Lincosamides, Streptogramins, Phenicols, and Pleuromutilins: Mode of Action and Mechanisms of Resistance

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Lincosamides, streptogramins, phenicols, and pleuromutilins (LSPPs) represent four structurally different classes of antimicrobial agents that inhibit bacterial protein synthesis by binding to particular sites on the 50S ribosomal subunit of the ribosomes. Members of all four classes are used for different purposes in human and veterinary medicine in various countries worldwide. Bacteria have developed ways and means to escape the inhibitory effects of LSPP antimicrobial agents by enzymatic inactivation, active export, or modification of the target sites of the agents. This review provides a comprehensive overview of the mode of action of LSPP antimicrobial agents as well as of the mutations and resistance genes known to confer resistance to these agents in various bacteria of human and animal origin.

For more than 70 years, antimicrobial agents have been indispensable for the control of bacterial infections in human and veterinary medicine. They inhibit bacteria mainly by interfering with cell-wall synthesis, nucleic acid synthesis, or protein synthesis. Their efficacy is, however, hampered by the continuous development of resistance not only by the target bacteria but also by members of the physiological microbiota in both humans and animals. Lincosamides, streptogramins, phenicols, and pleuromutilins (LSPPs) represent four classes of antimicrobial agents that inhibit protein synthesis by interacting with the 50S subunit of bacterial ribosomes. Bacterial resistance to LSPP antimicrobial agents can be a result of the acquisition of endogenous mutations or horizontally transmitted resistance genes (Schwarz et al. 2006). The mechanisms known so far, associated with resistance to LSPP antimicrobial agents, commonly fall into three categories: enzymatic inactivation, active efflux, and/or structural changes at the ribosomal target site. Many of the resistance genes known so far that confer LSPP resistance are located on mobile genetic elements, which facilitate their dissemination across strain, species, and even genus boundaries. The understanding of the mode of action of antimi-

crobial agents has in the past led to the development of derivatives of known antimicrobial agents, designed to escape preexisting bacterial resistance mechanisms (Schwarz and Kehrenberg 2006). As such, the knowledge of not only the mode of action but also of the mode of resistance is a key parameter in the understanding of the complex interaction of antimicrobial agents and bacteria.

In the present review, we summarize the current knowledge of the mode of action of LSPP antimicrobial agents and provide an overview of the genetic basis of resistance to these agents in bacteria of human and animal origin.

USE OF LSPP ANTIMICROBIAL AGENTS IN HUMAN AND VETERINARY MEDICINE

Lincosamides

Lincosamides consist of three components: an amino acid (aa) (L-proline substituted by a 4′-alkyl chain) and a sugar (lincosamine), connected by an amide bond (Fig. 1A) (Bryskier 2005a). The first lincosamide, lincomycin, was isolated in 1962 from *Streptomyces lincolnensis* ssp. *lincolnensis* found in a soil sample from Lincoln, NE (MacLeod et al. 1964; Bryskier 2005a). In 1967, lincomycin was licensed in the United States for the treatment of infections caused by Gram-positive bacteria. Although approved for use in human medicine, lincomycin is rarely used nowadays. In veterinary medicine, lincomycin is approved for use in various infections in swine, dogs, and cats. In combination with spectinomycin, it is not only approved for ruminants, pigs, and poultry (Giguère 2013) but also for dogs, cats, and carrier pigeons.

As lincomycin has only a limited spectrum of activity, various chemical modifications were introduced to improve the pharmacokinetics of lincomycin and to expand its antibacterial spectrum. The 7-chloro-7-deoxylincomycin derivative, clindamycin, proved to be the most effective. Clindamycin was approved by the Food and Drug Administration (FDA) in 1970 in the United States. The antimicrobial spectrum of clindamycin includes staphylococci, group A and B streptococci, *Streptococcus pneumoniae*, most anaerobic bacteria, and *Chlamydia trachomatis* (Smieja 1998). Moreover, clindamycin also shows activity against several protozoa, such as *Plasmodium* spp. and *Toxoplasma* spp. (Bryskier 2005a). However, it shows little, if no, activity against most aerobic Gram-negative bacilli, *Nocardia* spp., *Mycobacterium* spp., as well as *Enterococcus faecalis* and *Enterococcus faecium* (Giguère 2013). In veterinary medicine, clindamycin must not be used in food-producing animals, but may be used in dogs and cats under the Animal Medicinal Drug Use Clarification Act of 1994 (AMDUCA) in the United States and under similar regulations in other countries. Clindamycin is often used in human medicine for the treatment of infections caused by anaerobic bacteria. Because of its activity against anaerobes, it can lead to disruption of the intestinal microbiota and *Clostridium difficile* overgrowth causing diarrhea and colitis (Gerding et al. 1995). A similar situation has been observed in horses in which lincosamide administration can cause *C. difficile*–associated disease (CDAD), that is, a severe to fatal enterocolitis (Diab et al. 2013; Giguère 2013). It should be noted that lincosamides are also highly toxic to rabbits, guinea pigs, and hamsters, where toxins produced by *Clostridium perfringens* and other clostridia have been implicated in lincomycin- and clindamycin-induced enteritis (Morris 1995).

A new lincosamide, pirlimycin, was approved in 2000 in the United States and in 2001 in the European Union (EU). Pirlimycin represents a *cis*-4-ethyl-L-picecolic acid amide of clindamycin (Ahonkhai et al. 1982). It is exclusively approved for veterinary applications, that is, as an intramammary infusion for the control of staphylococci and streptococci associated with bovine subclinical mastitis.

Streptogramins

Streptogramins (pristinamycin, virginiamycin, mikamycin, and quinupristin–dalfopristin) consist of two structurally different components, A and B (Fig. 1B). The A components, such as pristinamycin IIA, virginiamycin M, mikamycin A, or dalfopristin, are polyunsaturated macro-
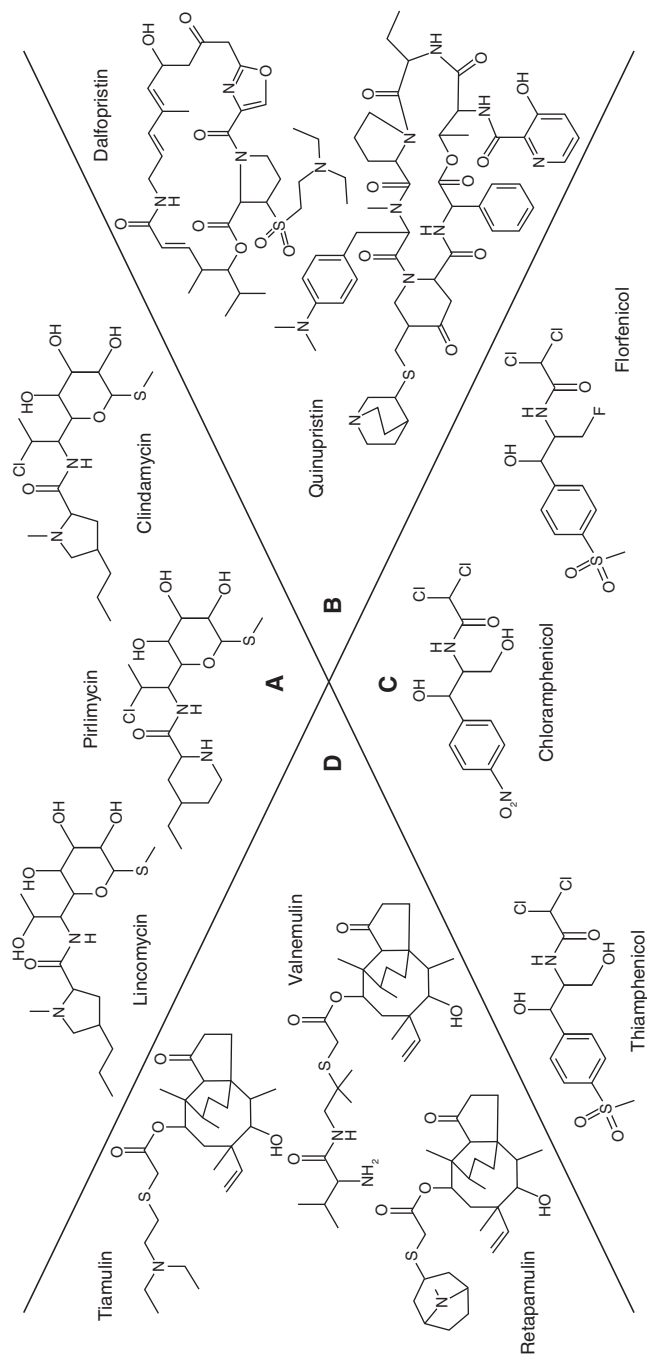
Figure 1. Structural formulas of the lincosamides, streptogramins, phenicols, and pleuromutilins (LSPP) antimicrobial agents. (*A*) The lincosamides lincomycin, clindamycin, and pirlimycin, (*B*) the streptogramin A dalfopristin and the streptogramin B quinupristin, (*C*) the phenicols chloramphenicol, thiamphenicol, and florfenicol, and (*D*) the pleuromutilins tiamulin, valnemulin, and retapamulin.

lactones. The B components, such as pristinamycin IB, virginiamycin S, mikamycin B, or quinupristin, are cyclic hexadepsipeptides (Allignet et al. 1996; Giguère 2013).

Pristinamycin was identified in culture filtrates of *Streptomyces pristinaespiralis* in 1962, virginiamycin in *Streptomyces virginiae* in 1955, and mikamycin in *Streptomyces mitakaensis* in 1956 (Vazquez 1966). Natural mixtures of streptogramins have been available for clinical use in Europe since the mid-1950s. Streptogramins cannot cross the outer membrane of most Gram-negative bacteria and are primarily effective against Gram-positive bacteria (Wright 2007). They have been used topically or orally in the treatment of skin, bone, and respiratory infections, mainly caused by staphylococci (Allignet et al. 1996). The first semisynthetic injectable streptogramin compound, quinupristin–dalfopristin, was approved in 1999. Quinupristin–dalfopristin represents one of the few potential antimicrobial agents for the treatment of infections in humans caused by multiresistant *E. faecium*, especially in cases of vancomycin- and/or linezolid-resistant isolates and also for multiresistant staphylococci, including methicillin-resistant *Staphylococcus aureus* (MRSA) (see who.int/iris/bitstream/10665/43765/1/9789241595742_eng.pdf). Therefore, it is considered as a last resort drug for human use (see ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/11/WC500118230.pdf).

Virginiamycin has been developed largely as a growth promoter (Giguère 2013) but its use for that purpose was phased out in the EU by the end of 2005. Since January 1, 2006, antimicrobial agents are not allowed to be used as growth promoters in the EU anymore. Streptogramins are currently not approved for therapeutic purposes in veterinary medicine in the EU, but virginiamycin may be used for specific indications in swine and horses in North America (Giguère 2013).

Phenicols

Chloramphenicol was isolated from *Streptomyces venezuelae* in 1947. It was the first phenicol antimicrobial agent and also the first natural product found to contain a nitro group (Fig. 1C). Because of the relative simplicity of its structure, chloramphenicol has been produced synthetically since 1950 (Schwarz et al. 2004). Thiamphenicol is a derivative of chloramphenicol, in which the *p*-nitro group has been replaced by a sulfomethyl group. Florfenicol is a fluorinated derivative of thiamphenicol in which the hydroxyl group at C3 has been replaced with fluorine (Dowling 2013).

Based on its activity against a wide range of Gram-positive and Gram-negative bacteria, chloramphenicol was initially considered a promising antimicrobial agent (Shaw 1983). However, serious adverse effects have been observed since the mid-1960s. These included a dose-unrelated irreversible aplastic anemia, a dose-related reversible bone marrow suppression, or the Gray syndrome in neonates and infants. In addition, hypersensitivity to chloramphenicol has been observed occasionally (Schwarz et al. 2004). Based on these adverse effects and the availability of less toxic antimicrobial agents with a similar spectrum of activity, chloramphenicol is now used in human medicine only for the treatment of a small number of life-threatening infections or for topical applications, for example, in eye infections. In veterinary medicine, chloramphenicol is still used in pets and non-food-producing animals. It was banned from use in food-producing animals in the EU in 1994 and in many other countries soon thereafter.

Thiamphenicol shows lower antimicrobial activity than chloramphenicol and, therefore, has rarely been used in human and veterinary medicine. Both, thiamphenicol and florfenicol do not cause dose-unrelated irreversible aplastic anemia, but may cause dose-dependent bone marrow suppression in animals (Dowling 2013). Florfenicol is exclusively approved for use in food-producing animals. Since 1995, florfenicol has been approved in numerous countries for the treatment of respiratory disease, pododermatitis, and keratoconjunctivitis in cattle, swine respiratory disease, air sacculitis in broiler chickens, but also for various diseases in fish. The application of florfenicol in horses is not recommended (Dowling 2013).

Pleuromutilins

Pleuromutilins are diterpene antimicrobial agents (Fig. 1D). They were discovered as natural antimicrobial agents in 1950/1951 (Bryskier 2005b; Novak and Shlaes 2010). The first pleuromutilin was found in the basidiomycete *Pleurotus mutilus*, later renamed as *Clitopilus scyphoides* (Bryskier 2005b). Tiamulin and valnemulin are semisynthetic pleuromutilins. Tiamulin was the first pleuromutilin to be approved for veterinary use in 1979, followed by valnemulin in 1999 (Novak and Shlaes 2010). Both tiamulin and valnemulin are exclusively used in veterinary medicine, mainly for the control of bacterial infections in pigs and poultry. They are particularly active against anaerobic bacteria, *Mycoplasma* spp., and selected Gram-positive and Gram-negative bacteria (Giguère 2013). In swine, tiamulin is commonly used for the treatment of swine dysentery caused by *Brachyspira hyodysenteriae*, swine pneumonia caused by *Actinobacillus pleuropneumoniae* and *Mycoplasma hyodysenteriae*, colonic spirochaetosis caused by *Brachyspira pilosicoli*, and proliferative enteropathy caused by *Lawsonia intracellularis*. In poultry, both pleuromutilins are commonly used for the treatment of *Mycoplasma gallisepticum* infections. Pleuromutilins are not approved for use in ruminants and should also not be administered to horses (Giguère 2013).

In 2007, the first pleuromutilin antimicrobial agent, retapamulin, was approved for use in humans (Novak and Shlaes 2010). However, the use of retapamulin is limited to topical treatment of impetigo caused by methicillin-susceptible *S. aureus* (MSSA) or *Streptococcus pyogenes* in patients of 9 months or older.

MODE OF ACTION OF LSPP ANTIMICROBIAL AGENTS

Early studies of the mechanisms of action of various antimicrobial agents relied on their effects in various functional assays. The interpretations of these studies were influenced by the lack of knowledge of their binding sites, and whether or not the effect assayed for was a major/minor or a direct/indirect effect. Details and summaries of these studies for translational inhibitors have been reviewed by Wilson (2009). After solution of the bacterial ribosome structure in the year 2000 (Ban et al. 2000), the unraveling of mechanisms of action for protein synthesis inhibitors increased tremendously because the binding sites of the antimicrobial agents could now be determined. The ribosome crystals were coincubated with the antimicrobial agents and thereafter the detailed position of binding could be determined for each antimicrobial agent. From the position in the ribosomes, the overall mode of action could then be deduced.

LSPP antimicrobial agents have very diverse chemical structures as shown by the examples presented in Figure 1. Despite their differences, they have all been found to bind at the peptidyltransferase center (PTC) of bacterial ribosomes. The ribosome structures with the bound antimicrobial agents have been obtained from various bacteria (*Escherichia coli*, *Deinococcus radiodurans*, *Thermus thermophilus*, and *S. aureus*) and one archaeon *Haloarcula marismortui*, and they show some minor differences in antibiotic binding. Details and summaries of these structures have been described by Wilson (2011, 2014) and Eyal et al. (2015). It is possible to transfer the binding positions from one structure to another to compare their binding sites as presented in Figure 2, which shows binding of one example from each antimicrobial class placed together. It is thus obvious that LSPP antimicrobial agents have overlapping binding sites and that each of them occupies specific sites in the ribosome in the area where the ribosome extends the nascent peptide chain. The binding site is in the bottom of the cleft of the 50S ribosomal subunit where the 3′-ends of aminoacyl-tRNA and peptidyl-tRNA are positioned for peptide transfer. The site is composed exclusively of RNA, and mainly RNA from the central part of domain V of 23S RNA (Nissen et al. 2000), and it is highly conserved in all bacteria. The same site in the ribosome also binds the oxazolidinones including linezolid.

Streptogramin B antimicrobial agents bind at a site adjacent to the PTC in the beginning of the nascent peptide exit tunnel (Fig. 2) and over-
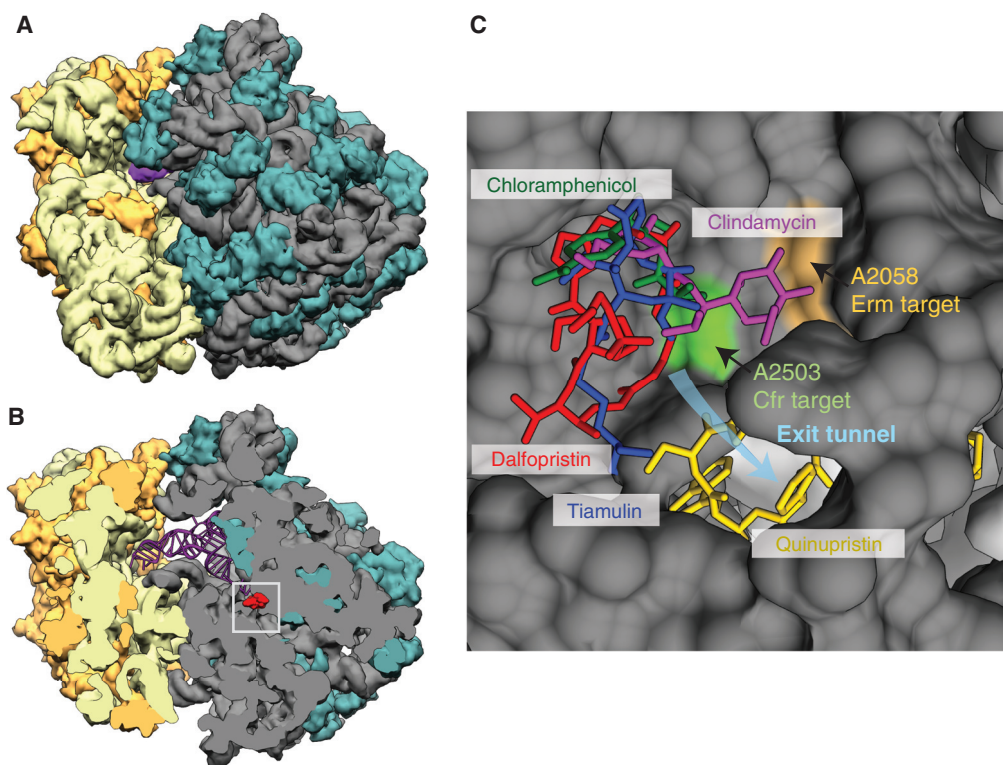
Figure 2. Lincosamides, streptogramins, phenicols, and pleuromutilin (LSPP) binding to the ribosome. (*A*) A model of the *Escherichia coli* bacterial ribosome with the two ribosomal subunits (based on RCSB Protein Data Bank [PDB] 4V9D; see rcsb.org, last accessed August 9, 2015), 30S with RNA in light yellow and proteins in darker yellow, 50S with RNA in grey and proteins in greenish. The magenta is the anticodon tip of tRNA in P-site. (*B*) A cut-view of the 50S subunit from A with a streptogramin A molecule (dalfopristin from PDB 4U26) in red to mark the peptidyltransferase center (PTC) area. The square marks the approximate area shown as a blow-up in *C*. (*C*) The overlapping binding sites of lincosamides, streptogramins, phenicols, and pleuromutilins exemplified by clindamycin (in pink) from PDB 4V7T, quinupristin (in yellow), and dalfopristin (in red) from PDB 4U26, chloramphenicol (in green) from PDB 4V7V, and tiamulin (in blue) from PDB 1XBP, respectively, and all placed in the *E. coli* structure. All PDB structures are from rcsb.org/pdb/home/home.do (Berman et al. 2000). The colored nucleotide bases indicate the positions methylated by Erm and Cfr methyltransferases. Nucleotides "in front" of the antimicrobial agents have been removed to be able to see the agents in their binding site.

lap the binding site of macrolides (reviewed in Poehlsgaard and Douthwaite 2005). The environment of the PTC seems to facilitate binding of a range of antimicrobial agents, which in this way interfere with the peptide transfer process. They can thus either disturb the positioning of aminoacyl-tRNA or peptidyl-tRNA for peptide transfer or directly block some movements required during peptide transfer. The overall mechanism of action is the inhibition of peptide transfer by sterically blocking the transfer process. The exact effect depends on the access of the antimicrobial agents to the PTC (during the initiation or the nascent chain elongation), "on" and "off" rates of each agent, and their binding to the ribosomal A- and/or P-site.

The phenicols are small molecules and at least one of them, chloramphenicol, seems to have an alternative binding site in archael ribosomes as discussed by Dunkle et al. (2010), but still with its active site in the A-site of the PTC. The structural data on phenicol binding to the ribosome have been obtained with chloramphenicol, but florfenicol and thiamphenicol

probably bind similarly. Lincosamides bind adjacent to and a bit overlapping with chloramphenicol and also at the A-site of PTC (Fig. 2) (Dunkle et al. 2010). The streptogramins appear naturally in pairs and bind together and work synergistically. The A component binds in PTC and the B component binds right beside it at the entrance of the tunnel (Fig. 2). The binding and effect on translation has recently been revisited by Noeske et al. (2014). All pleuromutilins have a conserved tricyclic core that fits nicely in a "cave" in the A-site PTC of the ribosomes (Davidovich et al. 2007). They vary by extensions pointing toward the P-site and occupying different positions (Davidovich et al 2007).

The older literature focused on the effect of various assays such as the puromycin reaction, fMet-tRNA binding, A- and P-site tRNA binding, translocation, etc.; however, some of these assays were under unnatural conditions and sometimes contradicting results were obtained. Nowadays, focus is more on defining the binding sites and using this knowledge: (1) to develop derivatives that bind more strongly to the ribosome and/or that bind despite target site modifications providing resistance; (2) to avoid other resistance mechanisms; and (3) to improve solubility and pharmacokinetics of the antimicrobial agents without compromising their effect.

PREVALENCE OF RESISTANCE TO LSPP ANTIMICROBIAL AGENTS

To classify a bacterial isolate as resistant or susceptible to an antimicrobial agent, clinical breakpoints are necessary. The Clinical and Laboratory Standards Institute (CLSI) has published a wide range of clinical breakpoints applicable to bacteria from humans and from animals (CLSI 2015, 2016). Among the lincosamides, no clinical breakpoints are available for lincomycin, whereas those for pirlimycin are applicable only to *S. aureus* and certain streptococcal species from bovine mastitis. For clindamycin, clinical breakpoints are only available for human staphylococci, streptococci, and anaerobes as well as for canine staphylococci and β-hemolytic streptococci. For the streptogramin

combination quinupristin–dalfopristin, only clinical breakpoints for staphylococci, streptococci, and enterococci are available. Among the pleuromutilins, the only clinical breakpoint available is for tiamulin and applicable to porcine *A. pleuropneumoniae*. In contrast, clinical breakpoints for chloramphenicol are available for a wide variety of Gram-positive and Gram-negative bacteria of human origin, whereas those for florfenicol are only applicable to bovine and porcine respiratory tract pathogens. In addition to CLSI, the European Committee on Antimicrobial Suceptibility Testing (EUCAST) provides slightly divergent clinical breakpoints (see eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_6.0_Breakpoint_table.pdf). Although there are numerous national resistance monitoring programs, those in human medicine often cover only pathogens from specific infections, mainly food-borne diarrheal diseases, whereas those in veterinary medicine usually monitor indicator bacteria and commensal bacteria, and only a very limited number of pathogenic bacteria (Silley et al. 2012).

Table 1 shows some examples of resistance prevalences for LSPP antimicrobial agents among pathogenic bacteria from the national resistance monitoring programs DANMAP (see danmap.org), GE*RM*-Vet (see www.bvl.bund.de/DE/09_Untersuchungen/untersuchungen_node.html;jsessionid=76F5E0F5BC375CA81F9CEF5BE44991E1.2_cid322), NARMS (see cdc.gov/narms/reports), NORM/NORM-VET (see vetinst.no/eng/Publications/NORM-NORM-VET-Report), as well as some long-term surveillance studies (Portis et al. 2012; Lindeman et al. 2013).

MECHANISMS OF RESISTANCE

In general, antimicrobial resistance can be based on two different mechanisms: the acquisition of mutations or resistance genes. Resistance-mediating mutations usually occur in genes or regions that represent the target site of the antimicrobial agents and prevent efficient binding of the antimicrobial agents to these sites. However, mutations may also enhance expression of

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Table 1. Resistance rates to LSPP antimicrobial agents from selected national resistance monitoring programs and surveillance studies

LSPP antimicrobial agent	Bacteria	Origin	Year	Isolates tested	Resistant isolates (%)	MIC (mg/L)	Country	References[a]
Clindamycin	*Staphylococcus aureus* (MSSA)	Human—blood culture	2013	1155	1.5	≥ 1	Norway	NORM/ NORM-VET
	S. aureus (MRSA)	Human	2013	1528	18.9	≥ 1	Norway	NORM/ NORM-VET
	S. aureus (MSSA)	Human—bacteraemia	2014	381	8.4	≥ 1	Denmark	DANMAP
	S. aureus (MRSA)	Human	2014	1932	33.0	≥ 1	Denmark	DANMAP
	Staphylococcus pseudintermedius	Dog—clinical	2013	201	18.0	≥ 0.5	Norway	NORM/ NORM-VET
	S. pseudintermedius	Dog—skin infections	2011	54	38.9	≥ 4	Germany	GE*RM*-Vet
Pirlimycin	*S. aureus*	Cattle—mastitis	2009	210	1.4	≥ 4	Germany	GE*RM*-Vet
	S. aureus	Cattle—mastitis	2010	342	3.0	≥ 4	United States, Canada	Lindeman et al. 2013
	Streptococcus dysgalactiae	Cattle—mastitis	2009	158	17.7	≥ 4	Germany	GE*RM*-Vet
	S. dysgalactiae	Cattle—mastitis	2010	257	7.0	≥ 4	United States, Canada	Lindeman et al. 2013
	Streptococcus uberis	Cattle—mastitis	2009	289	27.0	≥ 4	Germany	GE*RM*-Vet
	S. uberis	Cattle—mastitis	2010	289	25.0	≥ 4	United States, Canada	Lindeman et al. 2013
Quinupristin–dalfopristin	*S. aureus*	Poultry—clinical	2011	43	27.9	≥ 2	Germany	GE*RM*-Vet
	S. aureus	Horse—clinical	2011	33	0.0	≥ 2	Germany	GE*RM*-Vet
	S. pseudintermedius	Dog—skin infections	2011	54	0.0	≥ 2	Germany	GE*RM*-Vet
	Enterococcus faecium	Broiler meat	2014	177	2.8	≥ 8	Denmark	DANMAP
	E. faecium	Beef	2014	56	0.0	≥ 8	Denmark	DANMAP
	E. faecium	Pork	2014	23	0.0	≥ 8	Denmark	DANMAP
Tiamulin	*Actinobacillus pleuropneumoniae*	Swine—respiratory tract	2012	41	2.4	≥ 32	Germany	GE*RM*-Vet
Chloramphenicol	Nontyphoidal *Salmonella*	Human	2013	2178	3.9	≥ 32	United States	NARMS
	Salmonella Typhi	Human	2013	279	9.3	≥ 32	United States	NARMS
	Escherichia coli O157	Human	2013	177	2.8	≥ 32	United States	NARMS

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Table 1. *Continued*

LSPP antimicrobial agent	Bacteria	Origin	Year	Isolates tested	Resistant isolates (%)	MIC (mg/L)	Country	References[a]
	E. coli	Turkey—clinical	2012	159	18.9	≥32	Germany	GERM-Vet
	S. pseudintermedius	Dog—skin infections	2011	54	24.1	≥32	Germany	GERM-Vet
Florfenicol	*Pasteurella multocida*	Cattle—respiratory tract	2012	77	1.3	≥8	Germany	GERM-Vet
	P. multocida	Cattle—respiratory tract	2009	328	11.6	≥8	United States, Canada	Portis et al. 2012
	Mannheimia haemolytica	Cattle—respiratory tract	2009	304	8.6	≥8	United States, Canada	Portis et al. 2012
	Bordetella bronchiseptica	Swine—respiratory tract	2012	90	2.2	≥8	Germany	GERM-Vet
	A. pleuropneumoniae	Swine—respiratory tract	2012	41	0.0	≥8	Germany	GERM-Vet

TSPP, Lincosamides, streptogramins, phenicols, and pleuromutilins.

[a]Websites of the national resistance monitoring programs are given in the section "Prevalence of Resistance to LSPP Antimicrobial Agents."

efflux genes or alter the substrate spectrum of transporters and thereby cause resistance or decreased susceptibility. Resistance genes may confer antimicrobial resistance by either enzymatic inactivation, active efflux, or modifications at the target sites of the antimicrobial agents. Although mutations occur spontaneously and are vertically transferred during division of a cell that harbors the mutation, resistance genes are often associated with mobile genetic elements that are disseminated vertically during cell division, but also horizontally by the gene transfer processes.

Ribosomal Mutations Associated with Resistance to LSPP Antimicrobial Agents

All LSPP antimicrobial agents bind to a well-conserved area of the ribosome consisting mainly of RNA. Although the RNA sequence in the area is conserved and therefore not expected to allow many mutations, a number of mutations in domain V of 23S RNA causing resistance has been observed. It is also noteworthy that most bacteria have multiple 23S rRNA (ribosomal RNA) copies meaning that recombinations have to occur to obtain a full effect of such mutations. It has been shown that, for example, *S. aureus* under antimicrobial pressure will increase the number of mutated ribosomal RNA operons over time (Besier et al. 2008). Mutations occur constantly but maintaining 23S RNA resistance mutations depends on the number of copies of 23S RNA genes, how dominant the mutation is, and how "expensive" it is to contain a mutated RNA nucleotide in the exact position. In addition, it differs from species to species which mutations show up, and sometimes it is hard to prove that the resistance effect is because of the mutations observed as other mutations may contribute to or be the main effector. In addition, the identification of resistance mutations in clinical or animal isolates might be complicated by the lack of a wild-type comparator or the bacterial species might be hard to assay for resistance. The pub-

lished literature thus contains more- or less-proven examples of resistance mutations, and only the most proven and solid data has been included here. All 23S RNA mutations causing resistance to the LPPS antimicrobial agents have been found in the part of 23S RNA shown in the secondary structure model shown in Figure 3. This region encompasses the RNA located at the PTC and contains the nucleotides known to be involved in binding of antimicrobial agents to the PTC.

As can be seen, some of the RNA mutations cause resistance to multiple antimicrobial agents and some also to agents not mentioned in this review. Especially the adenine residues at positions 2058 and 2059 are well-studied nucleotides for macrolide and lincosamide resistance (Vester and Douthwaite 2001). Antibiotics with partly overlapping binding sites may also bind to some of the same nucleotides and, thus,

cross-resistance can be seen. However, the predictions of such a cross-resistance do not follow an easy recognizable pattern (Long et al. 2010) and, furthermore, multiple mutations can show enhanced effects (Douthwaite 1992; Long et al. 2010).

In addition to 23S rRNA, there are also a few ribosomal proteins located close to PTC, including L3 and L4 (Nissen et al. 2000), in which mutations have been correlated with antimicrobial resistance. L3 mutations associated with tiamulin resistance have been summarized by Klitgaard et al. (2015), and most data relate to the aa 144–151 region of L3 (*E. coli* numbering), which is also the region closest to PTC. The mutations have been found in *E. coli*, *Staphylococcus* spp., and *Brachyspira* spp., and especially mutations at positions 149–150 have been proven to confer pleuromutilin resistance (see Klitgaard et al. 2015, and references therein).
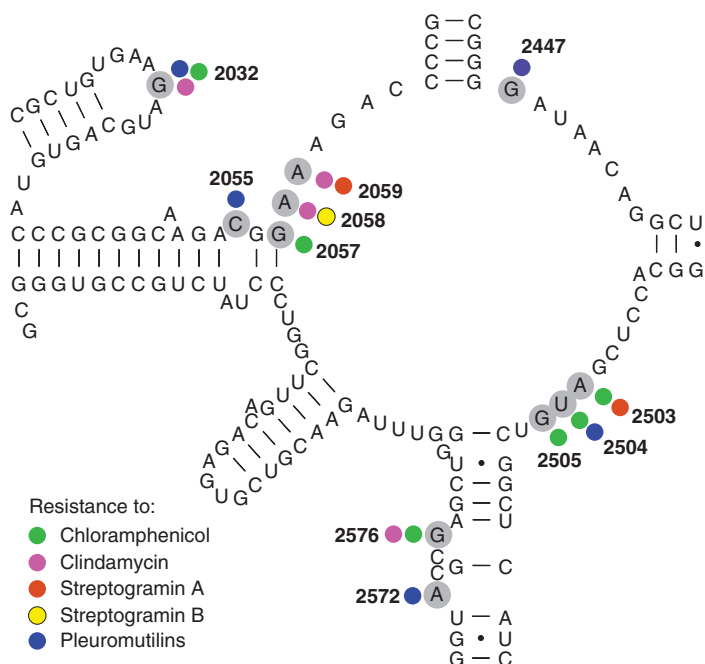
Figure 3. A secondary structure model of the peptidyl transferase loop of domain V of 23S rRNA (*Escherichia coli* sequence and numbering) with nucleotides providing antibiotic resistance marked with gray circles. Data are all from bacteria except the streptogramin A data that come from an archae (Porse and Garrett 1999). The bacterial data are from the following studies: Douthwaite (1992), Vester and Douthwaite (2001), Miller et al. (2008), Long et al. (2009, 2010), and Li et al. (2011), and references herein. The smaller circles indicate resistance to the various antibiotics and with the same color code as in Figure 2.

The same L3 region contains mutations associated with linezolid resistance and also examples of cross-resistance to linezolid and tiamulin (Klitgaard et al. 2015). Ribosomal protein L22 is located further down the tunnel, and a six aa insertion is reported to confer resistance to streptogramin B compounds in *S. pneumoniae* (Cattoir et al. 2007). As for the 23S RNA mutations, it is a cost-benefit matter whether a resistance-mediating mutation in a ribosomal protein "survives," and it is probably often followed by other "helper mutations" (also called compensatory mutations) as seen by Gentry et al. (2007). Increasing genome-sequencing capability and decreasing costs should facilitate more investigations on these contexts in the near future. In the current situation, investigators are often looking for what others have seen and might, therefore, ignore other positions that may be important for resistance.

Genes Conferring Resistance to LSPP Antimicrobial Agents by Enzymatic Inactivation

Enzymatic Inactivation of Lincosamides

Lincosamides are commonly inactivated by lincosamide nucleotidyltransferases, and their genes are found in various organisms. The known lincosamide nucleotidyltransferases show highest activity, as measured by the corresponding minimum inhibitory concentrations (MICs), against lincomycin, moderate activity against pirlimycin, and lowest activity against clindamycin (Lüthje and Schwarz 2006; Zhao et al. 2014).

The gene *lnu*(A) encodes a lincosamide nucleotidyltransferase of 161 aa (Brisson-Noël and Courvalin 1986). This gene is often located on small plasmids that usually contain only the *lnu*(A) gene and a plasmid replication gene. Several different types of *lnu*(A)-carrying plasmids have been described, many of them in *S. aureus* and coagulase-negative staphylococci (CoNS) of animal origin (Loeza-Lara et al. 2004; Lüthje and Schwarz 2007b; Lozano et al. 2012a).

The gene *lnu*(B) codes for a lincosamide nucleotidyltransferase of 267 aa and was first described in *E. faecium* of human origin (Boz-

dogan et al. 1999), and later identified in porcine *S. dysgalactiae* ssp. *equisimilis* (Lüthje and Schwarz 2007b). Recently, it has been detected as part of multiresistance gene clusters on plasmids or in the chromosomal DNA of *S. aureus*, *Staphylococcus hyicus*, *E. faecium*, *E. faecalis*, *Streptococcus agalactiae*, and *Erysipelothrix rhusiopathiae* of human and animal origin (Fig. 4) (Lozano et al. 2012a; Li et al. 2013, 2014b; Montilla et al. 2014; Silva et al. 2014; Wendlandt et al. 2014, 2015a; Zhang et al. 2015a).

The gene *lnu*(C) was first found in a human *S. agalactiae* isolate and codes for a 164-aa nucleotidyltransferase that inactivates lincomycin and clindamycin (Achard et al. 2005). It has also been discovered in the swine pathogen *Haemophilus parasuis* (Chen et al. 2010). The gene *lnu*(D) was detected in the chromosomal DNA of a *Streptococcus uberis* isolate from a case of bovine mastitis and codes for a nucleotidyltransferase of 164 aa (Petinaki et al. 2008). The gene *lnu*(E), which codes for a 173 aa protein, was found on a plasmid in *Streptococcus suis* in which it was interrupted by the integration of an IS*Enfa5*-*cfr*-IS*Enfa5* segment (Zhao et al. 2014). This gene was de novo synthesized, and after cloning and expression in *S. aureus* RN4220, shown to confer resistance to lincomycin. The gene *lnu*(F), originally referred to as *linF* codes for a 273-aa nucleotidyltransferase and was first identified on a plasmid from an *E. coli* isolate of human origin. Further analysis showed that the *lnu*(F) gene was part of a gene cassette located in a class 1 integron (Heir et al. 2004). A *lnu*(F)-related gene, originally reported as *linG*, which also encodes a 273-aa nucleotidyltransferase that shows 93% identity to Lnu(F), has been found as part of a gene cassette in a class 1 integron from a *Salmonella enterica* serovar Stanley isolate of human origin (Levings et al. 2006). Finally, a gene, described as *linA_{N2}*, has been detected on the mobilizable 11-kb transposon NBU2 in human clinical isolates of *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*. It codes for a nucleotidyltransferase of 171 aa, which shares only about 50% identity to Lnu(A) proteins (Wang et al. 2000).

From an evolutionary point of view, at least two groups of lincosamide nucleotidyltrans-

Figure 4. lsa(E)- and lnu(B)-carrying multiresistance gene clusters. Schematic presentation of the structural variability among *lsa*(E) and *lnu*(B)-carrying multi-resistance gene clusters found on plasmids or in the chromosomal DNA of *Enterococcus faecalis*, *E. faecium*, *Streptococcus agalactiae*, *Erysipelothrix rhusiopathiae*, MRSA (methicillin-resistant *Staphylococcus aureus*), and MSSA (methicillin-susceptible *S. aureus*). All genes are indicated by arrows with the arrowhead showing the direction of transcription. Insertion sequences are presented as gray boxes with the arrow inside indicating the transposase gene. All antimicrobial resistance genes are depicted as violet arrows. *aacA-aphD*, gentamicin/kanamycin/tobramycin resistance; *aadE*, streptomycin resistance; *aphA3*, kanamycin/neomycin resistance; *erm*(B), MLS_B resistance; *lnu*(B), lincosamide resistance; *lsa*(E), lincosamide/pleuromutilin/streptogramin A resistance; *spw*, spectinomycin resistance; *sat4*, strepto-thricin resistance. Genes involved in transposition are shown in yellow, whereas the genes involved in plasmid replication and plasmid recombination/mobilization are shown in green and blue, respectively. Genes with other functions are displayed in white. The Δ symbol indicates a truncated gene. A 1-kb scale is shown in the *upper right* corner. The gray-shaded regions show >99% sequence identity.

ferases can be differentiated. One group includes the proteins Lnu(A), Lnu(C), Lnu(D), and Lnu(E) that harbor a conserved domain at their amino terminus, which shows similarity to the aminoglycoside nucleotidyltransferase ANT(2″)-Ia (Petinaki et al. 2008). This observation points toward a divergent evolution from a common ancestor. The other group includes the proteins Lnu(B) and Lnu(F) with sequence similarity to the β-subunit of DNA polymerases. It is thus likely that they have developed from nucleotide polymerases, which are ubiquitous in bacteria (Morar et al. 2009).

Enzymatic Inactivation of Streptogramins

Inactivation of streptogramin A antimicrobial agents is commonly mediated by acetyltransferases. In staphylococci, three genes, *vat*(A) (Allignet et al. 1993), *vat*(B) (Allignet and El Solh 1995), or *vat*(C) (Allignet et al. 1998), which code for acetyltransferases of 219, 212, or 212 aa, respectively, have been described. These three *vat* genes have been identified on plasmids of different sizes, which occasionally also harbor additional resistance genes. In contrast to staphylococci from human sources, *vat* genes have rarely been detected in animal staphylococci. Solely, the *vat*(B) gene has been identified in two *Staphylococcus xylosus* isolates of poultry origin (Aarestrup et al. 2000). In enterococci, the plasmid-borne *vat*(D) gene from *E. faecium*, initially described as *satA*, was the first *vat* gene detected in this genus (Rende-Fournier et al. 1993). In another study, it was shown that the *vat*(D) gene, which codes for a 209-aa protein, is linked to the macrolide−lincosamide−streptogramin B (MLS$_B$)-resistance gene *erm*(B) and that both genes are cotransferred (Hammerum et al. 2001). The *vat*(E) gene, coding for an acetyltransferase of 214 aa and originally published as *satG*, was first identified in an *E. faecium* isolate from a sewage treatment plant in Germany (Werner and Witte 1999). A survey of streptogramin-resistant *E. faecium* from retail meat samples revealed some variability in the *vat*(E) genes. However, no correlation was seen between the number of aa substitutions and the MICs of quinupristin−dalfopristin (Simjee et al. 2001).

Besides Gram-positive bacteria, a novel type of *vat* gene, *vat*(F), which codes for a 221-aa protein, was detected in the chromosomal DNA of *Yersinia enterocolitica* (Seoane and García Lobo 2000). A *vat*(H) gene coding for a 216-aa acetyltransferase was detected together with the *vga*(D) gene on a plasmid in an *E. faecium* isolate of human origin (Jung et al. 2010). The origin of the VAT enzymes remains to be elucidated. Homologs and orthologs of the Vat enzymes, which are present in clinically resistant bacteria, are widely distributed in chromosomes of numerous environmental bacterial species, including species, such as *Y. enterocolitica*, which are intrinsically resistant to streptogramins (Wright 2007). It should be noted that streptogramin A−acetylating Vat proteins are related to CatB chloramphenicol acetyltransferases in their sizes, aa sequence, presumable active center, and tertiary structure (Murray and Shaw 1997), which points toward a common evolutionary origin.

So far, two genes, *vgb*(A) and *vgb*(B), have been described in staphylococci, which code for streptogramin B−specific lactone hydrolases of 298 and 295 aa, respectively (Allignet et al. 1988, 1998). Both genes are plasmid-borne. Plasmid pIP1714, which carries *vgb*(B), also harbors *vat*(C). This plasmid was isolated from a *Staphylococcus cohnii* ssp. *cohnii* found in the environment of a hospital where pristinamycin was extensively used (Allignet et al. 1998). There is little information about *vgb* genes in staphylococci from animals. The same two *vat*(B)-positive *S. xylosus* from poultry also carried a *vgb*(B) gene (Aarestrup et al. 2000). Orthologous and homologous *vgb* genes are present in the genomes of many environmental bacteria, including *Streptomyces coelicolor* and other *Streptomyces* spp., which belong to the same genus as the streptogramin producers (Wright 2007). Such genes may have served as precursors from which the *vgb* genes found in pathogenic bacteria have evolved.

Enzymatic Inactivation of Phenicols

Nonfluorinated phenicols, such as chloramphenicol and thiamphenicol, are commonly

inactivated by chloramphenicol *O*-acetyltransferases (CATs) (Shaw 1983; Schwarz et al. 2004). All CATs transfer an acetyl group from a donor molecule (e.g., acetyl-CoA) to the hydroxyl group at C3 of the phenicol molecule. This acetyl group is then shifted to the hydroxyl group at C1, and the hydroxyl group at C3 is available again for a second acetylation step. Neither mono- nor diacetylated phenicol molecules have antimicrobial activity (Murray and Shaw 1997). None of the CAT enzymes are able to inactivate florfenicol, because the hydroxyl group at C3 is replaced by fluorine and cannot act as an acceptor site for acetyl groups. There are two types of CATs, both of which have a trimeric structure composed of three identical monomers, and the respective *cat* gene codes for the monomer (Murray and Shaw 1997). The sizes of the classical CAT monomers vary between 207 and 238 aa, whereas CATs of the second type vary in their sizes between 209 and 219 aa (Schwarz et al. 2004).

The classical CATs, encoded by *catA* genes (Schwarz et al. 2004; Roberts and Schwarz 2009), represent a highly diverse group of enzymes whose members show an overall identity of approximately 44%. These enzymes have been detected in Gram-positive and Gram-negative aerobic and anaerobic bacteria and can be subdivided into at least 22 different groups when using a threshold value of ≥80% aa identity to define a group (Schwarz et al. 2004). The genes *catA1*, *catA2*, and *catA3* (also known as *catI*, *catII*, and *catIII*) are exclusively found in Gram-negative bacteria and are expressed constitutively. The CatA3 (CATIII) enzyme was the first to be crystallized, and the analysis of its crystal structure provided insight into the folding of the CAT monomers and helped to identify the aa that were important for the structure and function of the CAT enzyme (Leslie et al. 1988). Another three groups of classical CATs were named according to the plasmids (pC221, pC223/pSCS7, and pC194), on which their genes were first detected. Although initially found in staphylococci, the corresponding *cat* genes have in the meantime been identified in a number of Gram-positive genera. The expression of these *cat* genes is inducible by chlor-

amphenicol and is regulated by translational attenuation. The regulatory region comprises a reading frame for a short peptide and two inverted repeated sequences, and is located immediately upstream of the respective *cat* gene (Lovett 1990). Closely related CATP and CATD proteins were first identified in the Gram-positive anaerobe *Clostridium* spp. where they are located on transposons (Lyras and Rood 2000). However, these genes have also been identified in Gram-negative *Neisseria meningitidis* (Galimand et al. 1998; Shultz et al. 2003). Both genes are expressed constitutively. The remaining 15 groups of classical CATs are represented by individual enzymes whose genes have so far been detected in only a single species of either Gram-positive or Gram-negative bacteria (Schwarz et al. 2004).

The second type of CAT enzymes, encoded by *catB* genes (Murray and Shaw 1997; Schwarz et al. 2004; Roberts and Schwarz 2009), is only distantly related to the classical CATs. Their members are structurally similar to acetyltransferases involved in streptogramin A resistance (Murray and Shaw 1997). In general, these CAT enzymes confer lower MICs to chloramphenicol than the classical CATs. Hence, it was speculated that members of this second type of CAT might have a physiological role other than chloramphenicol resistance in their host bacteria (Murray and Shaw 1997). Using the same threshold value as for the classical CATs, at least five different groups can be distinguished, although all enzymes have approximately 77% identity with each other (Schwarz et al. 2004). Many of these *cat* genes are part of gene cassettes in class 1 and class 2 integrons in Gram-negative bacteria (Recchia and Hall 1995), whereas others have been identified on transposons.

In addition to CATs, inactivation of chloramphenicol by *O*-phosphorylation has been observed in the chloramphenicol producer *S. venezuelae* and is believed to contribute to self-defense of the host (Mosher et al. 1995). It is not known whether fluorinated chloramphenicol analogues can be inactivated by phosphorylation. Moreover, a chloramphenicol hydrolase gene has been found in the chloramphenicol producer *S. venezuelae* and considered to con-

tribute to self-defense of the host (Mosher et al. 1990). Moreover, the gene *estDL136* from a soil metagenome library was found to specify a hydrolase, which, when cloned in *E. coli*, inactivated both chloramphenicol and florfenicol (Tao et al. 2012).

Enzymatic Inactivation of Pleuromutilins

To the best of our knowledge, no pleuromutilin-inactivating enzymes have been described so far.

Genes Conferring Resistance to LSPP Antimicrobial Agents by Active Efflux

Active efflux can be based on multidrug transporters or specific transporters. In the following sections, multidrug transporters and specific transporters are described whose substrate spectrum includes one or more of the LSPP antimicrobial agents.

Resistance to LSPP Antimicrobial Agents by Multidrug Transporters

Usually, multidrug transporters confer an increase in the MICs of their substrates, but not necessarily to levels that are indicative of clinical resistance. Multidrug transporter systems assigned to the resistance/nodulation/cell division (RND) family have been reported to export phenicols from the bacterial cell. They include the AcrAB-TolC system in *E. coli* (McMurray et al. 1994), the MexAB-OprM and MexCD-OprJ systems in *P. aeruginosa* (Paulsen et al. 1996), as well as the OqxAB system in Enterobacteriaceae (Hansen et al. 2007), among others. Initially, intrinsic resistance of Enterobacteriaceae and other Gram-negative bacteria to macrolides, lincosamides, and streptogramins was considered to be based on the relative impermeability of the outer membrane to these compounds (Leclercq and Courvalin 1991). However, the observation that *E. coli* strains, such as CS1562 or AS19, are hypersusceptible to these antimicrobial agents caused by a deficiency of the TolC porin, suggests the involvement of RND pumps, which use TolC as

an outer membrane component, in intrinsic resistance to macrolides, lincosamides, and streptogramins.

In Gram-positive bacteria, several 12-trans-membrane segments (TMS) multidrug transporters of the major facilitator superfamily (MFS), such as Blt and Bmr proteins from *Bacillus subtilis* and NorA from *S. aureus*, have been reported to have a substrate spectrum that includes chloramphenicol (Paulsen et al. 1996). Another two closely related 12-TMS multidrug efflux proteins, MdfA and Cmr, which are able to export chloramphenicol, have been identified in *E. coli* (Nilsen et al. 1996; Edgar and Bibi 1997). Overexpression of the chromosomal multidrug exporter MdeA of *S. aureus* conferred a 16-fold increase in the MIC of virginiamycin, while no significant increase in the MICs of other LSPP antimicrobial agents tested was seen (Huang et al. 2004). In addition, the gene *lmr*(B) from *B. subtilis* coding for a 479-aa MFS protein (Kumano et al. 2003; Murata et al. 2003) was shown to confer resistance to multiple antimicrobial agents, including lincomycin, when overexpressed. The Lmr(P) protein of *Lactococcus lactis* specifies a 408-aa multidrug transporter of the MFS, which confers resistance to lincosamides, macrolides, streptogramins, and tetracyclines (Putman et al. 2001; Poelarends et al. 2002). A similar substrate spectrum was determined for the ATP-binding cassette (ABC) transporter encoded by the gene *lmr*(A) from *L. lactis* when expressed in a hypersusceptible *E. coli* strain (Putman et al. 2000; Poelarends et al. 2002). The 480-aa MFS protein LmrS from *S. aureus* has been reported to confer 4- to 16-fold increases in the MICs of numerous antimicrobial agents (including lincomycin, chloramphenicol, florfenicol, and linezolid) and other substances when expressed in *E. coli* (Floyd et al. 2010).

Active Efflux of Lincosamides by Specific Exporters

The 492-aa ABC transporter Lsa(B) conferred, in contrast to other Lsa proteins, only elevated MICs of lincosamides, which, however, are below the clinical breakpoint for resistance (Keh-

renberg et al. 2004). The *lsa*(B) gene was detected in close proximity to the multiresistance gene *cfr* on plasmids in *Staphylococcus sciuri* and *Staphylococcus warneri* (Kehrenberg et al. 2004, 2007). The *lmrA* gene of the lincomycin producer *S. lincolnensis* specifies an MFS protein of 481 aa, which exports lincomycin and it is believed to be part of a self-defense system of this organism (Peschke et al. 1995). A gene, designated *lmrB*, which codes for a 481-aa MFS exporter in *Corynebacterium glutamicum*, was shown to confer resistance to lincosamides but not to other antimicrobial agents tested (Kim et al. 2001). This Lmr(B) protein is only distantly related to the Lmr(B) protein in *B. subtilis* described above.

Active Efflux of Lincosamides, Pleuromutilins, and Streptogramin A Antimicrobial Agents by Specific Exporters

During recent years, several ABC transporters have been identified in staphylococci, streptococci, and enterococci, which confer combined resistance to lincosamides, pleuromutilins, and streptogramin A antimicrobial agents. A recent review provided detailed information about most of the corresponding multiresistance genes, such as *vga*(A), *vga*(A)$_V$, *vga*(A)$_{LC}$, *vga*(C), *vga*(E), *vga*(E)$_V$, *lsa*(A), *lsa*(C), *lsa*(E), *eat*(A)v, and *sal*(A) (Wendlandt et al. 2015b).

The ABC transporters specified by the genes *vga*(A) (Allignet et al. 1992), *vga*(A)$_V$ (Haroche et al. 2000), *vga*(A)$_{LC}$ (Novotná and Janata 2006; Gentry et al. 2008), *vga*(B) (Allignet and El Solh 1997), *vga*(C) (Kadlec and Schwarz 2009), *vga*(E) (Schwendener and Perreten 2011), and *vga*(E)$_V$ (Li et al. 2014a) show sizes of 522, 524, 522, 552, 522, 524, and 524 aa, respectively. All of these can export streptogramin A antimicrobial agents, whereas the Vga(A), Vga(C), and Vga(E) proteins also export lincosamides and pleuromutilins. The *vga*(A) genes are most widespread and can be located on the 5.5-kb transposon Tn*5406* (Haroche et al. 2002). Studies on clinical *S. aureus* isolates from France identified Tn*5406* inserted into the chromosomal att*554* site, a site that resembles the integration site in a type III SCC*mec*

cassette and on plasmids. The *vga*(A) genes can also be located on plasmids, ranging from small plasmids of 5.7 kb that harbor only the *vga*(A) gene (Kadlec et al. 2010) to large plasmids of 25–46 kb that carry additional resistance genes (Allignet and El Solh 1999; Weiß et al. 2014). The *vga*(C) gene was initially identified on a 14-kb multiresistance plasmid from a porcine livestock–associated (LA)-MRSA isolate (Kadlec and Schwarz 2009) but has also been identified on a small plasmid of 5.3 kb (Kadlec et al. 2010).

The *vga*(E) was shown to be part of the 11.5-kb transposon Tn*6133*, which was detected initially in a porcine LA-MRSA from Switzerland (Schwendener and Perreten 2011) and soon thereafter in LA-MRSA from cattle, chickens, and turkeys in Germany (Hauschild et al. 2012; Monecke et al. 2013). In 2014, a variant of the Vga(E) protein was described, which shared only 86% aa identity with the original Vga(E) protein (Li et al. 2014a). The Vga(E) variant also confers pleuromutilin–lincosamide–streptogramin A resistance, and the corresponding gene *vga*(E)$_V$ was located on a 5.6-kb plasmid in porcine *Staphylococcus simulans* and *S. cohnii* isolates. The *vga*(D) gene codes for a 525-aa protein and was found in *E. faecium* of human origin (Jung et al. 2010). Vga(D) was the first ABC transporter in *E. faecium*, which conferred resistance to streptogramin A antimicrobial agents. However, it is not known whether this ABC transporter also exports lincosamides and pleuromutilins.

The species-specific chromosomal gene *lsa*(A) from *E. faecalis* codes for an ABC transporter of 496 aa, which is believed to play a role in the intrinsic resistance of *E. faecalis* to lincosamides and streptogramins. Disruption of the *lsa*(A) gene was associated with an at least 40-fold decrease in MICs of quinupristin–dalfopristin, clindamycin, and dalfopristin, whereas complementation of the disruption mutant with an intact *lsa*(A) resulted in restoration of the MICs to wild-type levels (Singh et al. 2002). Another study reported that Lsa(A) not only confers resistance to lincosamide and streptogramin A, but also to pleuromutilins (Malbruny et al. 2011). The same resistance pattern is seen

for the *lsa*(C) gene from *S. agalactiae* that codes for an ABC transporter of 492 aa (Malbruny et al. 2011). The first *lsa* gene in staphylococci, *lsa*(E), which mediates combined resistance to lincosamides, pleuromutilins, and streptogramin A, has been described in human MRSA ST398 and MSSA ST9 (Wendlandt et al. 2013b). This gene codes for an ABC transporter protein of 494 aa and is part of a multiresistance gene cluster that is most likely of enterococcal origin (Fig. 4) (Wendlandt et al. 2013b). Several variants of this multiresistance gene cluster have been identified in MRSA ST398 of human origin and in MRSA/MSSA ST9 of human and pig origin in Europe and Asia (Lozano et al. 2012a; Li et al. 2013; Wendlandt et al. 2013a, 2014, 2015b). Moreover, as summarized in Figure 4, variants of the *lsa*(E)-containing multiresistance gene cluster have also been found in *E. faecalis* and *E. faecium* of human and swine origin in China (Li et al. 2014b; Si et al. 2015), in *S. agalactiae* of human origin in Argentina (Montilla et al. 2014), and also in *E. rhusiopathiae* of swine origin in China (Zhang et al. 2015a).

Although *E. faecalis* is intrinsically resistant to lincosamides, pleuromutilins, and streptogramin A by production of the ABC transporter Lsa(A), *E. faecium* is naturally susceptible. *E. faecium* harbors a gene *eat*(A) for an ABC transporter of 500 aa that shows only 66% aa identity to Lsa(A) and has no function in antimicrobial resistance. Despite this, an aa substitution (Thr450Ile) was found in the Eat(A) protein of isolates that were resistant to lincosamides, pleuromutilins, and streptogramin A antimicrobial agents. Single-nucleotide replacement and transfer of the mutated gene *eat*(A)$_V$ into a susceptible *E. faecium* isolate confirmed a role of this mutation in resistance to lincosamides, pleuromutilins, and streptogramin A (Isnard et al. 2013).

The gene *sal*(A) from *S. sciuri* codes for an ABC transporter of 541 aa, which was reported to confer resistance to streptogramin A and lincosamides (Hot et al. 2014). The *sal*(A) gene has exclusively been found in the chromosomal DNA of *S. sciuri* isolates inserted between the two housekeeping genes, *iscS* and

mnmA, of the staphylococcal core genome. A recent study confirmed that Sal(A) also confers pleuromutilin resistance (Wendlandt et al. 2015a).

Active Efflux of Macrolides and Streptogramin B by Specific Exporters

The gene *msr*(A) codes for a 488-aa ABC transporter protein that confers resistance to macrolides and streptogramin B (Ross et al. 1990) and is often found together with the gene *mph*(C) that codes for a macrolide phosphotransferase (Lüthje and Schwarz 2006). These two genes have been detected among CoNS and MRSA CC398 from humans in Germany and Spain, respectively (Gatermann et al. 2007; Lozano et al. 2012b), and on plasmids of the *S. aureus* clone USA300 (Kennedy et al. 2010). The 33-kb plasmid pMS97 has been reported to carry *msr*(A) and *mph*(C) together with the MLS$_B$-resistance gene *erm*(Y) (Matsuoka et al. 2003). In veterinary medicine, the *msr*(A) gene has been detected in *S. aureus* isolates of poultry (Nawaz et al. 2000) and dog origin (Lüthje and Schwarz 2007b), in canine *S. pseudintermedius* (Lüthje and Schwarz 2007b), and in various species of CoNS from cases of bovine mastitis (Lüthje and Schwarz 2006). Moreover, Jaglic et al. (2012) identified the gene *msr*(A) in not-further-specified CoNS isolates from turkeys, pigs, and cattle.

The species-specific and chromosomally located gene *msr*(C) of *E. faecium* codes for a 492-aa ABC transporter. Functional deletion of *msr*(C) resulted in a two- to eightfold decrease in MICs of the macrolides erythromycin, azithromycin, and tylosin, as well as of the streptogramin B quinupristin. This *E. faecium*–specific gene shows only 53% identity to *msr*(A) (Singh et al. 2001). The *msr*(D) from *S. pyogenes* (Gay and Stephens 2001) has been reported to confer macrolide and ketolide, but not streptogramin resistance (Poole 2005). The *msr*(E) gene found in various Gram-negative bacteria is also known to be involved in the efflux of macrolides (Kadlec et al. 2011), but it is not known whether this gene also confers resistance to streptogramin B antimicrobial agents.

Active Efflux of Phenicols by Specific Exporters

Specific exporters that transport only chloramphenicol or chloramphenicol and florfenicol out of the bacterial cell are mainly members of the MFS efflux proteins (Paulsen et al. 1996; Poole 2005). They commonly show 10 to 14 TMS (Butaye et al. 2003). Based on an 80% threshold value, at least 11 genetic groups of specific phenicol exporters can be distinguished, including four groups from soil and environmental bacteria (Schwarz et al. 2004; Roberts and Schwarz 2009). The *cmr* and *cmx* genes found in *Corynebacterium* spp. are located on plasmids, whereas the *cmx* gene is associated with transposon Tn*5564* (Schwarz et al. 2004). The genes *cmr* and *cmrA* from *Rhodococcus* spp. are also found on plasmids, with the *cmrA* being part of transposon Tn*5561* (Nagy et al. 1997). The *S. venezuelae cmlv* gene is believed to play a role in self-defense of the chloramphenicol producer (Mosher et al. 1990).

In clinically important bacteria, such as *S. enterica*, *E. coli*, *Klebsiella pneumoniae*, or *Pseudomonas aeruginosa*, several closely related *cmlA* genes have been identified on gene cassettes. Unlike other cassette-borne genes, *cmlA* is inducibly expressed via translational attenuation (Stokes and Hall 1991; Recchia and Hall 1995). The chloramphenicol exporter CmlB1, which shares 74%–77% identity with CmlA proteins, was identified on a plasmid from *Bordetella bronchiseptica* (Kadlec et al. 2007), a bacterium involved in respiratory tract infections in swine. The *cmlB1* gene is also inducibly expressed via translational attenuation. Neither CmlA nor CmlB1 proteins can efficiently export florfenicol from the bacterial cell, and bacteria carrying the corresponding genes are classified as florfenicol susceptible (Schwarz et al. 2004).

In contrast to the aforementioned genes, the *floR* gene codes for a MFS protein that can export both chloramphenicol and florfenicol (Schwarz et al. 2004). The *floR* gene (also referred to as *flo* or *pp-flo*) can be found in the chromosomal DNA of multiresistant *S. enterica* serovars, including Typhimurium DT104 and Newport, *Vibrio cholerae*, *E. coli*, *B. bronchisep-*tica, and *Acinetobacter baumannii*, or on plasmids of *E. coli*, *K. pneumoniae*, *Pasteurella multocida*, *Pasteurella trehalosi*, *A. pleuropneumoniae*, and *Stenothrophomonas maltophilia* (Schwarz et al. 2004). Recently, the *floR* gene has been identified as part of the chromosomally located integrative and conjugative element ICE*Pmu1* from *P. multocida*, which harbors a total of 12 antimicrobial resistance genes (Michael et al. 2012). A new *floR* gene variant, *floRv*, whose product showed only 84%–92% aa identity to the so-far known FloR proteins, has recently been identifed in a multidrug resistance genomic island of a porcine *S. maltophilia* isolate (He et al. 2015).

There are also a few chloramphenicol/florfenicol exporters in Gram-positive bacteria. The gene *fexA* is part of transposon Tn*558* and was first identified on a plasmid from *Staphylococcus lentus* (Kehrenberg and Schwarz 2005). Expression of *fexA* is inducible with either chloramphenicol or florfenicol. A translational attenuator, similar to those of *cat* genes from *Staphylococcus* spp. and *Bacillus pumilus*, is located immediately upstream of the *fexA* gene (Kehrenberg and Schwarz 2004). A *fexA* gene variant, *fexAv*, which conferred only chloramphenicol resistance, was detected in a canine *S. pseudintermedius* (Gómez-Sanz et al. 2013). In comparison to FexA, FexAv showed two aa substitutions Gly33Ala and Ala37Val. Both substitutions appear to be important for substrate recognition as site-directed mutagenesis to the original *fexA* gene, restored the chloramphenicol/florfenicol resistance phenotype. In 2010, a novel chloramphenicol/florfenicol resistance gene, designated *pexA*, was identified from an Alaskan soil sample (Lang et al. 2010). The gene *fexB*, which also confers resistance to chloramphenicol and florfenicol, was found on nonconjugative plasmids of *E. faecium* and *Enterococcus hirae* (Liu et al. 2012a).

Active Efflux of Phenicols and Oxazolidinones by a Specific Exporter

Most recently, a novel gene, designated *optrA* and coding for an ABC transporter of 655 aa, has been identified on a conjugative plasmid in

E. faecalis (Wang et al. 2015). In contrast to the MFS transporters described above, the *optrA* gene confers resistance, not only to chloramphenicol and florfenicol, but also to the oxazolidinones linezolid and tedizolid. The *optrA* gene was shown to be functionally active in *E. faecalis*, *E. faecium*, and *S. aureus*. A first survey conducted in China revealed that the *optrA* gene was more frequently found in enterococci of animal than of human origin (Wang et al. 2015). In a study including 1159 enterococcal isolates from five hospitals in China, the *optrA* gene was detected at a prevalence of 2.9%, and a distinct increase in *optrA* carriers was seen from 2010 to 2014 (Cai et al. 2015a). The analysis of the genetic environment of *optrA* in *E. faecalis* revealed a substantial heterogeneity (He et al. 2016). Besides in isolates from China, the *optrA* gene has also been identified in two clinical *E. faecalis* isolates from Italy (Brenciani et al. 2016). Finally, the analysis of 50 porcine staphylococci from China identified the *optrA* gene in a single *S. sciuri* isolate where it was located together with the genes *cfr*, *fexA*, *aadD*, *ble*, and *aacA-aphD* on a 60.5-kb nonconjugative multiresistance plasmid (Li et al. 2016).

Genes Conferring Resistance to LSPP Antimicrobial Agents by Target Site Modification

Target Site Modifications that Confer Resistance to Macrolides, Lincosamides, and Streptogramin B Antimicrobial Agents

Combined resistance to macrolides, lincosamides, and streptogramin B antimicrobial agents is commonly mediated by rRNA methylases that target the adenine residue at position 2058 in the domain V of the 23S rRNA. The adenine residue at position 2058 (Fig. 2) is located in the overlapping binding region of these three classes of antimicrobial agents. Methylation of this residue prevents macrolides, lincosamides, and streptogramin B antimicrobial agents from binding to their ribosomal target sites (reviewed in Weisblum 1995a; Roberts 2008). The corresponding *erm* genes, which code for these methylases, are often located on plasmids, transposons, or integrative and conjugative elements, which facilitate their dissemination across strain, species, and sometimes even genus boundaries. Based on a threshold value of <80% aa identity (Roberts et al. 1999), 46 classes of rRNA methylases are currently distinguished (see faculty .washington.edu/marilynr/ermweb1.pdf). Many *erm* genes are inducibly expressed via translational attenuation with 14- and 15-membered macrolides acting as inducers (Weisblum 1995b). Lincosamides and streptogramins, but also 16-membered macrolides usually do not act as inducers. Studies on *erm*(A) and *erm*(C) genes in staphylococci showed that constitutive expression can develop rapidly in the presence of such noninducers and is commonly a result of deletions, duplications, or point mutations in the translational attenuator (Schmitz et al. 2002a,b; Lüthje and Schwarz 2007a). Detailed information on the *erm* genes and their occurrence in the various bacteria can be seen in a review on macrolides and ketolides (Fyfe et al. 2016).

Target Site Modifications that Confer Resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A Antimicrobial Agents

The gene *cfr* was the first gene that conferred combined resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A (PhLOPS$_A$) antimicrobial agents. This gene was identified on a plasmid in a bovine *S. sciuri* and initially referred to as a chloramphenicol/florfenicol resistance gene (Schwarz et al. 2000). The clarification of the resistance mechanism, however, showed that *cfr* is a multiresistance gene, which confers resistance to the aforementioned classes of antimicrobial agents (Kehrenberg et al. 2005; Long et al. 2006). In addition, Cfr also provides decreased susceptibility to some large 16-membered macrolides, such as spiramycin and josamycin (Smith and Mankin 2008). The gene *cfr* encodes an rRNA methylase that targets the adenine residue at position 2503 in the domain V of the 23S rRNA (Fig. 2). This target site is lo-

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cated in the overlapping binding site of the PhLOPS_A agents and the additional Cfr-mediated methylation is believed to prevent these antimicrobial agents from binding to the ribosome. Further studies identified the *cfr* gene on a number of plasmids in *S. aureus*, *Staphylococcus hyicus*, and various CoNS species from pigs, cattle, horses, chickens, and ducks (Kehrenberg and Schwarz 2006; Kehrenberg et al. 2007, 2009; Wang et al. 2012d,e, 2013a; He et al. 2014). The gene *cfr* was also detected in *S. aureus* and CoNS from infections in humans (Toh et al. 2007; Mendes et al. 2008, 2013; Bonilla et al. 2010; Shore et al. 2010; Gopegui et al. 2012; Locke et al. 2012; Cui et al. 2013; LaMarre et al 2013; Feßler et al. 2014; Bender et al. 2015, Cai et al. 2015b). Most recently, the *cfr* gene was found to be integrated in a type IVb SCC*mec* cassette of an MRSA CC9 isolate of swine origin (Fig. 5) (Li et al. 2015).

Screening studies conducted in China revealed the presence of the gene *cfr* also in other Gram-positive bacteria such as *Bacillus* spp. (Dai et al. 2010; Zhang et al. 2011; Wang et al. 2012b), *Enterococcus* spp. (Liu et al. 2012b, 2013, 2014), *Macrococcus caseolyticus* and *Jeotgalicoccus pinnipedialis* (Wang et al. 2012c), and *S. suis* (Wang et al. 2013b). In most of these cases, the *cfr* gene was located on plasmids of variable sizes. Moreover, the *cfr* gene was also found in a small number of Gram-negative bacteria, for example, in the chromosomal DNA of a *Proteus vulgaris* isolate (Wang et al. 2011) or on plasmids in *E. coli* (Fig. 5) (Wang et al. 2012a; Zhang et al. 2014, 2015b).

A comparative analysis of the plasmids on which the *cfr* gene was found in the different bacteria revealed a high structural variability in the regions surrounding the *cfr* gene (Shen et al. 2013). Moreover, numerous insertion sequences, such as IS*21-558*, IS*256*, IS*1216*, IS*Enfa4*, IS*Enfa5*, and IS*26*, were found in close proximity to the *cfr* gene (Fig. 5). These insertion sequences may play a role in the transfer of *cfr* between different plasmids but also in the chromosomal integration of *cfr*-carrying segments. In addition, many *cfr*-carrying plasmids harbor additional resistance genes, which enable the coselection and persistence of the *cfr*

gene under a selection pressure imposed by non-PhLOPS_A antimicrobial agents.

Recently, a *cfr*-like gene, whose product showed only 75% aa identity to the original Cfr protein from *S. sciuri*, was detected in *C. difficile*, and claimed to be responsible for linezolid resistance (Marín et al. 2015; Schwarz and Wang 2015). In a different study, this gene, meanwhile designated *cfr*(B), has been shown to confer multiple antimicrobial resistance by the same mechanism as the original *cfr* gene (Hansen and Vester 2015). Most recently, the *cfr*(B) gene was also detected in *E. faecium* recovered from human specimens in the United States (Deshpande et al. 2015).

CONCLUDING REMARKS

For a number of years, fewer and fewer new antimicrobial agents have been approved for use in human and veterinary medicine despite rising trends in antimicrobial resistance in bacterial pathogens of human, veterinary, and zoonotic relevance. This is particularly true for veterinary medicine as all new antimicrobial agents approved during the last 25 years represent derivatives of already known substances. For the development of antimicrobial agents with improved binding to the ribosome, the exact mode of action of the antimicrobial agents, but also the knowledge about their binding sites, are indispensable prerequisites. Moreover, the knowledge about resistance-mediating mutations and the mechanisms specified by resistance genes are important aspects that need to be taken into account when developing new antimicrobial agents that may even overcome existing resistance mechanisms. Florfenicol is a good example to illustrate how knowledge about the most common phenicol resistance mechanism, namely, the enzymatic inactivation of chloramphenicol by chloramphenicol acetyltransferases, was used to generate an antimicrobial agent that was resistant to inactivation by the most widespread resistance mechanism and, in addition, did not show the adverse side effects of the parental substance. However, bacteria are versatile organisms that are able to quickly develop or
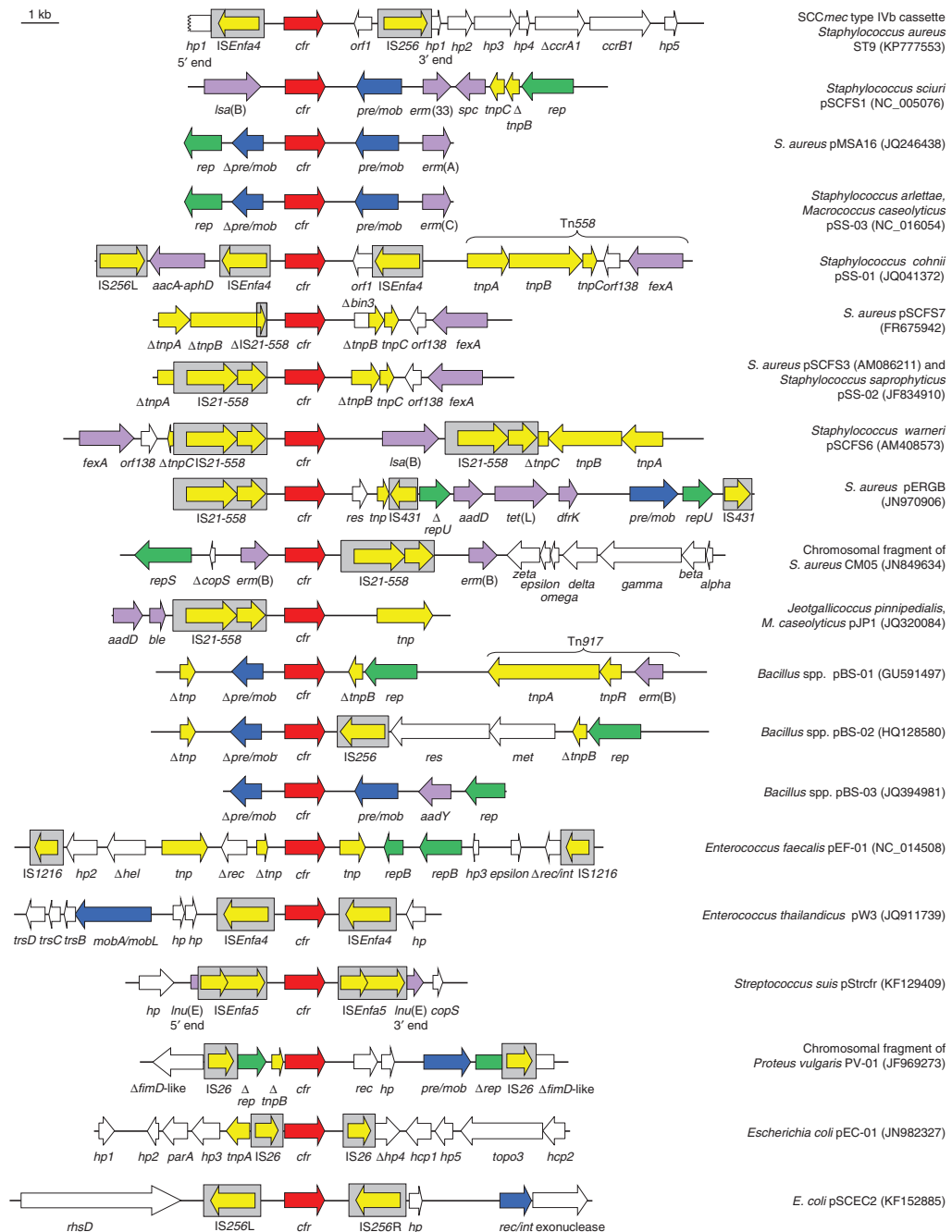
Figure 5. Schematic presentation of the structural variability in the regions surrounding the *cfr* gene on plasmids or in the chromosomal DNA of various Gram-positive and Gram-negative bacteria. All genes are indicated by arrows with the arrowhead showing the direction of transcription. Insertion sequences are presented as gray boxes with the arrow inside indicating the transposase gene. The *cfr* gene is shown as a red arrow, whereas all other antimicrobial resistance genes are depicted as violet arrows. Genes involved in transposition are shown in yellow, whereas the genes involved in plasmid replication and plasmid recombination/mobilization are shown in green and blue, respectively. Genes with other functions are displayed in white. The Δ symbol indicates a truncated gene. A 1-kb scale is shown in the *upper left* corner. *aadD*, kanamycin/neomycin resistance; *aadY*, streptomycin resistance; *aacA-aphD*, gentamicin/kanamycin/tobramycin resistance; *ble*, bleomycin resistance; *dfrK*, trimethoprim resistance, *erm*(A), *erm*(B), *erm*(C), *erm*(33), MLS$_B$ resistance; *fexA*, chloramphenicol/florfenicol resistance; *lsa*(B), elevated minimum inhibitory concentrations (MICs) of lincosamides; *spc*, spectinomycin resistance; *tet*(L), tetracycline resistance.

acquire new resistance mechanisms. In the case of the synthetic and new agent florfenicol, the first phenicol exporters and target site–modifying enzymes, that conferred florfenicol resistance, were identified only a few years after the introduction of florfenicol into use in animals (Kim and Aoki 1996; Arcangioli et al. 1999; Schwarz et al. 2000).

The introduction of any new antimicrobial agent into clinical use will create a new selection pressure under which bacteria develop and/or acquire sooner or later new resistance genes or resistance-mediating mutations. This will reduce the efficacy of the antimicrobial agents and create a demand for newer and better antimicrobial agents. Changes in the current practice of how we use antimicrobial agents not only in human and veterinary medicine, but also in horticulture and aquaculture, are unavoidable, and good antimicrobial stewardship practice should be adopted by everyone involved in antimicrobial use and application (Prescott 2014). The future will show whether new technologies, such as whole-genome sequencing, which have become widely available during the past few years, can successfully be used for the identification of new bacterial targets that serve for the development of future antimicrobial agents for specific bacterial pathogens and disease conditions (Prescott 2014).

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