Mechanisms of Bacterial Resistance to Antimicrobial Agents

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ABSTRACT During the past decades resistance to virtually all antimicrobial agents has been observed in bacteria of animal origin. This chapter describes in detail the mechanisms so far encountered for the various classes of antimicrobial agents. The main mechanisms include enzymatic inactivation by either disintegration or chemical modification of antimicrobial agents, reduced intracellular accumulation by either decreased influx or increased efflux of antimicrobial agents, and modifications at the cellular target sites (i.e., mutational changes, chemical modification, protection, or even replacement of the target sites). Often several mechanisms interact to enhance bacterial resistance to antimicrobial agents. This is a completely revised version of the corresponding chapter in the book *Antimicrobial Resistance in Bacteria of Animal Origin* published in 2006. New sections have been added for oxazolidinones, polypeptides, mupirocin, ansamycins, fosfomycin, fusidic acid, and streptomycins, and the chapters for the remaining classes of antimicrobial agents have been completely updated to cover the advances in knowledge gained since 2006.

INTRODUCTION

With regard to their structures and functions, antimicrobial agents represent a highly diverse group of low-molecular-weight substances which interfere with bacterial growth, resulting in either a timely limited growth inhibition (bacteriostatic effect) or the killing of the bacteria (bactericidal effect). For more than 60 years, antimicrobial agents have been used to control bacterial infections in humans, animals, and plants. Nowadays, antimicrobial agents are among the most frequently used therapeutics in human and veterinary medicine (1, 2). In the early days of antimicrobial chemotherapy, antimicrobial resistance was not considered as an important problem, since the numbers of resistant strains were low and a large number of new highly effective antimicrobial agents of different classes were detected. These early antimicrobial agents represented products of the metabolic pathways of soil bacteria (e.g., *Streptomyces*, *Bacillus*) or fungi (e.g., *Penicillium*, *Cephalosporium*, *Pleurotus*) (Table 1) and provided their producers with a selective advantage in the fight for resources and the colonization of ecological niches (3). This in turn forced the susceptible bacteria living in close contact with the antimicrobial producers to develop and/or refine mechanisms to circumvent the inhibitory effects of antimi-

TABLE 1 Origins of antimicrobial agents

Class	Antimicrobial agent	Producing organisms	Year(s) of isolation/description
β-Lactam antibiotics	Natural penicillins	*Penicillium notatum, Penicillium chrysogenum*	1929, 1940
	Cephalosporin C	*Cephalosporium acremonium*	1945, 1953
	Imipenem	*Streptomyces cattleya*	1976
	Aztreonam	*Gluconobacter* spp., *Chromobacterium violaceum*	1981
Glycopeptides	Vancomycin	*Amycolatopsis orientalis*	mid-1950s
	Teicoplanin, avoparcin	*Amycolatopsis coloradensis* subsp. *labeda*	1975
Macrolides	Erythromycin	*Streptomyces erythreus*	1952
	Spiramycin	*Streptomyces ambofaciens*	1955
Lincosamides	Lincomycin	*Streptomyces lincolnensis*	1963
Streptogramins	Streptogramin A+B	*Streptomyces diastaticus*	1953
	Virginiamycin A+B	*Streptomyces virginiae*	1955
Tetracyclines	Chlortetracycline	*Streptomyces aureofaciens*	1948
	Oxytetracycline	*Streptomyces rimosus*	1950
Phenicols	Chloramphenicol	*Streptomyces venezuelae*	1947
Aminoglycosides	Streptomycin	*Streptomyces griseus*	1943
	Neomycin	*Streptomyces fradiae*	1949
	Kanamycin	*Streptomyces kanamyceticus*	1957
	Gentamicin	*Micromonospora purpura*	1963
	Tobramycin	*Streptomyces tenebrarius*	1967
Aminocyclitols	Spectinomycin	*Streptomyces spectabilis*	1961
Pleuromutilins	Pleuromutilin, Tiamulin	*Pleurotus* spp.; synthetic	1951, 1976
Polypeptide antibiotics	Polymyxin B	*Bacillus polymyxa (aerosporus)*	1947
	Polymyxin E (colistin)	*B. polymyxa* var. *colistinus*	1949
	Bacitracin	*Bacillus licheniformis*	1943
Epoxide antibiotics	Fosfomycin	*Streptomyces fradiae, Streptomyces wedmorensis, Pseudomonas syringae*	1969
Pseudomonic acid antibiotics	Mupirocin	*Pseudomonas fluorescens*	1971
Steroid antibiotics	Fusidic acid	*Fusidium coccineum*	1960
Streptothricins	Nourseothricin	*Streptomyces noursei*	1963
Sulfonamides	Prontosil, sulfamethoxazole, etc.	Synthetic	1935
Trimethoprim	Trimethoprim	Synthetic	1956
Quinolones	Nalidixic acid	Synthetic	1962
Fluoroquinolones	Flumequine, enrofloxacin, etc.	Synthetic	1973
Oxazolidinones	Linezolid	Synthetic	1987, 1996

crobial agents. As a consequence, the origins of bacterial resistance to antimicrobial agents can be assumed to be in a time long before the clinical use of these substances. With the elucidation of the chemical structure of the antimicrobial agents, which commonly followed soon after their detection, it was possible not only to produce antimicrobial agents synthetically in larger amounts at lower costs, but also to introduce modifications that altered the pharmacological properties of these substances and occasionally also extended their spectrum of activity.

The increased selective pressure imposed by the widespread use of antimicrobial agents since the 1950s has distinctly accelerated the development and the spread of bacterial resistance to antimicrobial agents. In most cases, it took not longer than three to five years after the introduction of an antimicrobial agent into clinical use

until the first resistant target bacteria occurred ([1]). This is particularly true for broad-spectrum antimicrobial agents, such as tetracyclines, aminoglycosides, macrolides, and β-lactams, which have been used for multiple purposes in human and veterinary medicine, horticulture, and/or aquaculture. In contrast, this time span was extended to ≥15 years for narrow-spectrum agents, such as glycopeptides, which were used at distinctly lower quantities and only for specific applications. Multiple studies have also revealed that resistance to completely synthetic antimicrobial agents, such as sulfonamides, trimethoprim, fluoroquinolones, and oxazolidinones, can develop quickly ([4]–[7]). These observations underline the enormous flexibility of the bacteria to cope with less favorable environmental conditions by constantly exploring new ways to survive in the presence of antimicrobial agents.

This chapter summarizes the latest information on resistance mechanisms and the mobile elements involved. It is a completely revised and updated version of the chapter that was published in 2006 (8).

RESISTANCE TO ANTIMICROBIAL AGENTS

Resistance to antimicrobial agents can be divided into two basic types, intrinsic resistance and acquired resistance (1, 3, 8, 9). Intrinsic resistance, also known as primary or innate resistance, describes a status of general insensitivity of bacteria to a specific antimicrobial agent or class of agents. This is commonly due to the lack or the inaccessibility of target structures for certain antimicrobial agents, e.g., resistance to β-lactam antibiotics and glycopeptides in cell wall-free bacteria, such as *Mycoplasma* spp., or vancomycin resistance in Gram-negative bacteria due to the inability of vancomycin to penetrate the outer membrane. It can also be due to the presence of export systems or the production of species-specific inactivating enzymes in certain bacteria, e.g.,

the AcrAB-TolC system or the production of AmpC β-lactamase in certain *Enterobacteriaceae*. In addition, some bacteria, such as enterococci, can use exogenous folates and are thus not dependent on a functional folate synthesis pathway. As a consequence, they are intrinsically resistant to folate pathway inhibitors, such as trimethoprim and sulfonamides (9). Intrinsic resistance is a genus- or species-specific property of bacteria. In contrast, acquired resistance is a strain-specific property which can be due to the acquisition of foreign resistance genes or mutational modification of chromosomal target genes. Mutations that upregulate the expression of multidrug transporter systems may also fall into this category. Three different basic types of resistance mechanisms can be differentiated: (i) enzymatic inactivation by either disintegration or chemical modification of the antimicrobials (Table 2), (ii) reduced intracellular accumulation by decreased influx and/or increased efflux of antimicrobials (Table 3), and (iii) modification of the cellular target sites by mutation, chemical modification, or protection of the target sites, but also over-

TABLE 2 Examples of resistance to antimicrobials by **decreased intracellular drug accumulation** (modified from ref. 8)[a,b]

Resistance mechanism	Resistance gene(s)	Gene product	Resistance phenotype	Bacteria involved	Location of the resistance gene
efflux via specific exporters	*mef*(A)	10-TMS efflux system of the major facilitator superfamily	14-, 15-membered macrolides	*Streptococcus*, other Gram+ and Gram− bacteria	T, P, C
	tet(A-E, G, H, I, J, K, L, Z), *tetA*(P), *tet*(30)	12-, 14-TMS efflux system of the major facilitator superfamily	tetracyclines	various Gram+ and Gram− bacteria	P, T, C
	pp-flo, floR, floR$_V$	12 TMS efflux system of the major facilitator superfamily	chloramphenicol, florfenicol	*Photobacterium, Vibrio, Salmonella, Escherichia, Klebsiella, Pasteurella*	T, P, C
	cmlA	12 TMS efflux system of the major facilitator superfamily	chloramphenicol	*Pseudomonas, Salmonella, E. coli*	T, P, C
	fexA	14 TMS efflux system of the major facilitator superfamily	chloramphenicol, florfenicol	*Staphylococcus*	T, P, C
	fexB	14 TMS efflux system of the major facilitator superfamily	chloramphenicol, florfenicol	*Enterococcus*	P
efflux via multidrug transporters	*emrE*	4-TMS multidrug efflux protein	tetracyclines, nucleic acid binding compounds	*E. coli*	C
	blt, norA	12-TMS multidrug efflux protein of the major facilitator superfamily	chloramphenicol, fluoroquinolones, nucleic acid binding compounds	*Bacillus, Staphylococcus*	C
	mexB-mexA-oprM, acrA-acrB-tolC	multidrug efflux in combination with specific OMP's	chloramphenicol, β-lactams, macrolides, fluoroquinolones, tetracyclines, etc.	*Pseudomonas, E. coli, Salmonella*	C

[a]P = plasmid; T = transposon; GC = gene cassette; C = chromosomal DNA.
[b]TMS = transmembrane segments.

TABLE 3 Examples of resistance to antimicrobials by enzymatic inactivation[a]

Resistance mechanism	Resistance gene(s)	Gene product	Resistance phenotype	Bacteria involved	Location of the resistance gene[b]
Hydrolytic degradation	*bla*	β-lactamases	β-lactam antibiotics	Various Gram+, Gram−, aerobic, anaerobic bacteria	P, T, GC, C
	ere(A), *ere*(B)	esterases	macrolides	Gram+, Gram− bacteria	P, GC
	vgb(A), *vgb*(B)	lactone hydrolases	streptogramin B antibiotics	*Staphylococcus, Enterococcus*	P
Chemical modification	*aac, aad (ant), aph*	acetyl-, adenyl-, phosphotransferases	aminoglycosides	Gram+, Gram−, aerobic bacteria	T, GC, P, C
	aad (ant)	adenyltransferases	aminoglycosides/ aminocyclitols	Gram+, Gram−, aerobic bacteria	T, GC, P, C
	catA, catB	acetyltransferases	chloramphenicol	Gram+, Gram−, aerobic, anaerobic bacteria	P, T, GC, C
	vat(A-G)	acetyltransferases	streptogramin A antibiotics	*Bacteroides, Staphylococcus, Enterococcus, Lactobacillus, Yersinia*	P, C
	mph(A-G)	phosphotransferases	macrolides	Gram+, Gram− bacteria	P, T, C
	lnu(A-P)	nucleotidyltransferases	lincosamides	Gram+, Gram− bacteria	P
	tet(X), *tet*(37), *tet*(56)	oxidoreductases	tetracyclines	Gram− bacteria, unknown, *Legionella*	T, P

[a]Modified from reference 8.

[b]Abbreviations: P, plasmid; T, transposon; GC, gene cassette; C, chromosomal DNA.

expression of sensitive targets or the replacement of sensitive target structures by alternative resistant ones (Table 4) (1, 3, 8, 9).

The following subsections illustrate that bacterial resistance to antimicrobial agents varies depending on the agents, the bacteria, and the resistance mechanism. Resistance to the same antimicrobial agent can be mediated by different mechanisms. In some cases, the same resistance gene/mechanism is found in a wide variety of bacteria, whereas in other cases, resistance genes or mechanisms appear to be limited to certain bacterial species or genera. The data presented in the following subsections do not focus exclusively on resistance genes and mechanisms so far detected in bacteria of animal origin but also include resistance genes and mechanisms identified in bacteria from humans. For the best possible overview of the mechanisms and genes accounting for resistance to a specific class of antimicrobial agents, all data are presented under the names of the classes of antimicrobial agents.

Resistance to β-Lactam Antibiotics

A number of penicillins, alone or in combination with a β-lactamase inhibitor, as well as first- to fourth-generation cephalosporins, are licensed for use in veterinary medicine. No carbapenems or monobactams are currently approved for use in animals. Resistance to β-lactam antibiotics is mainly due to inactivation by β-lactamases (10) and decreased ability to bind to penicillin-binding proteins (PBPs) (11) in both Gram-positive and Gram-negative bacteria, but may also be based on decreased uptake of β-lactams due to perme-ability barriers or increased efflux via multidrug transporter systems (12, 13). Inactivation via β-lactamases is most commonly seen, with a wide range of β-lactamases involved. The evolution of β-lactamases which differ distinctly in their substrate spectra is believed to have occurred in response to the selective pressure imposed by the various β-lactam antibiotics that have been introduced into clinical use during the past decades (14).

Enzymatic inactivation of β-lactam antibiotics is based on the cleavage of the amino bond in the β-lactam ring by β-lactamases (10, 15, 16). At present, more than 1,000 β-lactamases have been described, most of which are variants of known β-lactamases that differ in their substrate spectra or their enzyme stability. Two classification schemes are currently in use. The initial classification scheme was based on the similarities in the amino acid sequences and divided the β-lactamases into two molecular classes, A and B (17), which were later expanded to four molecular classes, A to D (18). The second functional classification of β-lactamases was updated in 2010 by Bush and Jacoby (18) and is done on the basis of their substrate spectra and their susceptibility to β-lactamase inhibitors such as clavulanic acid (18). This system subdivides the β-lactamases into three groups, 1 to 3, with group 1 currently comprising 2 subgroups, group 2 comprising 12 subgroups, and group 3 comprising 2 subgroups.

Group 1 β-lactamases (molecular class C), for example, AmpC, CMY, ACT, DHA, FOX, and MIR, are cephalosporinases that are more active on cephalosporins than benzylpenicillin and are usually not inhibited by clavulanic acid. They are widespread among Gram-negative

TABLE 4 Examples of resistance to antimicrobials by **target modification** (modified from ref. [8])[a]

Resistance mechanism	Resistance gene(s)	Gene product	Resistance phenotype	Bacteria involved	Location of the resistance gene
methylation of the target site	*erm*	rRNA methylase	macrolides, lincosamides, streptogramin B compounds	various Gram+ bacteria, *Escherichia*, *Bacteroides*	P, T, C
protection of the target site	*tet*(M, O, P, Q, S, T)	ribosome protective proteins	tetracyclines	various Gram+ and Gram− bacteria	T, P, C
	vga(A)	ribosome protective ABC-F protein	lincosamides, pleuromutilins, streptogramin A-compounds	*Staphylococcus*,	P, T, C
	optrA	ribosome protective ABC-F protein	oxazolidinones, phenicols	*Enterococcus*, *Staphylococcus*	P, C
replacement of a sensitive target by an alternative drug-resistant target	*sul1, sul2, sul3*	sulfonamide-resistant dihydropteroate synthase	sulfonamides	various Gram− bacteria	P, I
	dfrA, dfrB, dfrD, dfrG, dfrK	trimethoprim-resistant dihydrofolate reductase	trimethoprim	various Gram+ and Gram− bacteria	P, GC, T, C
	mecA, mecC	penicillin-binding proteins with altered substrate specificity	penicillins, cephalosporins, carbapenems, monobactams	*Staphylococcus*	C
	vanA-E	alternative D-Ala-D-Lac or D-Ala-D-Ser peptidoglycan precursors	glycopeptides	*Enterococcus*, *Staphylococcus*	T, P, C
alteration of the LPS	*mcr-1* to *mcr-5*	phosphoethanolamine transferase	colistin	Enterobacteriaceae	T, P, C
mutational modification of the target site	—	mutation in the gene coding for ribosomal protein S12	streptomycin	several Gram+ and Gram− bacteria	C
	—	mutation in the 16S rRNA	streptomycin	*Mycobacterium*	C
	—	mutation in the 23S rRNA	macrolides	*Mycobacterium*	C
	—	mutation in the 16S rRNA	tetracyclines	*Propionibacterium*	C
	—	mutations in the genes for DNA gyrase and topoisomerase	fluoroquinolones	various Gram+ and Gram− bacteria	C
	—	mutation in the gene for the ribosomal protein L3	tiamulin	*E. coli*	C
mutational modification of regulatory elements	—	mutations in the *marRAB soxR* or *acrR* genes	fluoroquinolones	*E. coli*	C

[a]P = plasmid; T = transposon; GC = gene cassette; C = chromosomal DNA, I = integron.

bacteria. The *ampC* genes are commonly located on the chromosome but may also be found on plasmids. Some of these *ampC* genes are expressed inducibly; others are expressed constitutively ([18]). Point mutations in the promotor or attenuator region may increase β-lactamase production. Subgroup 1e enzymes are group 1 variants with greater activity against ceftazidime and other oxyimino-β-lactams as a result of amino acid substitutions, insertions, or deletions and include GC1 in *Enterobacter cloacae* and plasmid-mediated CMY-10, CMY-19, and CMY-37. They have been named extended-spectrum AmpC β-lactamases ([18]).

Group 2 β-lactamases (molecular classes A and D) represent diverse enzymes, most of which are sensitive to inhibition by clavulanic acid. Subgroup 2a (molecular class A) includes enzymes such as BlaZ from staphylococci, which can inactivate only penicillins. Subgroup 2b (molecular class A) comprises broad-spectrum β-lactamases, such as TEM-1, TEM-2, SHV-1, and ROB-1, which can hydrolize penicillins and broad-spectrum cephalosporins. Subgroup 2be represents extended-spectrum β-lactamases (ESBLs; e.g., variants of TEM and SHV families and CTX-M type enzymes), which can also inactivate oxyimino cephalosporins and mono-bactams. Subgroups 2a, 2b, and 2be enzymes are sensitive to inhibition by clavulanic acid. Due to their wide spectrum of activity, ESBLs are a serious cause of concern ([19]). Most currently known ESBLs belong to the TEM, SHV, CTX-M, or OXA families of β-lactamases. Less common ESBLs include BEL-1, BES-1, SFO-1, TLA-1, TLA-2, and members of the PER and VEB enzyme families. Details about the structure and function of these ESBLs, their location on mobile elements, their dissemination among bacteria of different species and

genera, and information on ESBL detection methods can be found in several reviews ([19]–[21]). Moreover, a continuosly updated database which lists the known ESBLs and inhibitor-resistant β-lactamases including TEM, SHV, OXA, CTX-M, CMY, IMP, and VIM types can be found at https://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources/. The enzymes of subgroup 2br (molecular class A) are also broad-spectrum β-lactamases, such as TEM-30, TEM-31, and SHV-10, which however, are not inhibited by clavulanic acid. Analysis of the β-lactamases of subgroups 2b, 2be, and 2br—in particular, those of the TEM and SHV types—revealed the presence of mutations which either extended the substrate spectrum or affected the enzyme stability ([10], [14], [19]). TEM enzymes that exhibit an extended spectrum and increased resistance to clavulanic acid inhibition are organized in subgroup 2ber and are called complex mutant TEM (CMT); these include TEM-50 (CMT-1) and TEM-158 (CMT-9) ([18]). Subgroup 2c (molecular class A) includes inhibitor-sensitive carbenicillinases such as CARB-3, PSE-1, and RTG, whereas the extended-spectrum carbenicillinase RTG-4 in subgroup 2ce shows enhanced activity against cefepime and cefpirome. The β-lactamases of subgroup 2d (molecular class D) (e.g., OXA-type enzymes) exhibit variable sensitivity to inhibitors and can hydrolyze oxacillin or cloxacillin. The extended spectrum of the enzymes in subgroup 2de (e.g., OXA-11 and OXA-15) is defined by their ability to hydrolize oxacillin or cloxacillin as well as oxymino β-lactams with a preference for ceftazidime. The subgroup 2df assembles OXA enzymes which are not inhibited by clavulanic acid and show carbapenem-hydrolyzing activities. The genes have been detected on plasmids and in the chromosome of Gram-negative bacteria ([18]). The β-lactamases of subgroups 2e and 2f represent cephalosporinases (e.g., CepA) or serine-carbapenemases (e.g., SME-1, IMI-1, KPC-2), which are sometimes inhibited by clavulanic acid ([18]).

While the β-lactamases of groups 1 and 2 have a serine residue in the catalytic center, the β-lactamases of group 3 (molecular class B) hydrolyze β-lactams by divalent cations (Zn^{2+}) and are referred to as metallo-β-lactamases (e.g., IMP-type, VIM-type, and NDM-type enzymes). Subgroup 3a consists of plasmid-encoded metallo-β-lactamases, which require two bound zinc ions for their activity. These enzymes can inactivate all β-lactams except monobactams and are insensitive to clavulanic acid but are inhibited by metal ion chelators such as EDTA. The metallo-β-lactamases in subgroup 3b preferentially hydrolyze carbapenems, especially if only one zinc-binding side is occupied ([18]). The location of many of the β-lactamase genes (*bla*) on either plasmids, transposons, or gene cassettes favors their dissemination ([20]–[22]).

Altered PBPs are often associated with resistance due to decreased binding of β-lactam antibiotics ([11]). PBPs are transpeptidases which play an important role in cell wall synthesis. They are present in most cell wall-containing bacteria, but they vary from species to species in number, size, amount, and affinity to β-lactam antibiotics ([11]). The acquisition of a novel PBP, such as the *mecA*-encoded PBP2a, which replaces the original β-lactam-sensitive PBP, is the cause of methicillin resistance in *Staphylococcus aureus*, *Staphylococcus pseudintermedius*, and coagulase-negative staphylococci ([23], [24]). Methicillin-resistant staphylococci are resistant to virtually all β-lactam antibiotics except cefpirome. The *mecA* gene, which codes for the alternative PBP2a, is part of a genetic element, designated *Staphylococcus* cassette chromosome *mec* (SCC*mec*) ([25]). So far, 11 SCC*mec* types have been described ([26]). In 2011, a new *mecA* homologue, *mecC* (formerly called *mec*$_{LGA251}$), which is part of a distinct SCC*mec* type (SCC*mec* XI), was identified in methicillin-resistant *S. aureus* (MRSA) ([27], [28]). The majority of known MRSA-carrying *mecC* belong to clonal complex (CC) 130, but other CCs (CC425, CC1943, CC599, CC49) have also been found to harbor *mecC*. In addition, *mecC* is not restricted to *S. aureus* but has been found in several staphylococcal species including *Staphylococcus saprophyticus*, *Staphylococcus sciuri*, *Staphylococcus stepanovicii*, and *Staphylococcus xylosus* ([29]–[31]). In addition to PBP2a, the Fem proteins are involved in expression of methicillin resistance. The FemAB proteins contribute to the formation of the pentaglycine crossbridge, which is a unique staphylococcal cell wall component ([32]). Inactivation of *femAB* has been found to completely restore susceptibility to β-lactams and other antimicrobial agents in MRSA strains ([33]). PBPs with low affinity for β-lactams have also been detected in streptococci and enterococci ([11]). Homologous recombinations in the genes coding for PBPs 1a, 2a, and 2b are assumed to result in mosaic proteins with decreased affinity to β-lactams in *Streptococcus pneumoniae* and *Neisseria* spp.. PBPs, which have a low affinity to β-lactams, have been reported to be overproduced in resistant strains of *Enterococcus faecium* and *Enterococcus faecalis*. It is noteworthy that alterations in PBPs do not necessarily result in complete resistance to all β-lactams but can also lead to elevated MICs of selected β-lactam antibiotics ([11]).

Reduced uptake of β-lactams is due to decreased outer membrane permeability and/or the lack of certain outer

membrane proteins, which serve as entries for β-lactams to the bacterial cell, and has been described in various *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and other bacteria (34–36). In *Escherichia coli* and *Klebsiella pneumoniae*, β-lactam resistance can be based on the decreased expression or the structural alteration of the porins OmpF (37) and OmpK36 (38), by which β-lactams cross the outer membrane. In *P. aeruginosa*, resistance to imipenem has been shown to be based on the loss of the porin OprD (39).

Several multidrug transporters such as the MexAB/OprM and the MexCD/OprJ systems in *P. aeruginosa*, the SmeAB/SmeC system in *Stenotrophomonas maltophilia*, and the AcrAB/TolC system in *Salmonella enterica* and *E. coli* (40, 41) are known to mediate the export of β-lactam antibiotics.

Resistance to Tetracyclines

Among this family of antimicrobial agents, oxytetracycline, chlortetracycline, and tetracycline have been used in veterinary medicine since the 1950s. More recently, doxycycline has been approved for dogs, cats, and pigs. Up to now, minocycline and glycylcyclines have not been licensed for use in animals. Based on aggregated data from a survey on sales of veterinary antimicrobial agents in 25 European countries, the sales of tetracyclines accounted for 37% of the total sales of veterinary antimicrobial agents in 2011 (42). Thus, it is not surprising that tetracycline resistance has become widespread among bacteria of veterinary importance, including in aquaculture (43–45). Tetracycline resistance is usually due to the acquisition of new genes (46). There are 33 efflux genes, which code for energy-dependent efflux of tetracyclines, 12 ribosomal protection genes, which code for a protein that protects bacterial ribosomes, 13 genes which code for enzymes that modify and inactivate the tetracycline molecule, and 1 gene [*tet*(U)] which specifies tetracycline resistance by an unknown mechanism. The products of different *tet* genes share ≤79% amino acid identity (47). An updated database listing the currently known *tet* genes and their occurrence in various bacteria is available at http://faculty.washington.edu/marilynr/. This website is updated twice each year. New *tet* gene names are approved by Dr. Stuart B. Levy, Tufts University, Boston. Antibiotic resistance genes are not randomly distributed among bacteria. This has been well documented with the distribution of *tet* genes (47–50).

The energy-dependent efflux of tetracyclines is mediated by membrane-associated proteins which exchange a proton for a tetracycline-cation complex (46, 51). These tetracycline resistance efflux proteins are part of the major facilitator superfamily and share amino acid and protein structure similarities with other efflux proteins (12). Of the 33 efflux genes, 14 [*tet*(A) to *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*(Y), *tet*(30), *tet*(31), *tet*(35), *tet*(41), *tet*(57)] are found only in Gram-negative genera. The remaining are found in both Gram-negative and Gram-positive genera or in bacteria of unknown source. The *tet*(B) gene has been identified in 33 Gram-negative genera, while the *tet*(L) gene has been identified in a total of 46 genera, including 24 Gram-negative and 22 Gram-positive genera (http://faculty.washington.edu/marilynr/). The Tn*10*-associated *tet*(B) gene codes for a unique efflux protein, which confers resistance to both tetracycline and minocycline but not to the new glycylcyclines (46). All the 32 other efflux proteins confer resistance to tetracycline but not to minocycline or glycylcyclines. Laboratory-derived mutations in the *tet*(A), *tet*(B), *tet*(K), and *tet*(L) genes have led to glycylcycline resistance, suggesting that bacterial resistance to tigecycline may develop over time and with clinical use (46, 52, 53). The *tet* efflux genes code for an approximately 46-kDa membrane-bound efflux protein.

The tetracycline efflux proteins present in Gram-negative bacteria commonly exhibit 12 transmembrane segments (TMSs), and upstream of the structural gene and read in the opposite direction is a specific *tet* repressor gene. Induction of the structural gene is based on the binding of a tetracycline-Mg^{2+} complex to the *tet* repressor protein which, in the absence of tetracycline, blocks transcription of the *tet* structural gene (54). The *tet*(A) ($n = 25$), *tet*(B) ($n = 33$), *tet*(C) ($n = 16$), *tet*(D) ($n = 22$), *tet*(G) ($n = 16$), *tet*(H) ($n = 12$), and *tet*(L) ($n = 22$) genes are most widespread among Gram-negative bacteria of human and veterinary origin, while *tet*(D) and *tet*(E) are often associated with aquaculture environments and fish (44). Their location on either transposons, such as Tn*1721* [*tet*(A)] (55), Tn*10* [*tet*(B)] (56, 57), or Tn*5706* [*tet*(H)] (58), and plasmids facilitates their spread within the Gram-negative gene pool. The Gram-positive *tet*(K) and *tet*(L) efflux genes are not regulated by repressors and confer resistance to tetracyclines, but not to minocycline. They code for proteins with 14 TMSs and are regulated by translational attenuation, which requires the presence of tetracyclines as inducers for the translation of the *tet* gene transcripts (59). These genes are generally found on small transmissible plasmids, which on occasion become integrated into the chromosome and occasionally may undergo interplasmidic recombination with other resistance plasmids (54, 59–61).

The ribosomal protection genes code for cytoplasmic proteins which protect the ribosomes from the action

of tetracycline both *in vitro* and *in vivo* and confer resistance to tetracycline, doxycycline, and minocycline ([46](#), [62](#)). These proteins have sequence similarity to the ribosomal elongation factors EF-G and EF-Tu and are grouped in the translation factor superfamily of GTPases ([63](#)). Their interaction with the ribosome causes an allosteric disruption of the primary tetracycline binding site(s), which then leads to the release of the tetracycline from the ribosome. This allows the ribosome to return to its functional normal posttranslocational conformational state, which was altered by the binding of tetracycline. A detailed review of the various experiments conducted to elucidate the mode of action of these proteins can be found in Connell et al. ([63](#)). The ribosomal protection genes are of Gram-positive origin and are found extensively among Gram-positive cocci. However, they have also been found in a number of Gram-negative genera. The first gene of this group, the *tet*(M) gene, has the widest host range of all *tet* genes, with 79 genera, of which 41 are Gram-positive and 38 are Gram-negative (http://faculty.washington.edu/marilynr/). This gene is located on conjugative transposons or integrative and conjugative elements, such as Tn*916* ([64](#), [65](#)). The other commonly found genes of both human and veterinary origin are *tet*(O) (20 Gram-positive, 18 Gram-negative genera), *tet*(Q) (eight Gram-positive, 11 Gram-negative genera), and *tet*(W) (11 Gram-positive, 22 Gram-negative genera). Recent work suggests that mutations within the *tet*(M) gene may confer increased resistance to tigecycline and thus may over time increase resistance to tigecycline in nature ([52](#)).

Enzymatic inactivation of tetracycline is mediated by 13 genes found in Gram-negative bacteria, nine of which [*tet*(47) to *tet*(55)] have recently been identified by soil functional metagenomic studies ([66](#)). The first inactivating gene described was the *tet*(X) gene ([67](#)) (which encodes an NADP-requiring oxidoreductase), which modifies and inactivates the tetracycline molecule in the presence of oxygen but was originally found only in a strict anaerobe, *Bacteroides*, where oxygen is excluded. The *tet*(X) gene has now been identified in 13 Gram-negative genera (http://faculty.washington.edu/marilynr/). This gene confers weak intrinsic resistance to tigecycline. The tigecycline activity can be improved by at least four different amino acid substitutions in the Tet(X) protein to obtain clinically relevant tigecycline resistance levels without loss of activity to other tetracyclines and was thought to be alarming for the future of tigecycline therapy ([52](#)). The gene *tet*(37) has been identified from the oral cavity of humans but is unrelated to the *tet*(X) or other genes in this class, and the function of the corresponding enzyme depends on oxygen ([68](#)). No bacterial host has been identified which carries *tet*(37). A third gene, *tet*(34), with similarities to the xanthine-guanine phosphoribosyl transferase gene of *Vibrio cholerae*, has also been identified in four Gram-negative genera ([69](#)). The recently described gene *tet*(56) has been identified in one Gram-negative genus ([66](#)), while the genes *tet*(47) to *tet*(55) have been isolated from grasslands and agricultural soils by functional genomics, where the genes were cloned into *E. coli* and shown to inactiviate tetracycline ([66](#)). With this recent study, the number of new genes coding for inactivating enzymes from the environment has greatly increased and may also be found in the future in bacteria of veterinary importance.

The *tet*(U) gene has been identified in the three Gram-positive genera: *Enterococcus*, *Staphylococcus*, and *Streptococcus*. However, it is still not clear if the gene confers tetracycline resistance in any of the bacteria it has been identified in.

A mutation in the 16S rRNA consisting of a single base exchange (1058G → 1058C) has been identified in tetracycline-resistant *Propionibacterium acnes* ([70](#)). Position 1058 is located in a region which plays an important role in the termination of peptide chain elongation as well as in the accuracy of translation.

Mutations which alter the permeability of the outer membrane porins and/or LPSs in the outer membrane can also affect resistance to tetracycline. A permeability barrier due to the reduced production of the OmpF porin, by which tetracyclines cross the outer membrane, has been described in *E. coli*. Mutations in the *marRAB* operon, which also regulates OmpF expression, may play a role in this type of tetracycline resistance ([13](#)).

Different types of multidrug transporters mediating resistance to tetracycline in addition to resistance to a number of structurally unrelated compounds have been described, for instance, in *E. coli* (EmrE), *S. enterica* (AcrAB/TolC), and *P. aeruginosa* (MexAB/OprM, MexCD/OprJ) ([12](#), [40](#), [41](#)).

Resistance to Macrolides, Lincosamides, and Streptogramins (MLS)

Several macrolide antibiotics, such as erythromycin, spiramycin, tylosin and tilmicosin, tulathromycin, gamithromycin, and tildipirosin, as well as lincosamide antibiotics, such as clindamycin, lincomycin, and pirlimycin, are approved for use in animals. Since the ban of growth promotors in the European Union, no streptogramin antibiotics are licensed for veterinary use in the European Union, but they may be used in other countries. The 16-membered macrolide antibiotics tylosin

and spiramycin were previously used as feed additives for animal growth promotion but remain as therapeutics for veterinary use for the control of bacterial dysentery, respiratory disease, and mastitis. Erythromycin, the first macrolide, was introduced into clinical use over 60 years ago and has good activity against Gram-positive cocci and other Gram-positive bacteria and activity against some Gram-negative bacteria such as *Campylobacter* spp.. *Enterobacteriaceae* and *Pseudomonas* spp. have been considered to be innately nonsusceptible to erythromycin due to multidrug transporters which have 14-membered macrolides as substrates (71). A number of Gram-negative aerobic, facultatively aerobic, and anaerobic genera carry a variety of acquired macrolide-lincosamide and/or streptogramin resistance genes (http://faculty.washington.edu/marilynr/). The data over the past 20 years clearly show that both Gram-positive and Gram-negative bacteria may become MLS resistant by acquisition of new genes normally associated with mobile elements. Acquired resistance mechanisms include specific efflux pumps, rRNA methylases that reduce binding of the antibiotic to the 50S subunit of the ribosome, or a variety of genes that inactivate the antibiotics (72–77). MLS antibiotics, though chemically distinct, are usually considered together because they share overlapping binding sites on the 50S ribosomal subunit, and a number of resistance genes confer resistance to more than one class of these antibiotics (72–74).

Target site modification occurs by rRNA methylases, which are encoded by *erm* genes. The *erm* genes were the first acquired genes that were identified to confer resistance to macrolides, lincosamides, and streptogramin B (MLS_B) antibiotics (74, 75). These genes are found in Gram-positive, Gram-negative, aerobic, and anaerobic genera. Currently, 43 rRNA methylases have been characterized. Each of these enzymes adds one or two methyl groups to a single adenine (A2058 in *E. coli*) in the 23S rRNA moiety which prevents binding of the antibiotic to the target site and thus confers MLS_B resistance to the host bacterium (http://faculty.washington.edu/marilynr; 74–76). The *erm* genes may be expressed all the time (constitutively) or inducibly via translational attenuation (77). This means that the gene is turned on in the presence of low doses of specific antibiotics (74–77); the type of expression depends on a regulatory region upstream of the *erm* gene and on which the antibiotic is able to cause induction (75, 77). In staphylococci, erythromycin and other 14- and 15-membered macrolides are able to induce *erm* gene expression, whereas 16-membered macrolides, lincosamides, and streptogramin B antibiotics are considered noninducers (77). Laboratory selection of *S. aureus* produced mutants that had

structural alterations in the translational attenuator region due to deletions, tandem duplications, point mutations, and the insertion of IS256 (78–80). Similar mutations have also been detected in naturally occurring strains carrying the *erm* genes (81).

Efflux genes include 21 ATP transporters and 5 major facilitator superfamily transporters. These genes confer a variety of resistance patterns including resistance to carbomycin, erythromycin, lincomycin, oleandomycin, spiramycin, tylosin, streptogramin A, streptogramin B, and pleuromutilins, alone or in varying combinations (http://faculty.washington.edu/marilynr; 71–74, 82). Recent work by Sharkey et al. (83) suggests that Vga(A) and Lsa(A) are ABC-F proteins, which lack transmembrane domains, not confer resistance by active efflux, but instead mediate resistance through ribosome protection (83). Further work is needed to determine if other proteins in these classes represent the same mechanism of resistance. The *vga*(A) and *vga*(B) have G+C contents of 29 to 36% and their gene products share 59% identical amino acids. The *msr*(A) gene confers inducible resistance to 14- and 15-membered macrolides and streptogramin B (MS_B) and is found in staphylococci. The hydrophylic protein made from the *msr*(A) gene contains two ATP-binding motifs characteristic of the ABC proteins (74, 83, 84, 85). The *msr*(A) gene confers lower levels of erythromycin resistance than the rRNA methylases (86). There are two groups of major facilitator superfamily transporters: one group encompasses *lmr*(A) and *lmr*(B), which code for lincomycin-specific efflux pumps, and the second group includes *mef*(A), *mef*(B) and *mef*(C) genes, which code for specific efflux pumps for 14- and 15-membered macrolides. The *mef*(A) gene was first described in the 1990s from *Streptococcus* spp. (87), but more recently it has been shown to be present in old isolates of pathogenic *Neisseria* spp. (88) and is now found in 30 different genera. It was the most common acquired macrolide resistance gene in a collection of 176 randomly collected commensal Gram-negative bacteria (89). Downstream of the *mef*(A) gene is a gene for an ABC protein that has now been shown to independently confer macrolide resistance and has been named *msr*(D) (90). In contrast, the *mef*(B) gene is found in *Escherichia* spp. and the *mef*(C) gene in *Photobacterium* spp. and *Vibrio* spp..

The 28 inactivating enzymes identified so far encode three esterases, two lyases, 16 transferases, and seven phosphorylases (http://faculty.washington.edu/marilynr; 74, 82). The esterases [Ere(A), Ere(B), and Ere(D)] hydrolyze the lactone ring of the macrolides. The esterases have been found in both Gram-negative and Gram-

positive bacteria, and their genes are often associated with plasmids, though the *ere*(A) gene has been associated with both class 1 and class 2 integrons ([91](#)). The lyase gene, *vgb*(A), has been identified in the genera *Enterococcus* and *Staphylococcus*, while the *vgb*(B) gene has been identified in *Staphylococcus*. These enzymes inactivate quinupristin by opening the lactone ring ([92](#)). The newest inactivating enzymes have been identified as transferases which confer resistance by adding an acetyl group to streptogramin A, thereby inactivating the antibiotic. Sixteen genes have been found in both Gram-positive and/or Gram-negative genera as described below (http://faculty.washington.edu/marilynr; [74](#), [82](#)). The nine lincosamide nucleotidyltransferases [*lnu* genes] confer resistance to lincosamides but not to macrolides by modification and inactivation of the antibiotic. The *lnu*(A) gene has been identified in five Gram-positive genera, and the *lnu*(B) gene in four Gram-positive genera. The gene *lnu*(C) was identified in *Streptococcus* and *Haemophilus*. The gene *lnu*(E) was found in *Streptococcus* and *Enterococcus*, while *lnu*(F) was identified in *Aeromonas*, *Comamonas*, *Desulfobacterium*, *Escherichia*, *Leclercia*, *Morganella*, *Proteus*, and *Salmonella*. The gene *lnu*(D) is associated with *Streptococcus*, *lnu*(G) with *Enterococcus*, and *lnu*(H) with *Riemerella*. Furthermore, *lnu*(P) has been identified in *Clostridium*. Seven virginiamycin *O*-acetyltransferases (*vat* genes) have been identified, six of which are associated with mobile elements in *Enterococcus*, *Lactobacillus*, *Staphylococcus*, and/or *Bacteroides*. Each gene was found in only one or two of these genera. In contrast, *vat*(F) is chromosomally encoded in *Yersinia enterocolitica*.

There are seven enzymes, encoded by *mph* genes, which confer resistance by phosphorylation of erythromycin. The *mph*(A) gene is unqiue because it confers resistance to azithromycin, while *mph*(B) and *mph*(C) confer resistance to spiramycin ([93](#)). Six phosphorylases, encoded by the genes *mph*(A), *mph*(B), *mph*(D), *mph*(E), *mph*(F), and *mph*(G), have been found exclusively in Gram-negative species. To date, the gene *mph*(A) is found in 11 genera, while *mph*(D) and *mph*(E) are each found in six genera. The *mph*(B) gene is present in four genera, while *mph*(F) is found in *Pseudomonas*. The *mph*(G) gene has been found in the fish pathogens *Photobacterium* and *Vibrio* spp. The *mph*(C) gene, which was originally characterized in *Staphylococcus* spp., has now been identified in a clinical *S. maltophilia* isolate (http://faculty.washington.edu/marilynr/).

Usually, mutational changes that affect the 23S RNA, ribosomal proteins, and/or innate efflux pumps may lead to moderate changes in susceptibility ([76](#), [82](#)). Various mutations have been identified in the 23S rRNA ([94](#)). Originally, mutations at either the A2058 or A2059 position (*E. coli* numbering) were found in pathogens that had one or two copies of the 23S rRNA, such as *Mycobacterium* or *Helicobacter* ([95](#)). Resistance to tylosin, erythromycin, and clindamycin in *Brachyspira hyodysenteriae* was also associated with an A → T substitution at the nucleotide position homologous with position 2058 of the *E. coli* 23S rRNA gene ([96](#)). Variations at positions 2058 and 2059 in the 23S rRNA have also been described in erythromycin-resistant *Streptococcus pyogenes*, *S. pneumoniae*, *Campylobacter coli*, *Campylobacter jejuni*, and *Haemophilus influenzae* ([97](#), [98](#)). An A → G substitution at position 2075 of the 23S rRNA was detected in *C. coli* from poultry and pigs which exhibited high-level erythromycin resistance ([98](#)). Mutations in ribosomal proteins L4 and/or L22 have been identified which confer elevated MICs of the newer agent telithromycin and/or of other members of the MLS group. Clinical Gram-positive bacteria have been found with the same mutations as mutants created in laboratories. Missense mutations, deletions, and/or insertions may alter the expression of innate pumps which then may alter resistance to the MLS antibiotics. A detailed discussion can be found in reference [71](#).

Resistance to Aminoglycosides and Aminocyclitols

Various aminoglycoside antibiotics, including gentamicin, kanamycin, amikacin, neomycin, (dihydro)streptomycin, paromomycin and framycetin, are licensed for use in both human and veterinary medicine. Among the aminocyclitol antibiotics, spectinomycin is approved for use in humans and animals, whereas apramycin is used exclusively in veterinary medicine. The main mechanism of resistance to aminoglycosides and aminocyclitols is enzymatic inactivation ([99](#)–[102](#)). In addition, reduction of the intracellular concentrations of aminoglycosides and modification of the molecular target can also result in resistance to aminoglycosides ([103](#)). Decreased intracellular concentration can result from either reduced drug uptake or from active efflux mechanisms. Chromosomal mutations conferring high-level resistance to streptomycin have also been described ([13](#)) and are the main resistance mechanism in mycobacteria.

Enzymatic inactivation of aminoglycosides and aminocyclitols is conferred by any of the three types of modifying enzymes: *N*-acetyltransferases (AACs), *O*-nucleotidyltransferases (also referred to as *O*-adenyltransferases [ANTs]), or *O*-phosphotransferases (APHs) ([99](#)–[102](#)). Acetyl-coenzyme A serves as a donor

of acetyl groups in acetylation reactions at amino groups, while ATP is used for the adenylation and phosphorylation reactions at hydroxyl groups. For each of these three classes of aminoglycoside-modifying enzymes, numerous members are known which differ more or less extensively in their structure. Most modifying enzymes exhibit a narrow substrate spectrum. Several reviews have listed the known enzymes involved in modification of aminoglycosides/aminocyclitols and their molecular relationships (49, 99–102). However, new genes for aminoglycoside/aminocyclitol-inactivating enzymes or variants of already known ones are constantly being reported. Unfortunately, a continuously updated database for the currently known aminoglycoside/aminocyclitol-inactivating enzymes is not available. Another problem is the lack of an unambiguous nomenclature. There are at least two alternatively used designations for genes coding for the same modifying enzyme: one designation, for example, *aph(3″)-Ib*, refers to the type of modification (*aph*) and the position where the modification is introduced (*3″*) and lists the subtype of the gene (*Ib*); the other designation, for example, *strA*, is easier to handle, refers only to the corresponding resistance phenotype (*str* for streptomycin resistance), and indicates the subtype (A).

So far, four classes of AACs are known which acetylate the amino groups at positions 1, 3, 2′, and 6′ (99–103). To date, at least 80 AACs have been identified, most of which vary in their substrate spectra. The vast majority of the AAC enzymes were identified in Gram-negative bacteria. Combined resistance to apramycin and gentamicin is due to the enzyme AAC(3)-IV; the corresponding gene emerged after the introduction of apramycin into veterinary use. It was first detected in *E. coli* and *Salmonella* from animals (104) and was found later in *E. coli* from humans as well (105–107). A gene for a bifunctional enzyme, which displays acetyltransferase AAC(6′) and phosphotransferase APH(2″) activities, is usually found on Tn*4001*-like transposons, which are widely spread among staphylococci, streptococci, and enterococci (108–111).

To date, five classes of ANTs are categorized depending on the position of adenylation (6, 9, 4′, 2″, and 3″) on the aminoglycoside molecule (99–102). The ANT(2″) and ANT(3″) enzymes are more frequent among Gram-negative bacteria, whereas the ANT(4′), ANT(6), and ANT(9) enzymes are usually found in Gram-positive bacteria (101). The different ANT enzymes also vary considerably in their substrate spectra. Among the seven phosphotransferases [APH(2″), APH(3′), APH(3″), APH(4), APH(6), APH(7″), and APH(9)] which modify the aminoglycosides at positions 2″, 3′, 3″, 4, 6, 7″, and 9 (101), numerous

variants have been identified which confer distinctly different resistance phenotypes. Most *aac*, *ant*, and *aph* genes are located on mobile genetic elements, such as plasmids, transposons, and gene cassettes (99–103, 112, 113).

The gene *apmA* codes for an acetyltransferase, which confers resistance to apramycin and decreased susceptibility to gentamicin. It has been detected on plasmids of variable sizes in MRSA ST398 (114–116). In staphylococci, spectinomycin resistance is mediated by the adenyltransferase genes *spc*, *spd*, and *spw* (117–121).

Multidrug efflux systems, such as MexXY in *P. aeruginosa* and AmrAB in *Burkholderia pseudomallei* (40), or the multidrug transporter AcrD in *E. coli* (122) can export aminoglycosides. The transporter MdfA from *E. coli* (123) has also been reported to mediate the efflux of the aminoglycosides kanamycin, neomycin, and hygromycin A.

Decreased uptake of aminoglycosides may be based on a mutation in lipopolysaccharide (LPS) phosphates or on a change in the charge of the LPS in *E. coli* and *P. aeruginosa*, respectively (124). Since the entry of aminoglycosides across the cytoplasmic membrane is mainly based on the electron transport system, anaerobic bacteria and facultative anaerobic bacteria exhibit relatively high insensitivity to aminoglycosides (13).

Methylation of the ribosomal target (16S rRNA) is responsible for high-level aminoglycoside resistance. It is also an emerging mechanism of great concern in clinically relevant Gram-negative bacteria. The first plasmid-mediated gene identified was the 16S rRNA methylase *armA* (125). To date, nine additional genes that encode methylases have been reported: *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rtmD2*, *rmtE*, *rmtF*, *rmtG*, and *npmA* (126). The *rmt* genes confer resistance to gentamicin and amikacin, whereas *npmA* confers resistance to gentamicin, neomycin, amikacin, and apramycin, but not to streptomycin (127).

Mutations in the gene *rpsL* for the ribosomal protein S12 have been shown to result in high-level streptomycin resistance (128). Single base-pair substitutions at different positions in the gene *rrs*, which encodes 16S rRNA in mycobacteria, have been described to be involved in either streptomycin resistance (129) or resistance to amikacin, kanamycin, gentamicin, tobramycin, and neomycin, but not to streptomycin (130). In *Mycobacterium tuberculosis*, mutations in the gene *rpsL*, which encodes the ribosomal protein S12, can cause high-level streptomycin resistance. Overexpression of the acetyltransferase-encoding gene, *eis*, has mainly been associated with resistance to kanamycin. Mutations in the *gidB* gene, which encodes a 7-methylguanosine methyltransferase, are also associated with resistance to

aminoglycosides in mycobacteria. It has been suggested that loss of function of this gene confers resistance (130).

Resistance to Sulfonamides and Trimethoprim

Various sulfonamides, trimethoprim, and combinations of sulfonamides and trimethoprim are licensed for use in humans and animals. There are no restrictions on the use of any of these compounds in food animals. Sulfonamides and trimethoprim are competitive inhibitors of different enzymatic steps in folate metabolism. In this regard, sulfonamides represent structural analogs of *p*-aminobenzoic acid and inhibit the enzyme dihydropteroic acid synthase (DHPS), whereas trimethoprim inhibits the enzyme dihydrofolate reductase (DHFR). Various mechanisms of intrinsic and acquired resistance to sulfonamides and trimethoprim have been described in bacteria (131–135).

Permeability barriers and efflux pumps play a relevant role by either preventing the influx or promoting the efflux of both compounds. Intrinsic resistance to both compounds in *P. aeruginosa* was initially thought to be based on outer membrane impermeability. However, the multidrug exporter system MexAB/OprM was found to be mainly responsible for resistance to sulfonamides and trimethoprim in *P. aeruginosa* (136). For other bacteria, such as *K. pneumoniae* and *Serratia marcescens*, impaired membrane permeability is still considered to play a role in sulfonamide and trimethoprim resistance (132, 133).

Naturally insensitive DHFR enzymes and folate auxotrophy play an important role in intrinsic resistance to sulfonamides and trimethoprim. DHFR enzymes which exhibit low affinity for trimethoprim and thus render their hosts intrinsically resistant to trimethoprim are known to occur in several bacterial genera including *Clostridium*, *Neisseria*, *Brucella*, *Bacteroides*, and *Moraxella* (13). Bacteria such as enterococci and lactobacilli which can utilize exogenous folates also show intrinsic resistance to trimethoprim and sulfonamides.

Mutational or recombinational changes in the target enzymes have been observed in a wide variety of bacteria. Mutations in the chromosomal *dhps* gene that lead to sulfonamide resistance by single amino acid substitutions can be generated under *in vitro* conditions but also occur *in vivo*. Such mutations have been identified in *E. coli*, *S. aureus*, *Staphylococcus haemolyticus*, *C. jejuni*, and *Helicobacter pylori* (132). In *S. pneumoniae*, two amino acid duplications which change the tertiary structure of the DHPS have been found to be responsible for sulfonamide resistance (137). Recombinational events between the naturally occurring gene coding for

a susceptible DHPS and that of a horizontally acquired resistant DHPS are believed to account for sulfonamide resistance in *Neisseria meningitidis* (132). Trimethoprim resistance has also been shown to be due to a single amino acid substitution in the DHFR protein in *S. aureus* (138) and *S. pneumoniae* (139). Mutations in the promoter region of chromosomal *dhfr* genes have been described to occur in *E. coli* and resulted in overexpression of the trimethoprim-susceptible DHFR (132). Mutations in both the promoter region and the *dhfr* gene have been identified in trimethoprim-resistant *H. influenzae* (140).

The replacement of sensitive enzymes by resistant enzymes usually causes high-level resistance (131–135). To date, three types of resistant DHPS enzymes encoded by the genes *sul1*, *sul2*, and *sul3* have been described to occur in Gram-negative bacteria (141–144). The gene *sul1* is part of class 1 integrons and thus is often associated with other resistance genes. As part of transposons, such as Tn*21*, and conjugative plasmids, it is spread into various Gram-negative species and genera (141, 143). The *sul2* gene often occurs together with the Tn*5393*-associated streptomycin resistance genes *strA-strB* on conjugative or nonconjugative plasmids (142, 143). The gene *sul3* was originally found on a conjugative plasmid from porcine *E. coli*, where it was flanked by copies of the insertion sequence IS*15Δ/26* (144). Meanwhile, it has also been identified in *E. coli* from humans and animals other than pigs, as well as in *S. enterica* from animal and food sources (144–147).

More than 40 DHFR (*dfr*, formerly also referred to as *dhfr*) genes have been identified. The genes occurring in Gram-negative bacteria are subdivided on the basis of their structure into two major groups, *dfrA* and *dfrB* (148). The 33 *dfrA* genes code for DHFR enzymes of 152 to 189 amino acids (aa), whereas the eight *dfrB*-encoded DHFR enzymes consist of only 78 aa. The *dfrA* genes have been detected more frequently than the *dfrB* genes. Additionally, there are *dfr* gene groups in Gram-positive bacteria that currently consist of only one gene each. The gene *dfrG* codes for an enzyme of 165 aa and has been detected in the chromosome of *S. aureus* (149). In *S. haemolyticus* and *Listeria monocytogenes*, the gene *dfrD*, which codes for an enzyme of 162 aa, has been identified on plasmids (150, 151). The gene for the 163-aa DHFR DfrK was first detected on plasmids in *S. aureus* and linked to the *tet*(L) gene (152). Since then, *dfrK* has been found as part of transposon Tn*559* in the chromosomal DNA of staphylococci and enterococci (153, 154) and on small plasmids from *Staphylococcus hyicus* that confer only trimethoprim resistance (115). In staphylococci, the composite transposon Tn*4003* has been iden-

tified on various multiresistance plasmids. Tn*4003* is composed of a central *dfrA* gene (also known as *dfrS1*) bracketed by copies of the insertion sequence IS*257* (155).

Transferable trimethoprim resistance genes have been identified in a wide variety of Gram-negative bacteria; several of these genes are part of plasmids, transposons, or gene cassettes (112, 133, 135, 156) and thus are easily disseminated across species and genus borders. Several studies showed the relationships between the *dfr* genes (133, 135, 156).

Resistance to Quinolones and Fluoroquinolones

Quinolones and fluoroquinolones are potent inhibitors of bacterial DNA replication. While early quinolones such as nalidixic acid and pipemidic acid have not been used in veterinary medicine, oxolinic acid and flumequine (the first fluorinated quinolone) have been used in food-producing animals, including fish, worldwide (1, 157). Since the first of the newer fluoroquinolones, enrofloxacin, was licensed for use in animals in the late 1980s (158), several other fluoroquinolones have been approved for veterinary use in recent years, including marbofloxacin, orbifloxacin, difloxacin, ibafloxacin, danofloxacin, and pradofloxacin. Two major mechanisms account for resistance to fluoroquinolones: mutations in the genes for DNA topoisomerases and decreased intracellular drug accumulation (159–163). In addition, plasmid-mediated (fluoro)quinolone resistance genes have been described in the past decade (163).

Mutational alteration of the target genes *gyrA* and *gyrB* (coding for the A and B subunits of the DNA gyrase) as well as *parC* and *parE* (coding for A and B subunits of the DNA topoisomerase IV) is frequently seen in (fluoro)quinolone-resistant bacteria. Both enzymes are tetramers consisting of two A and B units. The mutations in *gyrA* are commonly located within a region of ca. 130 bp which is referred to as the "quinolone resistance-determining region" (164). Mutations resulting in changes of Ser-83 (to Tyr, Phe, or Ala) and Asp-87 (to Gly, Asn, or Tyr) have been detected most frequently. In addition, double mutations at both positions and various other mutations have been described in Gram-positive and Gram-negative bacteria of human and veterinary importance (157, 160–162, 165). Stepwise mutations in *gyrA* and *parC* can result in an incremental increase in resistance to quinolones (157). Moreover, various mutations may also have different effects on resistance to the various fluoroquinolones (166). The complex interplay between individual mechanisms may also have different effects on fluoroquinolone resistance (167).

Multidrug efflux systems also conferring fluoroquinolone resistance have been identified in various Gram-positive and Gram-negative bacteria, such as *P. aeruginosa* (MexAB/OprM, MexCD/OprJ), *S. aureus* (NorA), *S. pneumoniae* (PmrA), *Bacillus subtilis* (Blt), *E. coli*, and *S. enterica* (AcrAB/TolC); for reviews see references 162, 168, and 169. Since the basal level of expression of these efflux systems is low, upregulation of their expression is required to confer resistance to fluoroquinolones and other antimicrobials. In *E. coli* the level of production of the AcrAB-TolC efflux system is under the control of several regulatory genes, in particular the global regulatory systems *marRAB* and *soxRS*, but also *acrR* (168–171). Mutations in these regulatory systems may lead to overproduction of the AcrAB-TolC efflux pump and expression of the multidrug resistance phenotype (172, 173). Besides overproduction of the AcrAB-TolC efflux pump, it has been recently shown using macroarrays that *E. coli* strains constitutively expressing *marA* showed altered expression of more than 60 chromosomal genes (174).

Interplay between several resistance mechanisms may lead to high-level resistance to quinolones and to other antibiotics when multidrug efflux pumps and decreased outer membrane permeability are involved (167, 175). For *in vitro* selected quinolone-resistant *E. coli* mutants, it has been shown that first-step quinolone-resistant mutants acquire a *gyrA* mutation. Second-step mutants reproducibly acquire a multidrug resistance phenotype and show enhanced fluoroquinolone efflux. In some third-step mutants, fluoroquinolone efflux is further enhanced and additional topoisomerase mutations are acquired. In clinical *E. coli* isolates from humans and animals, the situation appears to be the same, where high-level fluoroquinolone resistance is reached when mutations at several chromosomal loci are acquired (167, 175). It is noteworthy that inactivation of the AcrAB efflux pump renders resistant *E. coli* strains, including those with target gene mutations, hypersusceptible to fluoroquinolones and certain other unrelated drugs (175). Thus, in the absence of the AcrAB efflux pump, gyrase mutations fail to produce clinically relevant levels of fluoroquinolone resistance (176). The same observation has been made for *P. aeruginosa*, in which deletion of the MexAB-OprM efflux pump, which is the homolog of the AcrAB-TolC efflux pump in this species, resulted in a significant decrease in resistance to fluoroquinolones even for strains carrying target gene mutations (177). In high-level fluoroquinolone-resistant *S. enterica* serovar Typhimurium DT204 strains, carrying multiple target gene mutations in *gyrA*, *gyrB*, and

parC, inactivation of AcrB or TolC resulted in a 16- to 32-fold decrease of resistance levels to fluoroquinolones ([178](#), [179](#)).

Decreased drug uptake in Gram-negative bacteria is due to the *marRAB*-mediated downregulation of OmpF porin production. OmpF is an important porin for the entry of quinolones and fluoroquinolones into the bacterial cell ([180](#), [181](#)). Moreover, mutations in different gene loci (*cfxB*, *norB*, *nfxB*, *norC*, or *nalB*) are also associated with decreased permeability ([182](#), [183](#)).

Plasmid-mediated quinolone resistance mechanisms have been described in recent years in addition to the aforementioned chromosomal resistance mechanisms ([184](#)–[186](#)). These plasmid-mediated quinolone resistance mechanisms usually confer low-level (fluoro)quinolone resistance by (i) target protection, (ii) acetylation, and (iii) efflux pumps. The protection of the DNA gyrase is mediated by *qnr* genes. Six *qnr* gene families (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qnrVC*) with multiple alleles per gene have been described and are organized in a database (www.lahey.org/qnrStudies). The *qnr* genes are associated with several mobile genetic elements and located on plasmids of varying sizes and different incompatibility groups ([185](#), [186](#)). The gene *aac(6′)-Ib-cr* codes for an aminoglycoside acetyltransferase, which is able to acetylate the amino nitrogen on the piperazinyl ring of quinolones such as ciprofloxacin and norfloxacin ([185](#), [186](#)). Efflux pumps are encoded by the genes *qepA* and *oqxAB*. The OqxAB efflux pump has a wide substrate specificity and is found not only on plasmids but also in the chromosomal DNA. These plasmid-mediated resistance genes have been found in several Gram-negative bacteria. The plasmid-located gene for an efflux pump *qacBIII* is able to confer decreased susceptibility to ciprofloxacin and norfloxacin in *S. aureus* and is the first plasmid-mediated quinolone resistance gene in Gram-positive bacteria ([185](#), [186](#)).

Resistance to Phenicols

Two members of the phenicols, chloramphenicol and its fluorinated derivative florfenicol, are currently approved for use in animals. The predominant mechanism of chloramphenicol resistance in Gram-positive and Gram-negative bacteria is enzymatic inactivation ([187](#)–[189](#)). In addition, efflux systems that mediate either resistance to only chloramphenicol or combined resistance to chloramphenicol and florfenicol have also been identified ([188](#)). Furthermore, permeability barriers and multidrug transporters play a role in certain Gram-negative bacteria ([8](#), [12](#), [188](#), [190](#)). Detailed reviews on the different genes and mechanisms accounting for bacterial resistance to chloramphenicol and florfenicol have been published ([48](#), [188](#)).

Enzymatic inactivation of chloramphenicol is commonly achieved by chloramphenicol acetyltransferases (CATs) which transfer acetyl groups from acetyl-CoA to the C3 position of the chloramphenicol molecule. Subsequent transfer of the acetyl group to the C1 position and transfer of a second acetyl group to C3 results in mono- or diacetylated chloramphenicol derivatives, both of which are unable to inhibit bacterial protein biosynthesis ([187](#)–[189](#)). Two distinct types of CAT enzymes, which differ in their structures, are known: the classical CATs (type A) and a novel type of CAT (type B) ([187](#), [188](#)). All type A and type B CATs have a trimeric structure composed of three identical monomers. The *cat* gene codes for a CAT monomer, the size of which varies between 207 and 238 aa (type A CATs) and 209 and 212 aa (type B CATs) ([188](#)). Using the cutoff as set for the classification of tetracycline and MLS resistance genes ([47](#), [74](#)), 16 classes of *catA* determinants and at least another five classes of *catB* determinants can be differentiated ([48](#)). Among the *catA* genes, those formerly referred to as *catI*, *catII*, and *catIII* are most widespread among Gram-negative bacteria ([48](#), [191](#)–[193](#)). They are associated with either nonconjugative transposons such as Tn*9* or plasmids. Expression of these *catA* genes is constitutive. Various *catA* genes, indistinguishable from or closely related to those present on the *S. aureus* plasmids pC221, pC223/pSCS7, and pC194 ([48](#), [193](#)–[196](#)), have been detected in coagulase-positive and -negative staphylococci, but also in members of the genera *Streptococcus*, *Bacillus*, and *Listeria*, respectively. Expression of these mostly plasmid-borne *catA* genes is inducible by chloramphenicol via translational attenuation ([197](#)), whereas the Tn*4451*-borne *catA* genes of *Clostridium* spp. are expressed constitutively ([198](#)). The *catB* genes—also referred to as *xat* (xenobiotic acetyltransferase) genes—differ distinctly from the *catA* genes but are related to acetyltransferase genes, such as *vat*(A-E), involved in streptogramin resistance ([187](#)). Some of the *catB* genes have been found exclusively on the chromosome of either *Agrobacterium tumefaciens*, *P. aeruginosa*, or *V. cholerae*, whereas others proved to be part of transposons (Tn*2424*, Tn*840*) or plasmid-borne integrons. Studies of the level of *catB*-mediated chloramphenicol resistance revealed a distinctly lower level of chloramphenicol resistance compared to that conferred by type A CATs ([187](#)).

In addition to inactivation via CATs, enzymatic inactivation of chloramphenicol can also occur by *O*-phosphorylation or by hydrolytic degradation to

p-nitrophenylserinol ([48](#)). Since these mechanisms have so far only been seen in the chloramphenicol producer *Streptomyces venezuelae* and in a soil metagenome library, they are believed to play a role as self-defense mechanisms ([48](#), [188](#)).

A total of 11 classes of specific exporters which mediate either chloramphenicol or chloramphenicol/florfenicol resistance have been identified ([48](#), [188](#)). Among them, seven classes are represented by 10- to 12-TMS chloramphenicol exporters of soil bacteria of the genera *Streptomyces*, *Rhodococcus*, and *Corynebacterium* or of bacteria of unknown origin, whereas four classes of 12-TMS exporters were found among Gram-negative bacteria of medical importance ([188](#), [199](#)). Among these latter classes, one class represents the *cmlA* subgroup, and the others represent the *floR* subgroup. The gene *cmlA*, which codes for a chloramphenicol exporter, is a Tn*1696*-associated cassette-borne gene which, however, is inducibly expressed via translational attenuation ([200](#)). Genes related to *cmlA* are mainly found in *Enterobacteriaceae* and *Pseudomonas*. Genes related to *floR* have been identified in *Photobacterium*, *Vibrio*, *Klebsiella*, *E. coli*, and various *S. enterica* serovars and in *Pasteurella multocida* as part of the chromosomally located ICE*Pmu1* ([48](#), [201](#)–[208](#)). In *Vibrio* and *Salmonella*, the gene *floR* has been detected as part of chromosomal multiresistance gene clusters ([207](#), [209](#)), and in *E. coli*, as part of conjugative and nonconjugative multiresistance plasmids ([201](#), [202](#)). In *S. maltophilia* of porcine origin, a novel *floR* variant, *floR*$_V$, has been identified as part of a chromosomal genomic island ([210](#)). Another class of phenicol exporters is represented by FexA, the first specific chloramphenicol/florfenicol exporter of Gram-positive bacteria ([211](#)). The gene *fexA*, located on the transposon Tn*558* ([212](#)) from *Staphylococcus lentus*, codes for a 14-TMS exporter of the major facilitator superfamily and is expressed inducibly via translational attenuation. A second phenicol exporter, FexB, which exhibited 56.1% amino acid identity with the FexA protein, has been exclusively identified in enterococci ([213](#)).

Multidrug transporter systems that export chloramphenicol have been described to occur in several Gram-negative bacteria, including the systems MexAB/OprM and MexCD/OprJ in *P. aeruginosa*, AcrAB/TolC in *E. coli* and *S. enterica*, CeoAB/OpcM in *Burkholderia cepacia*, and ArpAB/ArpC and TtgAB/TtgC in *Pseudomonas putida* ([40](#), [181](#)).

Permeability barriers based on the reduced expression of the OmpF porin in *S. enterica* serovar Typhi or a major outer membrane protein in *H. influenzae* ([188](#)) have also been described to confer chloramphenicol resistance. The *mar* locus which is found in various *Enterobacteriaceae* can contribute to chloramphenicol resistance in two ways: on one hand, it can activate the AcrAB/TolC efflux system, leading to increased efflux of chloramphenicol, and on the other hand, MarA can activate the gene *micF*, whose transcripts represent an antisense RNA that effectively inhibits translation of *ompF* transcripts, which results in a decreased influx of chloramphenicol ([180](#), [181](#)).

Mutations in the major ribosomal protein clusters of *E. coli* and *B. subtilis*, but also mutations in the 23S rRNA of *E. coli*, have been described to mediate chloramphenicol resistance ([214](#)).

Resistance to Oxazolidinones

Oxazolidinones are a class of synthetic antibiotics that are highly active against Gram-positive bacteria. Currently, two oxazolidinones, linezolid and tedizolid, are exclusively approved for use in human medicine and are considered last-resort antimicrobial agents for the treatment of infections caused by MRSA, vancomycin-resistant enterococci, and penicillin-resistant *S. pneumoniae*.

Initially, point mutations within either the 23S rRNA and/or the genes coding for the ribosomal proteins L3 (*rplC*), L4 (*rplD*), and L22 (*rplV*) were recognized as the main mechanisms of reduced oxazolidinone susceptibility ([215](#)–[217](#)). Mutations in clinical staphylococcal and enterococcal isolates, including G2247T, T2500A, A2503G, T2504C, G2505A, and G2576T, usually were found in the vicinity of the peptidyltransferase center ([217](#)). Mutations in the gene *rplC*, including F147L and A157R, resulted in at least 2-fold increases of the oxazolidinone MICs of laboratory and clinical staphylococci ([218](#)). The mutations in the *rplD* gene, which resulted in amino acid exchanges K68N or K68Q, and the insertions $_{71}$GGR$_{72}$, $_{65}$WR$_{66}$, and $_{68}$KG$_{69}$ lead to oxazolidinone resistance in *S. pneumoniae* ([219](#)). Little is known about the effects of L22 mutations on linezolid resistance; whether the amino acid exchange A29V in L22 detected in two linezolid-resistant MRSA isolates is responsible for the linezolid resistance in these isolates remains to be investigated ([220](#)).

The gene *cfr* from *S. sciuri* was the first transferable oxazolidinone resistance gene ([221](#)). Initially described as a novel chloramphenicol/florfenicol resistance gene, the elucidation of the resistance mechanism revealed that *cfr* codes for an rRNA methylase, which confers resistance not only to phenicols but also to lincosamides, oxazolidinones, pleuromutilins, and streptogramin A by methylating the adenine residue at position 2503 in 23S

rRNA, which is located in the overlapping ribosomal binding site of these antibiotics (222, 223). The gene *cfr* is mainly located on plasmids in staphylococci and has been distributed across species and genus boundaries. In recent years, it has been detected in both Gram-positive and Gram-negative genera, including *Bacillus*, *Enterococcus*, *Escherichia*, *Jeotgalicoccus*, *Macrococcus*, *Staphylococcus*, *Streptococcus*, and *Proteus* (48, 224, 225). Recently, variants of the *cfr* gene have been identified, including *cfr*(B) in *E. faecium* and *Clostridium difficile* (226, 227) and *cfr*(C) in *Campylobacter* and *Clostridium* (228).

An ABC-F protein, which is able to mediate resistance to chloramphenicol and florfenicol as well as the oxazolidinones linezolid and tedizolid, is encoded by the gene *optrA*, which has been identified on a conjugative plasmid in *E. faecalis* (229). The insertion sequence IS*1216E* and the transposon Tn*558* have been identified in the *optrA* flanking regions on plasmids and on the chromosome of enterococci from humans, pigs, and chickens (230). Moreover, the gene *optrA* has also been detected on plasmids and in the chromosomal DNA of porcine *S. sciuri* (231, 232). Since then, this gene has been identified in genomes of clinical isolates of staphylococci, enterococci, and streptococci (233).

Resistance to Glycopeptides

Since the ban of the growth promotor avoparcin in 1996, no glycopeptide antibiotics are approved for use in animals. Glycopeptide antibiotics, such as vancomycin and teicoplanin, act by binding to the D-alanine-D-alanine termini of peptidoglycan precursors, thereby preventing transglycosylation and transpeptidation of the bacterial cell wall (234, 235).

Modification of the target site is the common mechanism of bacterial resistance to glycopeptides. So far, four D-Ala-D-Lac operons (*vanA*, *vanB*, *vanD*, and *vanM*) and five D-Ala-D-Ser operons (*vanC*, *vanE*, *vanG*, *vanL*, and *vanN*) have been described in enterococci (236). They differ in their levels of resistance to vancomycin and teicoplanin (235). In the D-Ala-D-Lac operons, the terminal dipeptide D-alanine-D-alanine is replaced by D-alanine-D-lactate, whereas in the D-Ala-D-Ser operons, it is replaced by D-alanine-D-serine. These replacements reduce the ability of glycopeptides to bind to the peptidoglycan precursors and result in the case of D-lactate in high-level and in the case of D-serine in low-level glycopeptide resistance.

The VanC operon is responsible for the intrinsic resistance of *Enterococcus gallinarum*, *Enterococcus casseliflavus*, and *Enterococcus flavescens* to glycopeptides (235). Similar to VanC, VanD and VanE, both

from *E. faecalis*, have been reported not to be transferable (235). In contrast, the *vanA* and *vanB* operons are associated with transposons which can be located on conjugative and nonconjugative plasmids in enterococci. The VanA phenotype is associated with the nonconjugative transposon Tn*1546*, which contains a total of nine reading frames, five of which are essential for high-level glycopeptide resistance (237). Among these, the two genes *vanR* and *vanS* code for a response regulator protein and a sensor protein, respectively, involved in regulatory processes. Three genes are directly involved in resistance: *vanH*, *vanA*, and *vanX*. The gene *vanH* codes for a cytoplasmatic dehydrogenase that produces D-lactate from pyruvate, whereas the gene *vanX* codes for a D,D-dipeptidase which cleaves the D-alanine-D-alanine, and the gene *vanA* codes for a ligase that joins the remaining D-alanine with D-lactate. While glycopeptide resistance is often found in enterococci (238, 239), transfer studies showed that conjugative transfer of VanA-mediated vancomycin resistance from *E. faecalis* to *S. aureus* is possible under *in vitro* conditions (240). In 2002, the first patients infected with *vanA*-carrying high-level vancomycin-resistant *S. aureus* isolates were detected in the United States (241). Genes homologous to enterococcal glycopeptide resistance genes *vanA* and *vanB* have also been detected among members of the genera *Paenibacillus* and *Rhodococcus* (242). Moreover, a new glycopeptide resistance operon, *vanOHX*, has recently been identified in *Rhodococcus equi* (236).

Impaired membrane permeability renders Gram-negative bacteria intrinsically resistant to glycopeptides, large molecules which can cross the outer membrane only poorly, if at all (235).

Resistance to Pleuromutilins

The pleuromutilins tiamulin and valnemulin are mainly used in veterinary medicine for the control and specific therapy of gastrointestinal and respiratory tract infections in swine and to a lesser extent in poultry and rabbits. Retapamulin is used as an ointment to treat bacterial skin infections in humans. Products for systemic use in humans with infections caused by multidrug-resistant bacteria are currently being developed.

The main target bacteria in veterinary medicine are *B. hyodysenteriae*, *Brachyspira pilosicoli*, *Lawsonia intracellularis*, and *Mycoplasma* spp. Resistance derives from chromosomal mutations in the 23S rRNA and *rplC* genes or mobile resistance genes located on plasmids or transposons, such as the *cfr* genes and certain *vga*, *lsa*, and *sal* genes (243, 244). The mechanism of resistance varies among bacterial species.

In *B. hyodysenteriae*, reduced susceptibility to tiamulin has been associated with point mutations in the V domain of the 23S rRNA gene (positions 2032, 2055, 2447, 2499, 2504, and 2572 in *E. coli* numbering) and/or the ribosomal protein L3 gene ([245], [246]). Mutation at nucleotide position 2032 appears to be related to pleuromutilin resistance and to decreased susceptibility to lincosamides ([246]). Tiamulin resistance in *B. hyodysenteriae* develops in a stepwise manner both *in vitro* and *in vivo*, suggesting that multiple mutations are needed to achieve high levels of resistance. The MICs of valnemulin are generally a few dilution steps lower than those of tiamulin ([247]). To date, data on the resistance mechanisms of *B. pilosicoli* and *L. intracellularis* are lacking, and data on resistance mechanisms of mycoplasmata are limited. A single mutation of the 23S rRNA gene caused elevated tiamulin and valnemulin MICs in *Mycoplasma gallisepticum*, but combinations of two or three mutations were necessary to produce high levels of resistance to these drugs ([248]).

Resistance in staphylococci can be due to point mutations in the V domain of 23S rRNA or in the *rplC* gene, encoding the ribosomal protein L3 ([249]). Transferable resistance in staphylococci can be caused by *vga* genes, encoding ABC transporters or ABC-F proteins, resulting in resistance to pleuromutilins, streptogramin A, and lincosamides. There are several *vga* genes which confer pleuromutilin resistance in addition to lincosamide and streptogramin A resistance: *vga*(A) and its variants, *vga*(C), and *vga*(E) and its variant. All these genes have been found on plasmids and transposons of staphylococci ([243]). Transferable resistance to five classes of antimicrobials (phenicols, lincosamides, oxazolidinones, pleuromutilins, streptogramin A) in staphylococci is mediated by the gene *cfr*. The *sal*(A) gene from *S. sciuri*, also encoding a putative ABC-F protein, has been shown to mediate combined resistance to lincosamides, pleuromutilins, and streptogramin A antibiotics ([244]).

E. faecalis is intrinsically resistant to pleuromutilins, streptogramin A antibiotics, and lincosamides by the production of the ABC-F protein Lsa(A). In *E. faecium*, acquired resistance to the above-mentioned antimicrobials is mediated by the gene *eat*(A)$_V$ which may encode a putative ABC-F protein ([83], [250]). The enterococcal gene *lsa*(E), which may also code for a putative ABC-F protein ([83]), has been detected in methicillin-susceptible *S. aureus* and in MRSA of human and animal origin ([251]).

Resistance to Polypeptide Antibiotics

There are three polypeptide antibiotics that are used in human and/or veterinary medicine: bacitracin, polymyxin B, and colistin (polymyxin E). Bacitracin inhibits cell wall synthesis and is active against Gram-positive bacteria. It used to be used as a growth promoter ([252]). Since the ban of antimicrobial growth promoters in 2006 in the European Union, it is no longer approved for veterinary use as growth promotor in the EU. In China, colistin has also been banned from use as growth promoter in food animals, as of April 2017. However, it is used for that purpose in other countries, and it is still approved for therapeutic purposes in the European Union and China. Polymyxin B and colistin (polymyxin E) disrupt the outer bacterial cell membrane of certain Gram-negative bacteria, such as most *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter baumannii*, whereas other Gram-negative bacteria, including *Proteus* spp., *Providencia* spp., *Morganella morganii*, *Serratia* spp., *Edwardsiella tarda*, and bacteria of the *B. cepacia* complex exhibit intrinsic resistance to these polypeptide antibiotics. Recently, colistin was included in the WHO list of critically important antibiotics ([253]). In human medicine, colistin is used as a last-line drug in the treatment of severe infections caused by multiresistant Gram-negative bacteria, whereas it is used in veterinary medicine for the treatment of enteric diseases, mainly in swine and poultry ([254]). So far, several mechanisms of resistance to polymyxins (polymyxin B and colistin) have been described ([255]). These include a variety of LPS modifications, such as modifications of lipid A with phosphoethanolamine and 4-amino-4-deoxy-L-arabinose, efflux pumps, the formation of capsules, and overexpression of the outer membrane protein OprH ([255]). Such resistance is chromosomally encoded, and hence spread entailed either *de novo* emergence or clonal expansion of resistant isolates.

The alteration of the LPS on its lipid A moiety is the primary mechanism of resistance to polycationic polymyxins. 4-Amino-4-deoxy-L-arabinose (L-Ara4N) or phosphoethanolamine is added to lipid A by enzymes such as ArnT and EptA, resulting in a decrease of the negative charge of the LPS, thereby lowering the affinity of the positively charged polymyxins to the outer membrane. The genes *arnT* (part of the operon *arnBCADTEF*) and *eptA*, which code for those enzymes, are controlled by chromosomally encoded two-component regulatory systems, such as PmrAB and PhoPQ. Mutations in the operons *pmrAB* and *phoPQ* may lead to an upregulation of *arnT* and *eptA* expression, resulting in polymyxin resistance. This has been described in *E. coli*, *K. pneumoniae*, *S. enterica*, and *P. aeruginosa* ([255]). Furthermore, mutations in the gene for the negative feedback regulator of PhoPQ, *mgrB*,

can activate the *arnBCADTEF* operon in *K. pneumoniae* ([256](#)). Other genes coding for phosphoethanolamine transferases are *eptB* in *E. coli*, *eptC* in *C. jejuni*, and *lptA* in *N. meningitidis*. Those phosphoethanolamine transferases add phosphoethanolamine to different positions on lipid A. The main polymyxin resistance mechanism in *A. baumannii* is mutations in the genes of the PmrAB system and the resulting overexpression of *pmrC*, which codes for an EptaA-like phosphoethanolamine transferase. In *P. aeruginosa* five two-component regulatory systems (PhoPQ, PmrAB, ParRS, CprRS, ColRS) have been identified, and mutations in the genes for those systems play a role in overexpression of the *arnBCADTEF-ugd* operon ([256](#)).

In addition, plasmid-mediated resistance to polymyxins was reported in 2016 ([257](#)). The gene *mcr-1* has been identified on a conjugative plasmid in *E. coli* of animal and human origin and codes for a phosphoethanolamine transferase. The *mcr-1* gene was first identified among isolates from China, but since then it has been detected in isolates of various *Enterobacteriaceae* from five continents ([254](#)). Furthermore, the gene *mcr-2* has been described in porcine and bovine *E. coli* isolates from Belgium ([258](#)). Most recently, another three *mcr* genes, designated *mcr-3* ([259](#)), *mcr-4* ([260](#)), and *mcr-5* ([261](#)), have been identified in *E. coli* and/or *S. enterica*. Several variants of *mcr-1* have been detected in *Enterobacteriaceae* ([259](#)), while *mcr-2* variants have been found in *Moraxella* spp. ([262](#)). Variants of the gene *mcr-3* have been identified in *Aeromonas* spp. ([263](#), [264](#)).

Polymyxin resistance due to the complete loss of LPS in *A. baumannii* is based on the inactivation of genes such as *lpxA*, *lpxC*, *lpxD*, and *lpsB*, the products of which are involved in LPS biosynthesis ([265](#)).

A variety of efflux pumps in several bacterial species have been described to be involved in polymyxin resistance in Gram-negative bacteria. Sensitive antimicrobial peptide proteins, encoded by the *sapABCDF* operon, and the resistance-nodulation-cell division transporter AcrAB-TolC seem to play a role in the susceptibility to polymyxins in *E. coli*, *S. enterica*, and *Proteus mirabilis* ([266](#)). The resistance-nodulation-cell division transporter VexB is involved in polymyxin resistance in *V. cholerae* ([267](#)). The efflux pump KpnEF, which has been described in isolates of *K. pneumoniae*, belongs to the small multidrug resistance protein family and is part of the Cpx regulon, which regulates capsule synthesis. Resistance to several antibiotics such as colistin, rifampicin, erythromycin, and ceftriaxone is influenced by KpnEF ([267](#)). Other efflux pumps of the small multidrug resistance protein family involved in polymyxin resis-

tance have been identified in *B. subtilis* (EbrAB) and in *A. baumannii* (AbeS) ([266](#)).

Capsule formation is another mechanism of polymyxin resistance. Capsule polysaccharides limit the interaction of polymyxins with their target sites, thus playing an important role in polymyxin resistance, not only in intrinsically resistant bacteria such as *N. meningitidis* and *C. jejuni*, but also in *K. pneumoniae*, *E. coli*, and *P. aeruginosa* ([268](#), [269](#)). Moreover, anionic bacterial capsule polysaccharides have been shown to neutralize the bactericidal activity of cationic polypeptide antibiotics and antimicrobial peptides ([269](#)).

Overexpression of the outer membrane protein OprH contributes to polymyxin resistance in *P. aeruginosa*. OprH is a basic protein that binds to divalent cation-binding sites of LPSs, making these sites unavailable for polymyxins ([270](#)).

Resistance to Mupirocin

Mupirocin is a topical antibiotic used mainly for decolonization of MRSA and methicillin-susceptible *S. aureus* in patients and in health care personnel, but also for treatment of local skin and soft tissue infections caused by *S. aureus* and streptococci ([271](#)). It is not approved for veterinary use. Mupirocin prevents bacterial protein synthesis by inhibiting the bacterial isoleucyl-tRNA synthetase. Low-level resistance against mupirocin results from point mutations in the native isoleucyl-tRNA synthetase gene, whereas the acquisition of genes coding for alternative isoleucyl-tRNA synthetases leads to high-level resistance. The gene *mupA* (also referred to as *ileS2*) has been identified on conjugative plasmids ([272](#)–[274](#)), while the gene *mupB* has been found on nonconjugative plasmids in staphylococci ([274](#), [275](#)).

Resistance to Ansamycins

Certain ansamycins, such as rifampicin and rifamycin, are used in veterinary medicine for the treatment of infections of horses caused by *R. equi*. Rifampicin inhibits the bacterial RNA polymerase by interacting with the β-subunit, which is encoded by the gene *rpoB*. Resistance to rifampicin and related compounds is mainly due to point mutations that cause amino acid substitutions in at least one of three rifampicin resistance-determining regions within the *rpoB* gene. It is noteworthy that not all amino acid substitutions have the same effect. In *R. equi*, mutations at different positions within the *rpoB* gene have been shown to correlate with different rifampicin MIC values ([276](#), [277](#)). Mutations of *rpoB* have been described in several bacteria,

such as *R. equi* ([276](#), [277](#)), *E. coli* ([278](#)), *Mycobacterium* spp. ([279](#)), *B. subtilis* ([280](#)), *S. aureus* ([281](#)), and *S. pseudintermedius* ([282](#)).

Furthermore, RNA polymerase binding proteins, such as RbpA in *Streptomyces coelicolor* and DnaA in *E. coli*, play a role in increased insensitivity to rifampicin ([283](#)).

Arr enzymes are ADP-ribosyltransferases, which are able to modify rifampicin by ADP-ribosylation and thus inactivate it. *M. smegmatis* carries the *arr* gene in its chromosomal DNA ([284](#)), homologues of which have been identified in the genomes of bacteria such as *S. maltophilia*, *Burkholderia cenocepacia*, and other environmental bacteria ([284](#)). The gene *arr*-2 has been identified in chromosomal DNA and on various plasmids as part of class 1 integrons or composite transposons in Gram-negative bacteria such as *P. aeruginosa*, *K. pneumoniae*, and *E. coli* ([283](#)). An *arr-3* gene was detected in a class 1 integron of *Aeromonas hydrophila* from a koi carp ([285](#)).

Other modification mechanisms are glucosylation and phosphorylation in *Nocardia* spp. and phosphorylation in *Bacillus* spp. ([283](#)).

Resistance to Fosfomycin

Fosfomycin interferes with the peptidoglycan synthesis of bacteria by inhibiting the enzyme MurA. The irreversible inhibition is due to alkylation of the catalytic cysteine of MurA ([286](#), [287](#)). The exchange of cystein for asparagine is a target alteration, leading to intrinsic fosfomycin resistance in bacteria such as *M. tuberculosis*, *Chlamydia trachomatis*, and *Borrelia burgdorferi* ([286](#), [287](#)). Fosfomycin reaches its target site through GlpT, a glycerol-3-phosphate transporter, or via UhpT, a glucose-6-phosphate transporter. Both substrates induce the expression of their transporter, which is regulated by cAMP. Mutations in the genes coding for GlpT and UhpT or their regulators may lead to defective or inactive transporters, resulting in fosfomycin resistance ([286](#), [287](#)).

Enzymatic inactivation of fosfomycin can be achieved by several fosfomycin-modifying enzymes. The main enzymes described are three types of metalloenzymes (FosA, FosB, and FosX) and two kinases (FomA and FomB). FosA and FosB are thiol transferases, while FosX is a hydrolase. The metalloenzymes open the oxirane ring of fosfomycin and thus render it inactive. FosA enzymes are glutathione-*S*-transferases which use Mn^{2+} and K^+ as metal cofactors. They add glutathione to the oxirane ring, thereby opening the ring and inactivating fosfomycin ([281](#)). Various *fosA* genes have been identi-

fied on plasmids or in the chromosomal DNA of Gram-negative bacteria such as *S. marcescens*, *E. coli*, *K. pneumoniae*, *E. cloacae*, and *P. aeruginosa* ([286](#), [287](#)). They have been described as parts of transposons such as Tn*2921* or flanked by copies of IS26 on plasmid pFOS18 ([288](#)). The gene *fosC2*, which also codes for a glutathione-*S*-transferase, has been identified on a gene cassette in a class 1 integron on a conjugative plasmid in *E. coli* ([289](#)). Other *fos* genes have been described in numerous bacteria, including *fosC* in *Achromobacter denitrificans* and *fosK* in *Acinetobacter soli* ([290](#)).

FosB enzymes are bacillithiol-*S*-transferases that use Mg^{2+} as a cofactor. Several *fosB* gene variants have been detected in the chromosomal DNA of Gram-positive bacteria, such as *Staphylococcus epidermidis* ([291](#)), *B. subtilis*, *Bacillus anthracis*, and *S. aureus* ([286](#), [287](#)). The gene *fosB3* has been identified on a conjugative plasmid in *E. faecium* ([292](#)), while the genes *fosB1*, *fosB5*, and *fosB6* were located on small plasmids in *S. aureus* ([293](#)). The gene *fosD* is related to *fosB* and has been found in avian *Staphylococcus rostri* ([294](#)).

FosX enzymes are Mn^{2+}-dependent epoxide hydrolases, which use water to break the oxirane ring ([286](#), [287](#)). Variants of the gene *fosX* have been detected in the chromosomal DNA of *Clostridium botulinum*, *L. monocytogenes*, and *Brucella melitensis* ([287](#)). The gene *fosX*^{CC} was detected as part of a multidrug-resistance genomic island in *C. coli* ([295](#)).

FomA and FomB are kinases that originate from the fosfomycin producer *Streptomyces wedmorensis*. They sequentially add phosphates to the phosphonate moiety of fosfomycin by using Mg^{2+} as a cofactor ([286](#)). Most likely, these enzymes represent part of the self-defense system of the fosfomycin producer ([286](#)). Another such kinase, originally called FosC and found in another fosfomycin producer, *Pseudomonas syringae*, is an ortholog of FomA ([296](#)).

Resistance to Fusidic Acid

Fusidic acid is a steroidal compound which was isolated from *Fusidium coccineum*. It exhibits antimicrobial activitiy against Gram-positive bacteria, such as staphylococci and *Corynebacterium* spp., as well as the Gram-negative *Neisseria gonorrhoeae*, *N. meningitidis*, and *Moraxella catarrhalis* ([297](#)). Fusidic acid is mainly used topically to treat skin infections caused by staphylococci, but it can also be administered systemically. Fusidic acid binds to elongation factor G and thus prevents polypeptide chain elongation during protein synthesis ([298](#)). Elongation factor G is encoded by the gene *fusA*. Several mutations in the gene *fusA* that cause resistance to

fusidic acid have been described in *S. aureus*, with L461K being the most prevalent (298). Protection of the target site and subsequent fusid acid resistance is conferred by the protein FusB, which prevents the interaction of fusidic acid with elongation factor G (299). The gene *fusB* has been identified on the widespread plasmid pUB101 in *S. aureus* (300). A *fusB*-related gene, *fusF*, has recently been identified in *Staphylococcus cohnii* (301). In contrast, the genes *fusC* and *fusD*, which also confer fusidic acid resistance by target protection, have been detected as part of a chimeric SCC*mec*IV-SCC$_{476}$ element in the chromosomal DNA of *S. aureus* (302) and in the chromosomal DNA of *S. saprophyticus* (303), respectively.

Resistance to Streptothricins

Streptothricins are antibiotics that consist of a streptolidine ring, a glucosamine, and a polylysine side chain. One of them, nourseothricin, was used as an antimicrobial feed additive in industrial animal farming in the former East Germany (304). Several *sat* genes have been identified which mediate streptothricin resistance by enzymatic inactivation via acetylation. In Gram-negative bacteria, particularly in *Enterobacteriaceae*, *sat* or *sat2* genes are usually located on gene cassettes in class 1 or class 2 integrons (305, 306). In staphylococci, the *sat4* gene is part of Tn*5405* and, as such, is commonly detected in staphylococci that also harbor *aphA3* and *aadE*. Furthermore, this gene has been detected in canine and feline *S. pseudintermedius* (307–309) and in MRSA of CC8 (ST254) from horses in Germany (310).

Resistance to Substances with Antimicrobial Activity Formerly Used as Growth Promoters

A number of substances with antimicrobial activity have been licensed as growth promoters for livestock. In the European Union, all growth promoters with antimicrobial activity were banned or withdrawn by 2006.

The mechanisms of resistance to the macrolides tylosin and spiramycin, the streptogramin virginiamycin, and the glycopeptide avoparcin were described in the sections "Resistance to Macrolides, Lincosamides, and Streptogramins (MLS)" and "Resistance to Glycopeptides." Hence, a brief summary of resistance to the remaining classes of growth promoters is given below. Two reviews (252, 311) are recommended for detailed insight into the various aspects of the use of growth promoters.

Bacitracin resistance was first described in the producer organism *Bacillus licheniformis*, in which an ABC transporter system, BcrABC, acts as a self-defense system by exporting the antibiotic from the producer cell (312). In *B. subtilis*, two independent but complementary-acting resistance mechanisms have been detected: an ABC transporter, YtsCD, that mediates the efflux of bacitracin, and a protein designated YwoA, which is believed to compete with bacitracin for the dephosphorylation of the C55-isoprenyl pyrophosphate (313). In *E. coli*, the gene *bacA*, which codes for an undecaprenyl pyrophosphate phosphatase, may account for bacitracin resistance (314). An ABC transporter, also termed BcrAB, that mediates bacitracin resistance was identified on a conjugative plasmid in *E. faecalis* (315).

Avilamycin resistance in the producer organism *Streptomyces viridochromogenes* Tü57 is based on the activity of an ABC transporter and two rRNA methyltransferases (316). In *E. faecalis* and *E. faecium*, resistance to avilamycin was initially described to be due to variations in the ribosomal protein L16 (317). Later, an rRNA methyltransferase, EmtA, which confers high-level resistance to avilamycin and evernimicin, was identified (318). Another two methylases—AviRa, which methylates 23S rRNA at the guanosine 2535 base, and AviRb, which methylates the uridine 2479 ribose—have been shown to confer avilamycin resistance (319). In addition, mutations at specific positions in the 23S rRNA also give rise to avilamycin resistance (320). Flavophospholipol (also known as flavomycin or bambermycin) has been reported to have a "plasmid-curing effect" on multiresistant *E. coli* under experimental conditions *in vitro* and *in vivo* (321). Cross-resistance to other antimicrobials has not been observed (252). Moreover, no genes or mutations conferring flavophospholipol resistance have been observed, to date.

Ionophores, such as salinomycin-Na and monensin-Na, are mainly used for the prevention of infections with parasites, such as *Eimeria* spp. (coccidiosis), *Plasmodium* spp., and *Giardia* spp. (252). Resistance or decreased susceptibility has been described in *S. hyicus*, coagulase-negative staphylococci from cattle and from *E. faecium* and *E. faecalis* from poultry and pigs. Genes or mutations accounting for acquired resistance to ionophores have not yet been described (252). Resistance to quinoxalines, such as carbadox and olaquindox, has been reported. An early study identified carbadox resistance to be associated with a conjugative multiresistance plasmid in *E. coli* (322). More than 20 years later, the genes *oqxA* and *oqxB*, which are responsible for olaqindox resistance, were cloned from a conjugative plasmid in *E. coli* (323). The corresponding gene products are homologous to several resistance-nodulation-

cell-division family efflux systems and use TolC as the outer membrane component. Interestingly, the OqxAB-TolC system also mediates resistance to chloramphenicol and ethidium bromide (323).

CONCLUSION

The development of antimicrobial resistance—by either mutations, development of new resistance genes, or the acquisition of resistance genes already present in other bacteria—is a complex process that involves various mechanisms. Numerous resistance genes specifying different resistance mechanisms have been identified in various bacteria. The speed of resistance development differs with regard to the bacteria involved, the selective pressure imposed by the use of antimicrobial agents, and the availability and transferability of resistance genes in the gene pools accessible to the bacteria. These basic facts apply to resistance development in bacteria from humans as well as in bacteria from animals. The loss of acquired resistance properties is often a cumbersome process which is influenced mainly by selective pressure, but also by the colocation of the resistance genes on multiresistance plasmids or in the chromosomal DNA and the organization of the resistance genes in multi-resistance gene clusters or integron structures. When organized in resistance gene clusters or integrons, loss of resistance genes may not be expected even in the absence of direct selective pressure. Because we know that the use of every antimicrobial substance can select for resistant bacteria, prudent use of antimicrobial agents is strongly recommended in both human and veterinary medicine, but particularly in food animal production to retain the efficacy of antimicrobial agents for the control of bacterial infections in animals.

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