tp in susceptible and R-plasmid-containing resistant cells. The enzymes involved are DHFR and DHPS, specified by either plasmids (PL) or the chromosome (CS).

The type II (stable) enzyme is specified by small nonconjugative plasmids such as RSF1010 (141) and pJP1 (402).

The biochemical properties of the *E. coli* chromosomal (Su-sensitive) DHPS have been compared with the plasmid-encoded enzymes (375). The chromosomal enzyme is about 10% larger than the plasmid-determined DHPS (375) and binds Su slightly more efficiently than the natural substrate *p*-aminobenzoic acid. In contrast, the plasmid-encoded enzymes bind Su 10,000-fold less efficiently. This degree of resistance is achieved without sacrificing the efficiency with which the natural substrate is bound. Plasmid-determined DHPS enzymes must be able to distinguish very precisely the carboxyl group of *p*-aminobenzoic acid from the sulfamido group of the inhibitor.

Between 10 and 50% of the DHPS activity in extracts of Su^r plasmid-carrying *E. coli* cells is resistant to Su in vitro (375). The resistant cells must be able to generate sufficient folic acid for growth, despite the fact that the chromosomal enzyme will continue to divert some pteridine into the pterin-sulfonamide analog. That pterin-sulfonamide is excreted into the medium prevents it from reaching a concentration which would inhibit dihydrofolate synthase activity.

Regulation of plasmid-specified DHPS. Very little has been reported on the regulation of plasmid-encoded DHPS expression. It is generally assumed that the enzymes are synthesized constitutively, although no systematic study of this has been published. One difficulty is the lability of type I DHPS, which makes accurate enzyme assays difficult.

The Su^r determinant of the type II DHPS-specifying plasmid RSF1010 is expressed coordinately with the adjacent streptomycin resistance (Sm^r) determinant, which specifies a 3″-*O*-phosphotransferase (164). Evidence for this was the finding that transposon A insertions in the Su^r determinant also reduced expression of Sm^r, presumably due to polarity (164). Thus, the two genes are transcribed from a single promoter in the order Su-Sm. In view of the report that some aminoglycoside-modifying enzymes are regulated by catabolite repression (93) (presumably at the level of transcription), it is possible that the type II DHPS could be regulated in a similar fashion. This hypothesis has not been directly tested.

The type I DHPS enzyme-specifying Su^r determinants of IncFII plasmids are also closely linked to a gene encoding a 3″-19-*O*-adenyltransferase which modifies Sm and spectinomycin (219, 263, 389). In this case there is no evidence for coordinate expression and the linkage may be entirely fortuitous.

Trimethoprim Resistance

Inhibitory effects of Tp. Trimethoprim (Tp) is a potent inhibitor of chromosomally encoded dihydrofolate reductase (DHFR), an enzyme involved in the metabolism of folic acid (Fig. 10). The drug is an analog of the natural substrate dihydrofolate. It binds 1,000-fold more strongly to the active site of the enzyme than does the natural substrate (11). A synergistic bactericidal effect is obtained when Su and Tp are combined; hence, the drugs are often administered together in treatment of infections.

Plasmid-specified Tp-resistant DHFR. The mechanism of resistance to Tp is analogous to that of sulfonamides, with plasmid-located genes specifying a Tp^r DHFR activity (10, 289, 352, 382). It has been encountered in many species of enteric bacteria (87, 88, 191). Epidemiological studies have indicated that the incidence of Tp^r is increasing (87, 117). Occasionally, Tp^r strains causing urinary tract infections have a mutation in the gene encoding thymidylate synthase (*thyA* in *E. coli*) (245, 358). This causes a thymine-requiring phenotype and resistance to Tp because one of the major cellular requirements for reduced folic acid is eliminated (358).

Three distinct Tp^r DHFR enzymes have been identified (116, 117, 289, 382). The enzymes can clearly be distinguished from one another and from the chromosomal DHFR by biochemical tests such as pH optima, stability, sensitivity to inhibitors, subunit structure, and immunological cross-reaction. These groupings have been confirmed by hybridization studies with cloned Tp^r DHFR genes (117).

The type I DHFR whose prototype is encoded by Tn*7* on R483 (17) is highly resistant to inhibition by Tp in vitro (50% inhibitory concentration, 57 μM) in comparison to the chromosomal enzyme (50% inhibitory concentration, 0.007 μM). It is composed of two identical subunits of 18,000 molecular weight each (116). In contrast, type II enzymes (prototypes encoded by R67 and R751::Tn*402*) are essentially completely resistant to Tp (50% inhibitory concentration, 70,000 μM). In addition, the R67-specified enzyme is structurally different from the type I enzymes because it is composed of four identical 8,500-dalton subunits (116, 356, 359). A variant of the type II enzyme specified by R388 has a slightly larger (10,500 dalton) subunit (116, 443). Both type I and type II DHFR enzymes determine very high levels of Tp resistance in *E. coli* (minimum inhibitory concentration, 2,000 μg/ml) compared with plasmid-free strains (minimum inhibitory concentration, 0.2 μg/ml). In contrast, plasmids specifying the recently discovered and less well-characterized type III DHFR determine moderate increases in Tp^r (minimum inhibitory concentration, 200 μg/ml) (394). The type III enzyme is also inhibited by lower concentrations of Tp in vitro (50% inhibitory concentration, 1.5 μM) (117).

DECREASED ACCUMULATION INVOLVING EFFLUX MECHANISMS

Resistance to Tetracyclines

Uptake of Tc by susceptible cells. At low concentrations the tetracyclines (Tc) are bacteriostatic antibiotics that inhibit protein synthesis by preventing the binding of aminoacyl tRNA to the ribosome A site (130). The drug also chelates cations, notably Mg^{2+}. A Tc-Mg chelation complex is involved in ribosome binding (130, 395).

The mechanism of entry of Tc into sensitive cells has been discussed in recent reviews (56, 61, 232). Tc's are broad-spectrum antibiotics with considerable activity against gram-negative bacteria. Passage of Tc through the outer membrane appears to be the rate-limiting step in drug accumulation by gram-negative organisms (256). Tc is relatively hydrophilic and appears to diffuse preferentially through *ompF* porins (60). Mutants deficient in *ompF* porin are only threefold more resistant to Tc, so the drug must be able to enter by other routes. Hydrophobic derivatives such as minocycline probably pass through the lipid interior of the outer membrane (60, 256).

Early work (reviewed by Chopra and Howe [61]) showed that Tc uptake is energy dependent. More recent studies indicate that Tc uptake in *E. coli* is biphasic (109, 257). The initial rapid phase is energy independent and probably represents binding of drug to the cell surface and passage by diffusion through the outer layers of the cell. The second slower phase of uptake is energy dependent and corresponds to transport across the cytoplasmic membrane. This transport is inhibited by energy poisons, uncouplers, and depolarizers, but not by inhibitors of membrane-bound ATPase (58, 232), suggesting that proton motive force is required. Studies on the accumulation of Tc in membrane vesicles prepared from susceptible *E. coli* cells confirmed that active Tc transport depends on proton motive force (256).

The identity of the Tc carrier in the bacterial cytoplasmic membrane has remained elusive. Indeed, until recently formal kinetic evidence for a carrier was lacking because of its low affinity for Tc (163). The earlier suggestion that Mg^{2+} permeases might be involved, with drug binding to Mg^{2+} while being transported (124), seems unlikely because *E. coli* mutants defective in Mg^{2+} transport accumulated Tc as well as the wild type (15). Other possible candidates

such as the dicarboxylic and glutamate transport systems are now known not to be involved (58, 61).

Naturally occurring Tc resistance determinants. Tc resistance is widely encountered in gram-positive and gram-negative bacteria. It is usually plasmid encoded and expressed inducibly, although chromosomally located and constitutively expressed Tc^r determinants have been reported.

(i) Tc resistance in gram-negative bacteria. Five plasmid-specified Tc^r determinants (TetA to TetE) have been distinguished in enteric bacteria by DNA hybridization experiments and resistance phenotypes (49, 63, 120, 260, 315). Despite the failure of these elements to form hybrids, recent DNA sequence analysis of the Tn*10* (TetB), Tn*1721* and RP1 (TetA), and pSC101 (TetC) determinants has revealed significant homologies between the *tet* structural genes. This is discussed below. The TetB determinant is the most frequently encountered in enteric coliform bacteria (232). It is also responsible for Tc^r in *H. influenzae* (188). Most strains of *Proteus mirabilis* express inducible high-level Tc^r from a chromosomal locus (232). The possibility that this is related to one of the plasmid-located determinants has not been tested. The plasmid-linked Tc^r determinant in *B. fragilis* is unusual because conjugation functions and expression of Tc^r may be coordinately regulated (301).

(ii) Tc resistance in gram-positive bacteria. Tc resistance in *S. aureus* is usually determined by small, closely related, multicopy plasmids (184). Chromosomally mediated Tc^r is rarer. Chromosomal and plasmid determinants have been distinguished on the basis of resistance phenotype (14, 62), but the possibility remains that they are genetically related. Many different soil bacilli harbor small Tc^r plasmids (26, 299), but no sequence homology between these and the *S. aureus* plasmid-linked determinants was found by hybridization experiments (299).

Tc resistance is very common in the streptococci. Three distinct Tc^r determinants have been found by hybridization studies (42). The *tetL* determinant is present on nonconjugative plasmids, including the amplifiable element of pAMα1 in *Streptococcus faecalis* (66, 431). The *tetM* genes are found in the chromosomes of *S. agalactiae*, *S. pneumoniae*, and *S. mutans* (391) and are also associated with the conjugative transposon Tn*916* (123) of *S. faecalis* (42). Tc^r strains of *Clostridium difficile* also harbor a chromosomal Tc^r element which promotes conjugational transfer (354), but it is not known whether this is related to Tn*916*. The *tetN* element is characteristic of large conjugative plasmids (42). Hybridization tests failed to reveal any homology between the streptococcal *tet* determinants and the TetA–D determinants of enteric bacteria, or the *tet* plasmids of *Staphylococcus aureus* and *B. sphaericus* (42).

Mechanism of Tc resistance. It is accepted that Tc resistance is due mainly to a decrease in drug accumulation (61, 124). A significant recent contribution to understanding the mechanism of Tc resistance was the discovery of a drug efflux mechanism (16, 258). Whether efflux alone is sufficient to promote resistance (as seems to be the case for cadmium and arsenate resistance) remains to be determined. It is possible that the decrease in drug accumulation reflects both decreased uptake and increased efflux mechanisms acting together. In addition, a ribosome protection mechanism may be involved (235, 236).

Two different experimental approaches have implicated an efflux mechanism as the major factor in Tc resistance. Using spectrofluorimetry to measure drug uptake under conditions where cells remained metabolically active, it was shown that resistant cells bound less Tc and that accumulated Tc was rapidly lost by an energy-dependent process when the cells were transferred to a drug-free medium (16). In other studies, inverted cytoplasmic membrane vesicles prepared from Tc^r bacteria accumulated Tc by an energy-dependent process (163, 258). The Tc transport system (which presumably determines efflux from whole cells) had a high affinity for Tc (163), was saturable and energy dependent, had different pH and Mg^{2+} requirements compared with transport into sensitive cells, and was competitively inhibited by minocycline (258). Clearly this represents a novel plasmid-encoded Tc-specific transport system.

It has been argued that reduced Tc accumulation is not sufficient to explain high-level Tc resistance, since resistant cells still take up a substantial quantity of drug (234, 236). There is no evidence for compartmentalization or inactivation of the drug in resistant cells (232). Several reports have suggested that ribosomes in Tc^r cells may be protected from the inhibitory effects of the drug (232, 236). However, the level of protection detected in vitro was small and the *tet* product involved has not yet been identified. In one study, polypeptide synthesis directed by endogenous message or by polyuridylic acid in unfractionated lysates of resistant bacteria was two- to threefold more resistant to Tc inhibition than in extracts of susceptible cells (236). There is some doubt about the validity of this finding. Attempts to repeat the experiments with unwashed and washed ribosomes have failed, and furthermore, toluene treatment of Tc^r cells completely eliminated the resistance of polypeptide synthesis to Tc inhibition (I. Chopra, personal communication).

There has also been some speculation about the *tet* repressor functioning as a resistance protein, possibly by binding Tc (18). The data that led to this suggestion were obtained from experiments with multicopy *tet* plasmids. Whether there is sufficient repressor in cells carrying a single copy of Tn*10* to significantly lower the Tc concentration is questionable. It is possible that binding of Tc to repressor in uninduced cells delays the inhibitory effect of the drug on protein synthesis sufficiently for the resistance gene to be transcribed and translated. Also, the proposal that the repressor is a membrane protein needs to be confirmed. Evidence for membrane association was presented for a hybrid repressor–β-galactosidase protein but not for the repressor itself (18).

Structure and function of the Tn*10* Tc resistance region. The Tc^r determinant of Tn*10* has been studied in most detail at the molecular level. Our current understanding of the genetic organization and regulation of the Tn*10* *tet* genes is summarized in Fig. 11. *tetR* (repressor) and *tetA* (resistance gene encoding TET protein) of Tn*10* are transcribed divergently from a common regulatory region that contains overlapping promoters (166; Fig. 11). The direction of transcription was deduced by studying polypeptides synthesized by cloned fragments and by *tet* mutant plasmids in minicells (18, 69, 193, 430). The genetic organization was confirmed by DNA sequence analysis (166). The regulation of *tet* gene expression is discussed below.

Two Tc-inducible polypeptides specified by Tn*10* have been unequivocally assigned to *tet* genes. These are the 23.5K repressor protein and the membrane-bound TET protein. The TET protein was originally identified in *E. coli* minicells harboring plasmid R222, which carries Tn*10*. Its molecular weight has been reported as 50,000 or 36,000 depending on the gel electrophoresis buffer system used (110, 193, 233, 438, 444). DNA sequence analysis of the Tn*10* *tet* region shows a single translational reading frame that spans the entire resistance gene region (166). This would encode a polypeptide of 43.3K (Fig. 11). The discrepancy between the molecular weight measured by electrophoresis and that deduced from the DNA sequence could be explained if the hydrophobic protein migrated aberrantly, as has been shown for other membrane proteins (276). Post-translational processing of the TET protein does not seem likely because the predicted N-terminal amino acid sequence does not suggest a leader sequence and because TET protein synthesized in minicells and in vitro migrated at the same rate (166, 438).

It has also been suggested that a Tc-inducible polypeptide of 15K may be specified by Tn*10* (438), but this protein has not been consistently

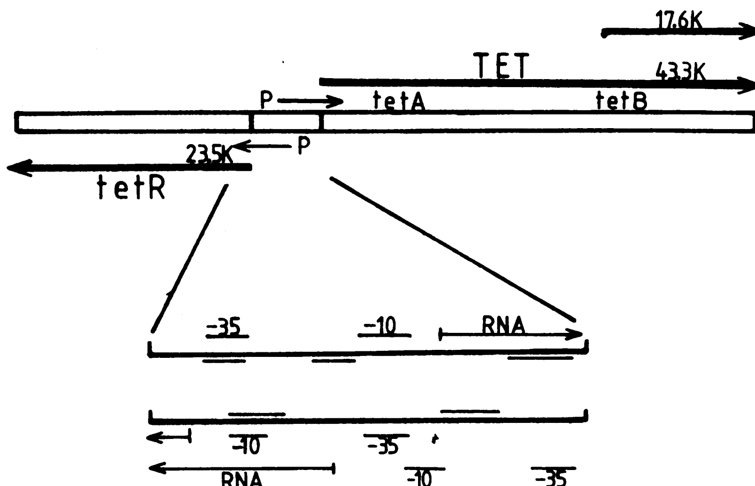


FIG. 11. Genetic organization and regulation of the Tc resistance genes of Tn*10*. The upper part shows the organization of the *tet* region of Tn*10*. The *tetR* gene is transcribed from right to left from promoters located in the central regulatory region. It encodes the 23K repressor protein, which binds to operator sites to prevent transcription of both *tetR* and the TET structural gene. DNA sequence analysis indicates that the TET resistance gene encodes a polypeptide of 43.3K which migrates with an apparent molecular weight of 36,000 in sodium dodecyl sulfate-polyacrylamide gels. The location of mutations which form complementation groups *tetA* and *tetB* is indicated. An expanded diagram of the regulatory region is shown at the bottom. The −35 and −10 regions of the promoters for the *tet* resistance gene and the *tetR* repressor gene are shown, as well as the transcriptional start points (→) and regions of the DNA sequence protected from DNase digestion by repressor binding (—).

seen in minicell experiments. A protein of this size was identified in the cytoplasmic membrane of resistant bacteria, using immunological methods (Chopra, personal communication). However, there is no evidence that this is a product of a *tet* gene required for expression of resistance. The Tn*10* *tet* DNA sequence reveals an open translational reading frame overlapping the promoter-distal part of the *tetA* coding sequence, which could encode a protein of 17.6K (166; Fig. 11). This might be responsible for the low-molecular-weight Tc-inducible polypeptide, but any function in resistance is dubious.

Genetic complementation tests performed between compatible plasmids carrying different Tc-sensitive mutations provided evidence for two complementation groups called *tetA* and *tetB* (68, 79) (Fig. 11). Both map within the *Hinc*II fragment known to carry the *tet* structural gene(s). Since there is a single coding sequence spanning the *tetA-tetB* region (166) and the complementation tests failed to generate a wild-type high-level Tc^r phenotype, it is likely that Tc resistance was generated by intragenic complementation resulting from the interaction of two different mutant TET proteins. The complementation groups may represent two functional domains within the TET protein rather than different polypeptide products of two cistrons. The membrane-bound TET protein may be a homomultimer.

The 25K Tc-inducible polypeptide seen in λTn*10*-infected UV-irradiated cells (444) probably corresponds to the 23.5K repressor protein identified by studying in vitro generated recombinant *tet* plasmids in minicells (18, 430). That the repressor is itself inducible by Tc indicates that it regulates its own expression.

The *tet* determinants of RP1 (TetA group; S. Waters and J. Grinsted, personal communication), Tn*1721* (TetA group; R. Schmitt, personal communication), and pSC101 (TetC group; 360) have a structural organization similar to that of the TetB group determinant of Tn*10*. Furthermore, significant DNA sequence homology exists between these determinants in the region specifying the resistance proteins. These Tet determinants have clearly evolved from a common ancestor, but their DNA sequences have diverged to an extent which prevents detectable hybridization (260). The predicted amino acid sequences of the TET proteins reveal the following homologies: RP1-Tn*10*, 45%; RP1-pSC101, 52%; Tn*10*-pSC101, 25% (Chopra, personal communication). The conserved amino acids occur in localized regions which could be important in the function of the protein.

The TetC determinant of pBR322 (derived from pSC101) may be organized in a slightly different fashion from the Tn*10* and Tn*1721* elements (372). According to the DNA sequences, the TET protein of pSC101 lacks 56 N-terminal amino acids from the corresponding region of the Tn*10* TET protein (Chopra, personal communication). However, the reported molecular weight of the pSC101 TET protein is 34,000 (379), which is only 2,000 smaller than that of the Tn*10* protein. The DNA sequence of pBR322 also indicates that a small protein could be encoded by a promoter-proximal reading frame which partly overlaps the coding sequence for the N-terminus of the TET protein (372). This region has some homology with the corresponding region of Tn*10* and could code for one of the Tc-inducible polypeptides specified by pSC101 in minicells (379). A frameshift mutation within the coding sequence for the ancestral TET protein could account for this change.

Regulation of *tet* gene expression. Molecular, genetic (18, 430, 438), and DNA sequence analyses (166) of the Tn*10* *tet* genes show that the repressor and structural genes are transcribed divergently from a common regulatory region. The repressor protein negatively regulates the transcription of the *tetA* gene as well as that of the *tetR* gene (15, 430). DNase protection experiments showed that the repressor binds to several parts of the regulatory region, some of which overlap the −35 region of *tet* promoters (166; Fig. 11). The Tn*1721* *tet* genes are organized and regulated in the same way (R. Schmitt, personal communication). In the presence of Tc the repressor is presumably detached from the operators, allowing transcription of both genes. Biochemical analysis of the interaction of purified repressor protein with Tc and with a small DNA fragment carrying the operators (165, 167) supports this interpretation.

DNA sequence analysis of the Tn*10* and Tn*1721* *tet* regions revealed two promoters capable of initiating transcription of *tetR*, both of which are active in vitro (166; W. Hillen and R. Schmitt, personal communications). The function of the two *tetR* promoters is not known, but it is possible that one is not fully repressed and provides a "basal" level of repressor synthesis in uninduced cells.

Another aspect of the expression of Tc^r by Tn*10* is the reduction in resistance level when *tet* genes are present in multiple copies (69). In contrast, TetA and TetC determinants express higher levels of resistance when the *tet* gene copy number is raised (43, 328, 329, 421). Most of the transposon Tn*5* insertions in the coding sequence for the TET protein prevented expression of high-level Tc^r from a single copy of the wild-type Tn*10* in the chromosome (43). Thus, amino-terminal fragments of the TET protein could still interfere with Tc resistance. Overproduction of the repressor was shown not to be

responsible for the negative gene-dose effect (43). One possible explanation is autogenous regulation of translation of the TET protein when it is overproduced. Alternatively, overproduction of the TET protein might simply be deleterious to the cell and prevent growth.

Resistance to Cadmium

Cadmium (Cd) ions are taken into sensitive bacterial cells by the energy-dependent manganese transport systems (397, 398, 419), where they cause rapid cessation of respiration by binding to sulfhydryl groups in proteins.

Resistance to Cd^{2+} is a common plasmid-specified function in *S. aureus* (103, 106, 281, 355). It has not been encountered in other bacterial genera. Two distinct Cd resistance determinants have been recognized: (i) CadA, which causes a 100-fold increase in resistance level due to a specific Cd^{2+} efflux system (295, 355, 398); and (ii) CadB, which specifies a lower level of resistance than CadA by an uncharacterized mechanism which does not involve decreased accumulation (295). Cd resistance is frequently associated with the large "penicillinase" plasmids (280, 281, 355). Some plasmids (e.g., pII147) specify both CadA and CadB resistance determinants, whereas others (e.g., pI258) carry only CadA determinants (280). A small multicopy CadA plasmid has also recently been described (106). Both CadA and CadB determinants are expressed constitutively and also confer resistance to zinc ions (295).

It has been known for some time that high-level resistance to Cd^{2+} (specified by CadA determinants) involves decreased accumulation (55, 56, 209, 419). It is now clear from the detailed studies of Tynecka et al. (397, 398) that Cd^{2+} resistance is caused by a plasmid-encoded efflux system. Experiments with uncouplers and ionophores showed that resistant strains expelled Cd^{2+} via a $Cd^{2+}/2H^+$ antiport system which is energized chemiosmotically by proton circulation across the membrane. Thus, one Cd^{2+} ion is exchanged for two protons and at external Cd^{2+} concentrations below 100 μM no inhibition of growth or respiration occurs. Attempts to demonstrate efflux of Zn^{2+} have so far failed (295).

Resistance to Arsenate

Arsenate ions enter bacterial cells via the phosphate transport systems (423). Arsenate is toxic to bacteria because it is an analog of phosphate and can inhibit enzymes such as kinases. Also, arsenylated sugars hydrolyze spontaneously, resulting in a loss of free energy in glycolysis (369).

Resistance to arsenate is determined by plasmids in *S. aureus* (280, 281, 347) and enteric bacteria (159, 354). Arsenate resistance is invariably inducible and is linked to arsenite and antimony(III) resistance determinants in both groups of bacteria. Resistance to the three ions is coordinately induced, with each ion being capable of acting as an inducer at subtoxic concentrations (347). In addition, bismuth(III) can induce these resistance determinants, but resistance to bismuth is not expressed (347). The arsenate resistance determinant is distinct from arsenite and antimony(III) resistance. Resistance to arsenate is known to be due to a specific efflux mechanism (see below), whereas resistance to arsenite and antimony(III) is poorly understood. In addition, the arsenate and arsenite resistance determinants are genetically separable (280).

The arsenate resistance determinants of both *S. aureus* and *E. coli* specify an efflux system which has recently been studied in some detail by measuring the loss of $^{74}AsO_4^{3-}$ from preloaded resistant cells (269a, 348). The efflux system is highly specific for arsenate. Unlike the uptake systems it does not recognize phosphate. In contrast to the Cd efflux mechanism, arsenate efflux is not driven chemiosmotically and is not dependent on membrane potential or a pH gradient across the membrane. Rather, it is driven by hydrolysis of ATP or some other high-energy cofactor.

ALTERATION TO THE ANTIBIOTIC TARGET SITE

Resistance to Antibiotics in the MLS Group

MLS antibiotics are a structurally diverse group which cause a bacteristatic effect by binding to the 50S ribosome subunit of susceptible bacteria and inhibiting protein synthesis (for a review, see reference 130). Resistance to MLS drugs in natural isolates is caused by methylation of adenine residues in 23S rRNA which prevents the drugs from binding to their target. The MLS resistance phenotype is often plasmid encoded (see below). Many gram-negative bacteria are intrinsically resistant to these drugs because of their impermeable outer membranes and possibly because of the level of methylation of rRNA (71, 380). However, *Bacteroides* spp. are susceptible to the lincosamide drug clindamycin. Mutations in three chromosomal loci cause higher resistance to macrolides (including erythromycin) in *E. coli*. Lesions in *eryA* and *eryB* alter ribosomal proteins L4 and L22, respectively (427), whereas *eryC* mutations probably affect maturation of 16S and 23S rRNA (288). The resistance phenotypes conferred by these mutations are narrower than naturally occurring MLS resistance.

Occurrence of MLS resistance in natural isolates. Resistance to MLS antibiotics is specified by plasmids in the gram-positive bacteria *S. aureus* and *Streptococcus* spp. (65, 173) and in the gram-negative *Bacteroides* spp. (420). Chromosomally located MLS resistance determinants have been found in *Bacillus licheniformis* (98) and *Streptococcus pneumoniae* (65) and in several species of *Streptomyces* which produce MLS antibiotics (125). The *S. aureus* pI258 determinant is located on a transposon Tn*551* (279) and the *Streptococcus faecalis* plasmid pAD2 has an interesting Em^r transposon Tn*917*, in which transposition is inducible by erythromycin (392).

DNA hybridization studies indicate that there are at least three distinct groups of MLS resistance determinants in gram-positive bacteria. The Em^r gene of Tn*551* has significant homology with the streptococcal determinants (415) but not with the *S. aureus* plasmid pE194. The Em^r determinant of *B. licheniformis* does not hybridize with either staphylococcal determinant (98). The relationship between these determinants and the *Bacteroides* sp. and *Streptomyces* sp. genes has not been tested. It is interesting to note that although homology was not detected between the Em^r regions of pE194 and pAM77 (which is related to the pI258::Tn*551* determinant) by Southern blotting, significant similarity was revealed by comparing the DNA sequences and the derived amino acid sequences of the methylase proteins (173).

Mechanism of resistance to MLS antibiotics. Resistance to MLS drugs is caused by specific N^6,N^6-dimethylation of adenine residues in 23S rRNA (216–218). The methylase encoded by plasmid pE194 has been purified to homogeneity and studied in vitro (344). It is a 29,000-dalton polypeptide which depends on *S*-adenosylmethionine for its activity. In vitro, it will methylate free 23S rRNA and the 50S ribosome subunit, but not the 70S ribosome. Access to the methylation site on the 50S subunit may be sterically blocked when it interacts with the 30S particle. Ribosomes with dimethylated 23S rRNA bind MLS antibiotics less effectively and are not inhibited by the drugs (218).

Induction of MLS resistance determinants. Some MLS resistance determinants in *S. aureus* and streptococci are inducible, whereas others [e.g., *S. aureus* (pI258)] are expressed constitutively. The number of antibiotics that can induce the MLS resistance phenotype is limited. The pE194 determinant can only be induced by erythromycin and oleandomycin (171, 345, 413, 414), whereas other staphylococcal determinants can be induced by these drugs and by carbomycin (3). This phenomenon has been described as "dissociated" resistance (48, 132) and means that resistance to the full range of MLS antibiotics can only be expressed after induction by one of the limited range of inducers, or when constitutive mutants are selected. The reason for this can now be explained in molecular terms (see below; 146, 171, 345).

The *Streptococcus pyogenes* MLS resistance determinant is induced by erythromycin and lincomycin (239, 240). However, this organism expresses an unusual zonal pattern of resistance to lincomycin (239, 240). Cells only grow at below 0.06 μg or between 60 and 250 μg of lincomycin per ml. Growth at intermediate lincomycin concentrations occurs only after induction with erythromycin or with constitutive mutants. At concentrations between 0.06 and 60 μg/ml, lincomycin cannot induce resistance and cell growth is inhibited.

Further variation in induction patterns and resistance phenotypes occurs among the various *Streptomyces* species that produce MLS antibiotics (125).

Regulation of expression of MLS resistance encoded by plasmid pE194: the translational attenuation model. The MLS resistance determinant (*ermC*) located on *S. aureus* plasmid pE194 has been subjected to detailed biochemical and genetic analysis in the laboratories of Dubnau (146, 345) and Weisblum (171, 172). Induction of *ermC*-encoded resistance by erythromycin and oleandomycin occurs by a mechanism that results in an increased rate of translation of *ermC* mRNA rather than by stimulating transcription of the gene. This induction occurs in the presence of inhibitors of transcription (345) and results in the mRNA becoming more stable, probably because of changes in its secondary structure.

The DNA sequence of the *ermC* regulatory region provided important clues about the regulatory mechanism (140, 171). The *ermC* message has two open translational reading frames, each preceded at the appropriate position by a ribosome binding site showing the strong homology to 16S rRNA typical of gram-positive bacteria (146, 171, 254). One reading frame could specify a short 19-amino acid peptide. The other determines the 29K methylase. There are six repeated sequences in this region of the message which have the potential to form a variety of hairpin loop structures (140, 146, 171, 172) (Fig. 12). In uninduced cells the ribosome binding site (SD2) or the translational initiation codon (AUG2) for the methylase or both may be masked by secondary structure. The translational attenuation model (140, 146, 171) suggests that induction is effected by altering the secondary structure of the transcript so that SD2 and AUG2 are unmasked (Fig. 12).

The model suggests that an Em-sensitive ribo-

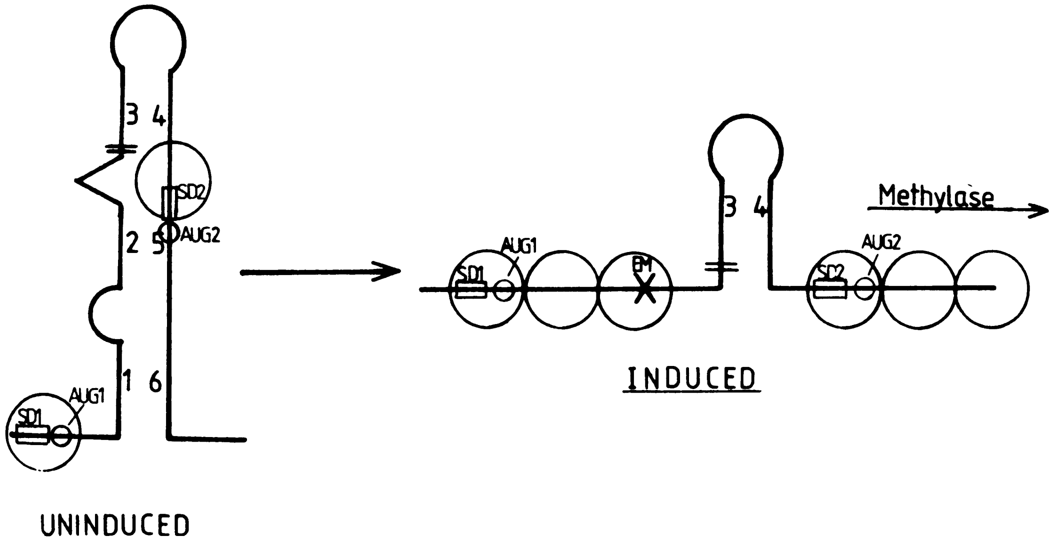


FIG. 12. Induction of Em resistance by translational attenuation. Possible secondary structures that could occur at the 5′ end of the *ermC* message are indicated. The open boxes represent the ribosome binding sites for the 19-amino acid leader peptide (SD1) and for the methylase (SD2). The small circles depict the translational start signals AUG1 and AUG2. The large circles are ribosome particles. The change in secondary structure of the mRNA which is thought to be induced by stalling of the ribosomes with bound Em during the course of translating the leader peptide is shown. This allows ribosomes to bind to AUG2 and to commence translating the methylase.

some stalls during translation of the leader peptide in the presence of the drug. This allows the mRNA to change structure and thus exposes SD2 and AUG2. Resistant ribosomes or sensitive ribosomes without bound drug will then translate the methylase. When sufficient ribosomes have been methylated, the stalling during translation of the leader will no longer occur and the mRNA will revert to the uninduced secondary structure.

One feature of this model is the inhibitory activity of antibiotics that can induce resistance. Em will only bind to free ribosomes, not to those engaged in protein synthesis on polysomes (296, 378). The Em-bound ribosome participates in limited translation before stalling occurs. It seems significant that the drugs capable of inducing resistance are those which allow translation to occur for the longest period before the ribosomes stall (210, 242, 243). Also, the structure of the amino acids being incorporated into the peptide may be important determinants of where the ribosomes stall. The bulky hydrophobic side chains of the proline at position 16 and the lysines at positions 18 and 19 in the leader peptide may favor stalling towards its carboxy terminus. The amino terminus has many residues which are free of hydrophobic side chains and which will continue to be incorporated into the polypeptide by Em-bound ribosomes (47, 242, 243).

If the ribosome stalls in repeat sequence 2, there is a possibility that a conformational change in mRNA structure will occur and that repeat sequences 3 and 4 will pair (Fig. 12). This rearrangement will expose the AUG2 for the methylase and will allow translation of the protein. It is conceivable that SD2 is occupied by a ribosome in a preinitiation complex which is poised to commence translation immediately when the conformational change occurs.

Structure of nascent transcripts and basal expression of methylase. The *ermC* gene is transcribed continuously in uninduced MLS^+ cells. The kinetic trapping model (Fig. 13; 146) proposes that the message folds in different configurations as transcription proceeds. Initially, a fairly stable structure (A in Fig. 13) is formed before the distal end of the regulatory region is transcribed. Thus, SD2 will be exposed immediately as it is transcribed, allowing ribosomes to bind and a short burst of translation of methylase protein to occur before the structure of the message changes. When repeat sequences 5 and 6 are transcribed, SD2 and AUG2 will become trapped because the mRNA will rearrange into a more stable form (B and C in Fig. 13).

The paradox of induction by a mechanism involving translational attenuation. The translational attenuation model proposes that Em induces translation of the methylase by stopping the translation of the leader peptide. This event is required to stimulate the mRNA structural change. How can this occur when many ribo-

somes in the cell have drug bound to them? First, basal expression of methylase renders 5% of the ribosomes in uninduced cells resistant. Thus, SD2 has a 1 in 20 chance of being bound by a resistant ribosome and some transcripts will be translated by such ribosomes immediately after induction. Second, at low drug concentrations many sensitive ribosomes will avoid being inactivated by Em before initiating translation of methylase. Once translation starts the drug cannot bind to the ribosome. Third, the ribosomes in the putative preinitiation complex at SD2 may not be able to bind Em. Thus, transcripts may be primed to begin translation as soon as attenuation occurs, ensuring that the first round of translation cannot be halted by the drug.

UNCHARACTERIZED RESISTANCE MECHANISMS

Resistance to Fusidic Acid in *S. aureus*

Fusidic acid is a steroid antibiotic which inhibits protein synthesis by interfering with the translocation protein EFG_1 (130). Chromosomal mutations which cause G factor to bind fusidic acid with decreased efficiency have been isolated in *E. coli* (130). Chromosomal mutations causing Fu^r can also be easily isolated in *S. aureus* (213). Despite this, Fu^r clinical isolates of *S. aureus* usually have plasmid-borne resistance determinants (213, 214), possibly because strains with chromosomal mutations are less virulent (57).

The expression of plasmid-encoded Fu^r in *S. aureus* is rapidly inducible (57). It does not protect ribosomes and it does not cause detectable drug modification or inactivation (57). Despite the failure to demonstrate decreased fusidic acid uptake by resistant cells, it was postulated that the resistance mechanism resulted in decreased permeability because of a change in the ratio of phosphatidylglycerol to lysylphosphatidylglycerol in resistant cells. There is no evidence that resistance is associated with CAT, as it is in enteric bacteria (406), but an intracellular binding mechanism cannot be ruled out.

Nonenzymatic Chloramphenicol Resistance in Gram-Negative Bacteria

Some Cm resistance determinants in gram-negative bacteria do not express CAT (128, 129, 271). As with the fusidic acid resistance determinant of *S. aureus*, the nonenzymatic Cm^r phenotype is inducible, does not cause detectable drug modification or inactivation, and does not protect ribosomes against Cm inhibition in vitro (128). A permeability mechanism operating at the level of the cytoplasmic membrane is suspected because spheroplasts still express resistance and also because cells carrying both enzymatic and nonenzymatic Cm resistance determinants do not acetylate the drug (128). The nonenzymatic Cm^r determinants of R26 and R55-1 do not confer high-level resistance to the fluorinated analogs of Cm (99).

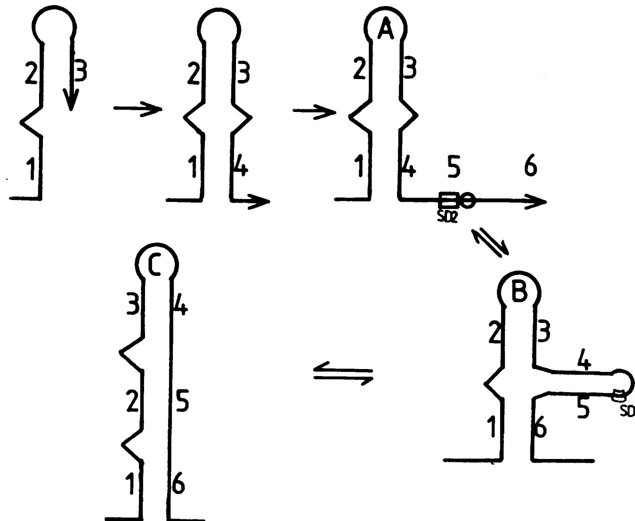


FIG. 13. Kinetic trapping model. Changes that could occur in the secondary structure of the 5′ end of the *ermC* message during the course of transcription are shown. Ribosomes might bind transiently to SD2 to allow a short burst of methylase synthesis (structure A) before more stable mRNA structures (B and C) can form and sequester SD2/AUG2 initiation signals upon completion of the transcription of the region.

Resistance to Fosfomycin

Fosfomycin interferes with bacterial cell wall synthesis by inhibiting UDP-*N*-acetylglucosamine-3-*O*-enolpyruvyl transferase (195). The drug enters the *E. coli* cell via the L-α-glycerophosphate (*glpT*) or the hexose-6-phosphate (*uph*) transport system (195, 317). Chromosomally specified resistance to fosfomycin is due to defects in one of these transport systems or to mutations in the target enzyme (195, 404).

In clinical isolates the most frequently encountered mechanisms of resistance to fosfomycin are mutations which impair drug transport (J. M. Ortiz, personal communication). Plasmid-specified fosfomycin resistance in *Serratia marcescens* has been reported (261). In one case a transposon was implicated (131). There was no impairment of drug transport in resistant cells (228). Recently, evidence has been obtained for an intracellular drug inactivation mechanism (J. Leon, J. M. Garcia-Lobo, and J. M. Ortiz, personal communication).

Resistance to Cadmium Ions Specified by the CadB Determinant

Unlike the CadA determinant which promotes efflux of Cd^{2+} from resistant cells (398), CadB does not alter the rate of Cd^{2+} accumulation in whole cells or in vesicles. In contrast, resistant cells bind more Cd^{2+} than sensitive cells, which suggests that an intracellular binding mechanism might be operating (295). An analogy was drawn between CadB resistance and metallothionein proteins of *Neurospora* sp. (229), yeast (118), and blue-green algae (cyanobacteria) (285) which bind Cd^{2+}, Zn^{2+}, and Cu^{2+} strongly.

Resistance to Arsenite and Antimony(III)

Arsenite and antimony(III) are toxic to bacteria because they bind to cysteine residues in proteins (2). Resistance to arsenite and antimony(III) is linked to and coordinately induced with arsenate resistance (347). Little is known about the mechanism of resistance to arsenite and antimony(III). Excretion of a chelating agent (e.g., dimercaptal) into the medium was ruled out (347) as was the possibility that arsenite was oxidized to arsenate and then eliminated by the arsenate efflux system (369). An intracellular binding mechanism seems unlikely because no difference in binding was found between resistant and sensitive cells (347).

Resistance to Silver Ions

The occurrence of plasmid-mediated resistance to silver ions in clinical isolates of gram-negative bacteria probably reflects the use of silver salts to treat burns (12, 253). Silver is toxic to bacteria because it interferes with respiration (29) and other cell surface-associated functions (122, 322).

The silver resistance determinant is expressed constitutively and confers a high level of resistance (346). Resistance to $AgNO_3$ is dependent on the presence of halide ions. Susceptible cells can extract Ag^{2+} from an $AgCl_2$ precipitate, whereas resistant cells cannot. These determinants also confer halide-independent resistance to silver-sulfadiazine. Thus, silver resistance appears to be due to a function which is probably located at the cell surface and which prevents cells from extracting silver, which is in an insoluble or slowly released form. The mechanism cannot exclude soluble silver salts.

Resistance to Other Heavy Metal Compounds

Plasmid-specified resistance to bismuth, lead, boron, chromium, cobalt, nickel, tellurium, and zinc compounds has been described previously (364, 365, 369), but nothing is known about the mechanisms involved.

Resistance to Ethidium Bromide

Some strains of *S. aureus* are resistant to ethidium bromide, proflavine, acriflavine, and acridine (107, 192). The determinant is often located on penicillinase plasmids. Little is known of the resistance mechanism, but decreased permeability has been suggested on the basis of binding experiments (192). It is possible that this determinant has been selected in the staphylococci by the use, until recently, of acriflavine as an antiseptic.

MECHANISMS FOR INCREASING EXPRESSION OF ANTIBIOTIC RESISTANCE DETERMINANTS

Amplification by Tandem Duplication

The activity of a genetic determinant can be increased by elevating the number of gene copies by tandem duplication (12). Chromosomal genes in eucaryotes (118, 197, 407) and procaryotes (12, 104), as well as plasmid-borne antibiotic resistance determinants in bacteria (292, 328, 329, 421, 431), can be amplified by this mechanism. Tandem duplications are often initiated by legitimate recombination between directly repeated DNA sequences which flank the gene (Fig. 14 and 15). These are promoted by host recombination enzymes. Some plasmid-borne antibiotic resistance determinants have evolved to facilitate duplications by having long (700 to 1,200 bp) direct repeat sequences to stimulate the recombination event. They include the drug resistance r-determinant region of IncFII R plasmids (50, 176, 303), the Tc^r genes of Tn*1721* (328, 421), and the kanamycin resistance determinant of Rts1 (179) (Fig. 16).

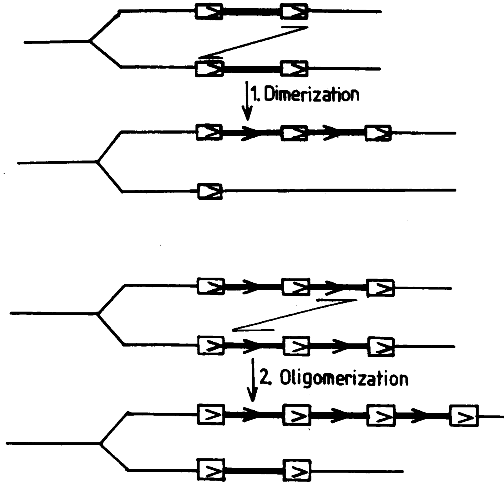


FIG. 14. Model for formation of tandem duplications by unequal crossing over. The open boxes represent the direct repeat sequences that flank amplifiable genes. The antibiotic resistance gene is indicated by the thicker line between the two repeats. Recombination is represented by the thin lines.

Amplification can be selected in the laboratory by prolonged exponential growth in liquid medium containing a high but subinhibitory concentration of drug. Cells expressing higher resistance levels will outgrow those carrying nonamplified resistance genes (292, 328, 329, 421, 431). Alternatively, the amplified cells which exist in the population can be directly selected by plating on agar containing a concentration of drug which is inhibitory to nonamplified cells (157, 421). When the drug is removed from the medium the plasmid population will return to the nonamplified state because tandem duplications are inherently unstable (12, 292, 421).

Two models have been proposed to explain how tandem duplications can occur (176, 262, 303, 431) (Fig. 14 and 15). A common feature is that the duplication is initiated by *recA*-dependent homologous (legitimate) recombination between directly repeated DNA sequences which flank the resistance gene. In model 1 (Fig. 14) the duplication is initiated during DNA replication and requires a double but unequal crossover event. Model 2 (Fig. 15) requires the resistance gene to be excised by a single crossover and then to integrate into another plasmid carrying an intact resistance determinant. Model 1 predicts that any small circular molecules present will be due to excision of the tandemly duplicated sequences. Indeed, this probably leads to loss of duplicated sequences during the reversal of amplification that occurs during growth in drug-free medium. In model 2 the excised circular resistance determinants are intermediates in the duplication process.

Amplification (or "transition") of the drug resistance (r-determinant) region of plasmid NR1 (Fig. 16) in *Proteus mirabilis* (157, 292, 321) requires the directly repeated copies of the 760-bp insertion sequence IS*1* which flank the resistance genes to provide homology for the initial duplication to take place (50, 176, 303). The scheme proposed for transition was essentially the same as model 2 (Fig. 15), with the added possibility that independent r-determinants might increase their copy number by replicating autonomously (176, 293, 303). However, it now seems unlikely that r-determinant monomers can replicate autonomously, at least in *E. coli* (52). A proposal that transition occurred by amplification of the entire plasmid population rather than by selective outgrowth of a few cells carrying preamplified plasmids (294) needs to be substantiated.

A more complicated type of transition was reported for plasmid NR84, which is related to NR1 (321). Several different regions of the r-determinant were amplified in different experiments (321). Perhaps insertion sequences IS*1* and IS*10* transposed to different sites in the plasmid before amplification to provide alternative direct repeats to the IS*1* elements that flank the r-determinant.

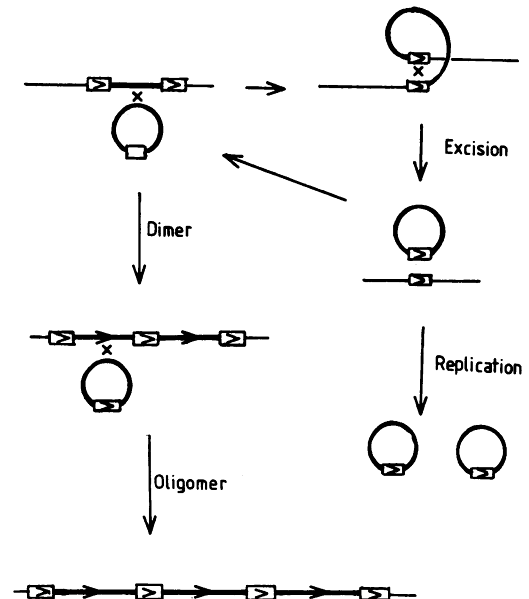


FIG. 15. Model for formation of tandem duplications by excision and reinsertion of the resistance gene. The open boxes represent the direct repeat sequences that flank amplifiable resistance genes. The antibiotic resistance gene is indicated by the thicker line. Recombination events are indicated by ×.

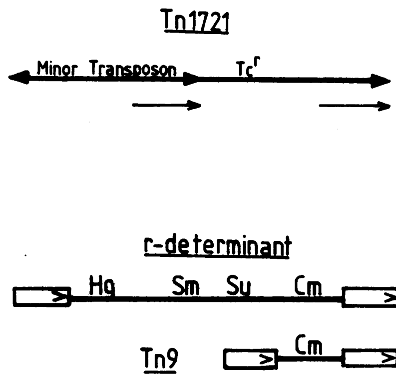


FIG. 16. Structure of amplifiable drug resistance determinants. The amplifiable Tc resistance determinant of transposon Tn*1721* is shown in the upper diagram. The filled-in arrows represent the 38-bp repeat sequences which mark the termini of the minor and major transposons of Tn*1721*. The lower line shows the position of the 1,200-bp direct duplication of part of the minor transposon which provides homology for the initiation of tandem duplications. The amplifiable drug resistance r-determinant region of the R plasmid NR1 and the related transposon Tn*9* are shown in the lower diagrams. The open boxes represent the directly repeated IS*1* sequences which flank the resistance genes. These elements confer resistance to Hg, Cm, Sm, and Su.

Transposon Tn*9* is flanked by direct repeats of IS*1* (Fig. 16), and appears to be derived from a plasmid related to NR1 by deletion of a substantial portion of the r-determinant (178). Transposons with similar structures have been isolated from the r-determinant of R100, which is identical to NR*1* (178). Unlike the r-determinant, these small IS*1*-flanked transposons can be amplified in *E. coli* (51, 262). Kanamycin resistance transposon Tn*2680* from plasmid Rts1 is flanked by 800-bp direct repeats and is also amplifiable (179).

The Tc^r gene contained within transposons Tn*1721* and Tn*1771* can be amplified by tandem duplication in *E. coli* (327–329, 421). Part of the region of the transposon which encodes transposition functions is repeated on the other side of *tet* to provide a 1,200-bp direct sequence duplication (327; Fig. 16). The amplified forms (carrying up to nine copies of *tet*) were only found in Rec$^+$ strains but could be transferred to and stably maintained in a RecA$^-$ mutant (421). This demonstrates that homologous recombination is required for both amplification and the reduction of duplications.

The *tet* gene of *Streptococcus faecalis* plasmid pAMα1 is the only reported example of an amplifiable determinant in gram-positive bacteria (66, 431). In this case the presence of directly repeated sequences flanking the *tet* genes have been inferred but not demonstrated directly.

Acquisition of Multiple Copies of a Transposon

Cultivation of *H. influenzae* cells harboring a plasmid with a Tc^r determinant located on a transposon similar to Tn*10* in subinhibitory concentrations of Tc resulted in the emergence of variants harboring plasmids with two, three, or four copies of the transposon (188). A similar process of duplication by intramolecular transposition of Tn*2660* on R6K occurred when mutants expressing elevated levels of ampicillin resistance were selected (170). Intramolecular transposition was at least 10-fold more frequent than intermolecular transposition. Thus, the transposition immunity mechanism of Tn*A*-like transposons only seems to apply to intermolecular transposition into a replicon already harboring that element and has little effect on intramolecular transposition (170, 410).

Increased Plasmid Copy Number

Plasmid JR66 specifies an aminoglycoside phosphotransferase [APH(3')]-II enzyme which modifies neomycin and kanamycin (Table 4). It has little activity on amikacin, and *E. coli* cells carrying JR66 are phenotypically sensitive to the drug. Amikacin-resistant mutants synthesizing increased levels of enzyme which had a substrate profile indistinguishable from the parental enzyme provided sufficient amikacin-modifying activity to confer resistance to the drug (28). The increased enzyme activity was caused by a gene-dose effect due to an increase in plasmid copy number, which was probably the effect of a chromosomal mutation (28). Mutations in plasmid-located replication control genes, which cause an increase in plasmid copy number, also conferred an increased level of drug resistance through a gene-dose effect (277, 381, 399).

Increased Transcription of Resistance Genes

A possible mechanism for increasing the expression of an antibiotic resistance gene is mutation in the promoter, which increases the efficiency of transcription. Such mutations may have occurred in the TEM β-lactamase gene promoter and could be responsible for TEM genes isolated from different host species being transcribed at different rates in *E. coli* (436).

Transcription could also be increased by deletions which link the structural gene to a more efficient promoter or by insertion of a promoter-carrying insertion sequence close to the gene.

ORIGINS AND EVOLUTION OF ANTIBIOTIC RESISTANCE DETERMINANTS

It has been thought for some time that R-plasmid-mediated antibiotic resistance determinants may have evolved before the antibiotic era

of the past 40 years in species of bacteria other than the pathogens and commensals, in which resistance is often encountered today (108). Bacteria in the genus *Streptomyces* have mechanisms to protect their potentially susceptible targets from the antibiotics which they produce. These genes may have been transferred to other bacteria as plasmid- or transposon-encoded drug resistance or both. Also, there is evidence that soil bacteria commonly harbor antibiotic resistance plasmids (26, 98, 108, 299, 337), presumably to help them combat antibiotics released in their environment by competitors. These genes could have transferred to clinically important bacteria either directly or via a number of intermediate hosts and plasmid vectors.

When a gene is transferred into a new host, changes may be selected to allow the protein specified to survive protease activity or because more efficient expression or secretion is required. Genetic drift will occur if amino acid substitutions can be tolerated without altering catalytic properties. Changes in the third "wobble" base of the triplet codons will also occur to change the base sequence of the gene. Semisynthetic antibiotics designed to evade existing resistance mechanisms impose additional selective pressure which may lead to variants of the resistance determinants being selected. Such changes have been selected in the laboratory (147, 291).

Close relationships between resistance determinants can be recognized by comparative techniques such as immunological cross-reaction and isoelectric focusing of the resistance gene protein products and by DNA-DNA hybridization. When substantial variation has occurred, homology may only be recognized by amino acid or DNA sequence analysis. When the nucleotide sequences of resistance genes have been compared, it has been possible to demonstrate evolutionary relatedness even though the DNA failed to hybridize in Southern blot experiments (166, 173, 260). The Southern blotting technique will not detect <50% base sequence homology. The definition of homology groups will also depend on the stringency of the conditions used in the hybridization reaction. Being assigned to different homology groups does not necessarily imply that the determinants have evolved independently. However, if a small degree of homology confined to the active site of an enzyme is found, it could be argued that this is due to convergent evolution. Convergence would certainly be suspected where no homology was detectable (8, 189).

Origin of MLS Resistance Determinants

The streptomycetes that synthesize MLS antibiotics (including *S. erythreus*, the producer of erythromycin) protect their ribosomes from the inhibitory effects of the drugs by N^6, N^6-dimethylation of adenine residues in 23S rRNA in the same way as MLS-resistant staphylococci and streptococci (125, 139, 386). The adenine methylase of *S. erythreus* was shown to be a determinant of MLS resistance when it was transferred into *S. lividans* (386, 387). DNA sequence analysis of Em^r determinants representing the two homology groups found in staphylococci and streptococci revealed significant homology despite their failure to hybridize in Southern blot experiments (173). It will be very interesting to discover whether such homology exists between the staphylococcal/streptococcal determinants and that of *S. erythreus* to show whether the two groups are evolutionarily related.

It is appropriate to mention here that streptomycetes which produce thiostreptone and related antibiotics protect their ribosomes by 2-*O*-pentose methylation of an adenosine residue in 23S rRNA (78, 385). However, as yet these determinants have not been found in other bacteria.

Origins of Aminoglycoside-Modifying Enzymes

Two mechanisms of resistance to autotoxic aminoglycoside antibiotics occur in producing bacteria. (i) Ribosomal resistance has been found in the gentamicin producer *Micromonospora purpurea* (297) and in the istamycin-producing *Streptomyces tenjimaniensis* (431). In both cases the spectrum of resistance conferred to other aminoglycosides was unique and drug-modifying activity was not detected in extracts. (ii) Resistance which is probably due to drug modification occurs in streptomycetes that produce kanamycin [AAC(6)?; 175], neomycin [APH(3′); 73, 175], streptomycin [APH(6); 175, 248, 362], hygromycin [APH(4)?; 221], and butirosin [APH(3′); 75]. In addition, the producers of the non-aminoglycosides viomycin and capreomycin protect themselves by drug phosphorylation (350).

Ribosomes from the streptomycetes are sensitive to the aminoglycoside antibiotic produced, even during the phase of antibiotic synthesis (45, 46, 175, 361, 362, 363). No active drug is detectable in the cytoplasm during the phase when antibiotic is accumulating in the medium (45). These organisms synthesize phosphotransferases and acetyltransferases, which can inactivate the drugs in vitro (175, 207, 221, 298, 362, 386, 407). In some cases the enzyme is not produced during the exponential phase of growth when the bacteria are phenotypically sensitive to the antibiotic (298, 362). Evidence that these drug-modifying enzymes can act as resistance determinants comes from transfer experiments which result in resistance being con-

ferred in another host (386, 387). This has been demonstrated for *S. fradiae* APH(3') and AAC(6) (which were required together to confer high-level resistance to neomycin; 386, 387) and *B. circulans* APH(3') (75).

An attractive hypothesis for the role of the phosphotransferases in *Streptomyces* spp. is that they are involved in the biosynthesis and excretion of the antibiotic, as well as in determining resistance to exogenous drug (207, 405). The penultimate biosynthetic precursor of aminoglycosides may be an inactive phosphate derivative which is excreted from the cell by an efflux mechanism coupled to phosphatase activity (237, 405). This is supported by the observations mentioned in the preceding paragraph and by the finding that the expression of APH(3') in *S. fradiae* is coregulated with the level of neomycin synthesis and resistance (207).

It thus seems plausible that some R-plasmid-mediated aminoglycoside-modifying enzymes are derived from the enzyme determinants in antibiotic-producing bacteria. The R-plasmid-specified APH(3') of neomycin- and kanamycin-resistant bacteria (Table 4; Fig. 4 and 5) may be related to the *S. fradiae* enzyme, the APH(6) of streptomycin-resistant staphylococci (Table 4; Fig. 7) may be derived from the *S. griseus* and *S. bikiniensis* determinants, and the APH(4) of hygromycin-resistant *E. coli* (Table 4) (R. Rao, personal communication) may be related to the *S. hygroscopicus* enzyme (221). It is also possible that some acetyltransferases evolved in this way. However, there have been no reports of adenyltransferases in antibiotic-producing bacteria.

Early attempts to substantiate this hypothesis by DNA hybridization and immunological cross-reaction failed to show any homology among the APH(3') determinants of gram-negative bacteria, staphylococci, *B. circulans*, and *S. fradiae* (73). However, recent DNA sequence analysis has revealed sufficient homology between the *S. fradiae* and *E. coli* determinants to suggest that they have a common origin (J. Davies, personal communication).

Origin of Tetracycline Resistance

The organisms that produce Tc (*Streptomyces rimosus* and *S. aureofaciens*) may have an inducible mechanism for protecting their ribosomes from the inhibitory effects of Tc. Ribosomes isolated from cells not engaged in Tc biosynthesis are more sensitive to inhibition by Tc in vitro than those isolated from cells actively synthesizing the antibiotic (95, 264). Furthermore, the level of ribosomal Tc resistance in vitro seemed to be correlated with the level of drug production (95, 264). It is conceivable that another component of Tc resistance in *Streptomyces* spp. could involve efflux of the drug. This would be analogous to the plasmid-specified Tc resistance mechanism. This could be examined by cloning the streptomycete Tc^r determinant(s), by elucidating the resistance mechanisms, and by comparing the DNA sequences.

Origins of Chloramphenicol Resistance

Ribosomes isolated from *Streptomyces venezuelae* (the producer of Cm) were found to be sensitive to inhibition by Cm in vitro, irrespective of whether they were isolated from cells actively engaged in producing the drug (238, 405). Furthermore, Cm was not detected in the cytoplasm (238, 405). This implies that the drug is actively exported from the cell. Possibly the penultimate step in Cm biosynthesis is an inactive form which is activated during the export process (405). In addition, the Cm^r determinant can be induced in non-antibiotic-producing cells. It seems possible that the inducible nonenzymatic Cm^r determinants encoded by some Cm^r R plasmids (128, 271) could have originated here.

The common R-plasmid-encoded Cm-inactivating enzyme CAT does not occur in *S. venezuelae*, although it is specified by other streptomycetes (341). There is no evidence that this mechanism evolved in the drug-producing organism. R-plasmid-specified CAT may have evolved from an acetyltransferase involved in activating sugars or amino acids for catabolic pathways.

Proteus mirabilis is phenotypically sensitive to Cm but it produces a small amount of CAT. Cm-resistant variants can be selected which express an elevated level of a CAT enzyme which is closely related to the type I enzyme commonly specified by enteric bacterial R plasmids (336). It is possible that the type I enzyme is derived from the *Proteus mirabilis* determinant.

Origins of β-Lactamases

Three evolutionary distinct types of β-lactamase have been identified on the basis of biochemical criteria and amino acid and DNA sequence comparisons (8, 24, 189). (i) Class A enzymes are penicillinases which are primarily associated with plasmid-specified β-lactam resistance. (ii) For class B, the only example is the *Bacillus cereus* type II enzyme. (iii) Class C includes the chromosomally encoded cephalosporinases of enteric bacteria and *P. aeruginosa*.

The R-plasmid-specified TEM β-lactamase of gram-negative bacteria and the plasmid-specified penicillinases of *S. aureus* have significant amino acid sequence homology and have clearly evolved from a common ancestor (8). They may be derived from the D-alanine carboxypeptidase as originally postulated by Tipper and Stro-

minger (390), since amino acid homologies exist around the serine residues in their active centers (412). However, it could be argued that this limited homology is due to convergent evolution (8).

Comparison of the DNA and amino acid sequences of the TEM and *E. coli ampC* determinants have shown that the enzymes are not related in evolutionary terms (9, 189, 371), although they have similar catalytic mechanisms involving a serine residue in the active site (112, 203). It will be interesting to see whether the plasmid-borne cephalosporinases CEP-1 and CEP-2 (Table 1) have homology with the chromosomally specified enzymes of gram-negative bacteria.

Origins of Sulfonamide and Trimethoprim Resistance

The DHFR and DHPS enzymes that confer resistance to the synthetic drugs Tp and Su were probably not widespread in soil or clinical bacteria before the antibiotic era. It is likely that these determinants became associated with R plasmids after the introduction of the drugs into clinical practice. However, their origins remain a complete mystery, despite the existence (in the case of Tp) of naturally resistant enzymes specified by mammalian cells and bacteriophages (443). It is possible that mutations in chromosomal genes encoding drug-sensitive enzymes could have resulted in drug resistance, although it is difficult to obtain high resistance levels in the laboratory. Alternatively, DHFR could have evolved from a reductase with another substrate. In the case of Tp^r DHFR this event occurred at least twice because there is no detectable homology between the genes encoding the type I and type II enzymes (443).

CONCLUDING REMARKS AND FUTURE PROSPECTS

One of the most important recent developments in the chemotherapy of bacterial infections is the introduction of antibiotic derivatives which evade existing (mainly plasmid-specified) resistance mechanisms. Examples of these are β-lactamase inhibitors, β-lactamase-resistant cephalosporins, and new aminoglycosides. The efficacy of these drugs may prove to be short-lived because existing resistance mechanisms might adapt to deal with them, or new mechanisms of resistance might evolve.

Methicillin is a semisynthetic derivative of penicillin which is not hydrolyzed by staphylococcal penicillinases. It was introduced 20 years ago to combat penicillin-resistant *S. aureus* strains which produced penicillinase. Methicillin-resistant strains which did not inactivate the drug soon emerged. The mechanism of methicillin resistance was poorly understood until it was recently shown to be associated with PBP (target) changes (155, 158). The response of *S. aureus* to methicillin may be repeated by other bacteria becoming resistant to new β-lactams. Indeed, strains of *P. aeruginosa* which are resistant to carbenicillin and cefsulodin because of mutations altering the affinity of PBPs have already been encountered (80, 136, 269, 316). Cephradine resistance in *S. aureus* also involves PBP changes (134). In *P. aeruginosa* the PBP changes may occur in combination with mutations which reduce the permeability of the outer membrane to β-lactams (269, 316). It is possible that the incidence of these mechanisms of resistance will increase as the new β-lactams are used more. Another possibility is the spread of plasmid-specified β-lactamases which can hydrolyze cephalosporins (e.g., CEP-1 and CEP-2; Table 1).

New aminoglycoside antibiotics which inhibit bacteria synthesizing the commonly encountered plasmid-specified modifying enzymes are now available. These include the 5-*epi* isomers of gentamicin and sisomicin, amikacin, and apramycin. Aminoglycoside resistance may also be controlled by inhibitors such as 7-hydroxytopolone, which interferes with AAD(2″) activity (4). However, it seems unlikely that aminoglycoside resistance will be completely contained in this way. The drugs have several sites presenting potential targets for modifying enzymes. It may prove impossible to block or remove all potential modification sites without detracting from antibiotic activity. Also, mechanisms have been described which indicate how bacteria can respond to challenge by new drugs: (i) by acquisition of an enzyme which is capable of modifying the drug; (ii) by mutations which alter the substrate specificity of an enzyme [derivatives of APH(3′) which rapidly modify and confer resistance to amikacin and other minor substrates can be selected (291)]; (iii) by increased expression of an enzyme with poor activity against the new drug, which could occur by increasing the rate of transcription or by a gene-dose effect as described above; (iv) the finding of mutations which impair the critical EDPI phase of drug transport by interfering with membrane energization in clinical strains of *P. aeruginosa* and *S. aureus* (34, 267); (v) by alteration of the ribosome target site. In clinical strains this mechanism has so far been confined to streptomycin resistance (213, 241, 396, 442). It is possible that multiple (stepwise) ribosomal changes conferring resistance to deoxystreptamine-containing aminoglycosides will emerge, possibly in combination with mutations affecting drug transport.

The diversity of the mechanisms by which

bacteria have adapted to antimicrobial drugs and the rapidity with which the resistance has spread are impressive. It seems likely that bacteria will evolve resistance to most, if not all, antibiotics with which they are challenged. Coordinated programs of antibiotic use in clinical and veterinary practice will help to increase the usefulness of drugs by reducing the rate with which resistance emerges.

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