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Modes and Modulations of Antibiotic Resistance Gene Expression

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INTRODUCTION

Bacteria may use various biochemical pathways to escape the lethal action of drugs: (i) decreased intracellular accumulation of the antibiotic by an alteration of outer membrane permeability, diminished transport across the inner membrane, or active efflux; (ii) alteration of the target by mutation or enzymatic modification; (iii) enzymatic detoxification of the drug; and (iv) bypass of the drug target. The coexistence of several of these mechanisms in the same host can lead to multidrug resistance (MDR). However, since antibiotic resistance usually affords a gain of function, there is an associated biological cost resulting in the loss of fitness of the bacterial host. Considering that antibiotic resistance is most often only transiently advantageous to bacteria, an efficient and elegant way for them to escape the lethal action of drugs is the alteration of resistance gene expression. It appears that the expression of bacterial resistance to antibiotics is frequently regulated, which indicates that modulation of gene expression probably reflects a good compromise between energy saving and adjustment to a rapidly evolving environment. Modulation of gene expression can occur at the transcriptional or translational level, following mutations or the movement of mobile genetic elements, and may involve induction by the antibiotic. In the latter case, the antibiotic can have a triple activity: as an antibacterial agent, as an inducer of resistance to itself, and, as in the case of tetracycline and gram-positive bacteria harboring conjugative transposons, as an inducer of the dissemination of a resistance determinant. We will review certain mechanisms, all reversible, that bacteria have elaborated to achieve antibiotic resistance by fine-tuning the expression of genetic information.

REGULATION OF RESISTANCE EXPRESSION BY TWO-COMPONENT SYSTEMS IN GRAM-POSITIVE BACTERIA

Two-Component Regulatory Systems

Bacteria live in precarious environments and must constantly adapt to external conditions by adjusting their structure, physiology, and behavior to survive. Many signaling proteins from both gram-positive and gram-negative bacteria are built from modular domains that promote information transfer within and between proteins (242). One such system, designated the "two-component regulatory system," comprises two proteins: a sensor usually located in the membrane that detects certain environmental signals and a cytoplasmic response regulator that mediates an adaptative response, usually a change in gene expression (Fig. 1) (110, 242). The terms "kinase" and "response regulator" are used since they seem to best represent the essential activities of these proteins. The large majority of histidine kinases are homodimeric proteins with an N-terminal periplasmic sensing domain coupled to a C-terminal cytoplasmic kinase domain (Fig. 1). The sensing domains are variable in sequence, reflecting the many different environmental signals to which histidine kinases are responsive and consequently the numerous specific functions that they regulate. Communication with the cytoplasmic transmitter domain involves the propagation of sensory information across the cytoplasmic membrane, presumably with the induction of conformational changes. The kinase domain that binds ATP and catalyzes the autophosphorylation of a histidine (Fig. 1) is more conserved. It is divided into two subdomains, a variable connecting linker and a second subdomain containing several highly conserved sequences designated H, N, D, F, and G boxes, which may play the role of catalytic center (Fig. 1) (71, 188). The phosphate group of the histidine residue is then transferred to a highly conserved aspartate residue in the receiver domain of the regulator (Fig. 1) (110, 242). Response regulators are characterized by a conserved domain of approximately 125 amino acids usually attached by a linker sequence to a domain with an effector function (Fig. 1) (110, 242). Prominent sequence features of regulators include two aspartate residues near the amino terminus, a lysine close to the carboxyl terminus, and a centrally located aspartate (Fig. 1). The effector domain generally has DNA binding activity, and in that instance, response regulator phosphorylation results in the activation of transcription (Fig. 1). In many instances, the response regulators act as transcriptional activators or repressors.

Several mechanisms control the rate of dephosphorylation of the phosphorylated response regulators. First, some of the regulators exhibit an autophosphatase activity with half-lives ranging from a few seconds to many minutes. Second, dephosphorylation can be mediated by the corresponding kinase. Finally, auxiliary regulatory proteins can also function as phosphatases to enhance the rate of dephosphorylation of the response regulators.

Resistance to Glycopeptides in Enterococci

The molecular target of glycopeptide antibiotics is the D-alanyl–D-alanine (D-Ala–D-Ala) terminus of intermediates in peptidoglycan synthesis. By binding to this dipeptide, vancomycin and teicoplanin inhibit the transglycosylation and transpeptidation reactions in peptidoglycan assembly (215).

Glycopeptide resistance in enterococci results from the production of modified peptidoglycan precursors ending in D-Ala–D-Lac (VanA, VanB, and VanD) or D-Ala–D-Ser (VanC, VanE, and VanG), to which glycopeptides exhibit low binding affinities, and from the elimination of the high-affinity precursors ending in D-Ala–D-Ala and synthesized by the host Ddl ligase (17, 218). In enterococci with the VanA, VanB, or VanD phenotype, the synthesis of D-Ala–D-Lac requires the presence of a ligase (VanA, VanB, or VanD) of altered specificity compared to the host Ddl ligase and of a dehydrogenase (VanH, $VanH_B$, or $VanH_D$) that converts pyruvate to D-Lac (Fig. 2) (19). In VanC-, VanE-, and VanG-type strains, the ligase genes (*vanC*, *vanE*, or *vanG*) encode a protein catalyzing the synthesis of D-Ala–D-Ser (218), and the production of D-Ser is due to a membrane-bound serine racemase (VanT, $VanT_E$, or $VanT_G$) (Fig. 2) (1, 11, 63).

The interaction of a glycopeptide with its normal target is prevented by the removal of precursors terminating in D-Ala (216). Two enzymes are involved in this process: a cytoplasmic

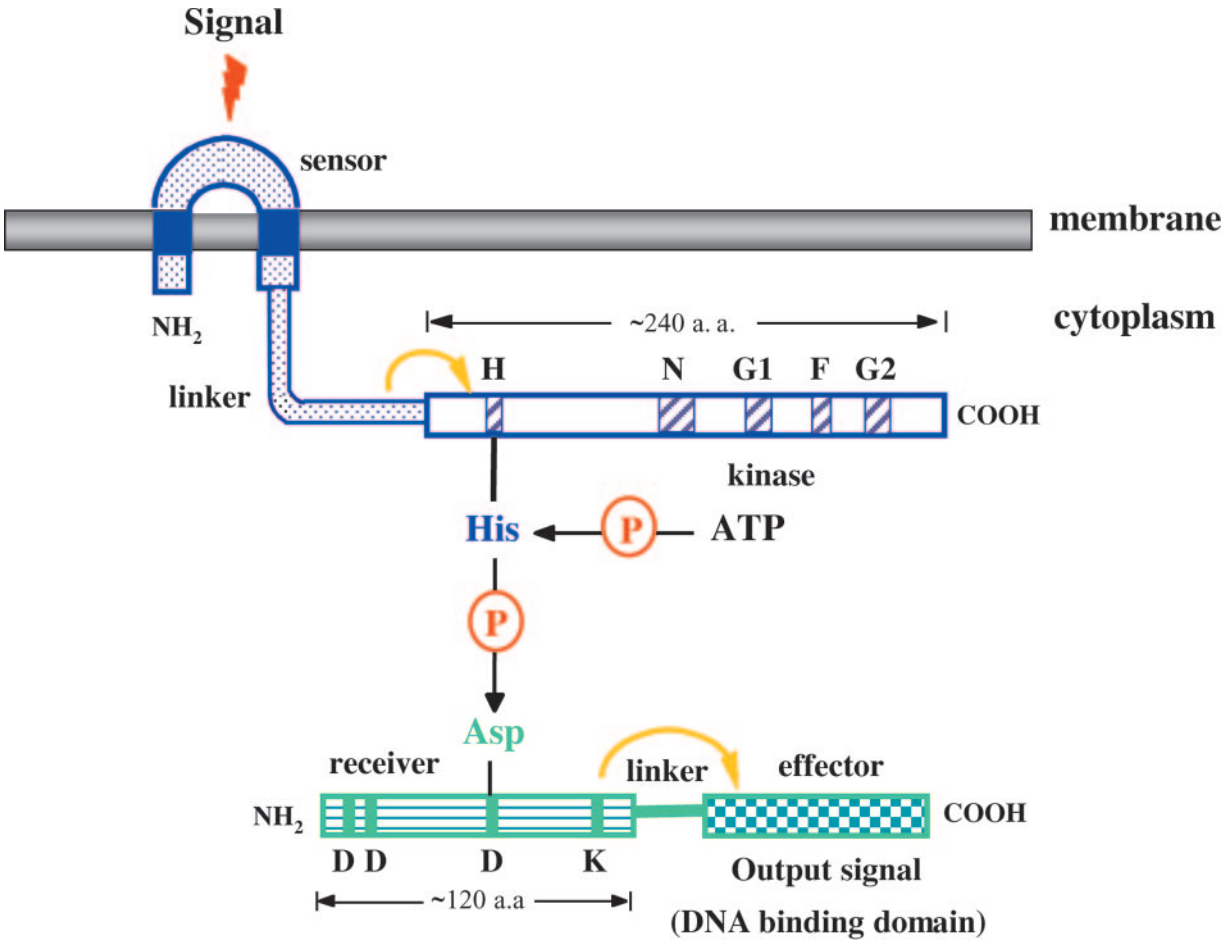


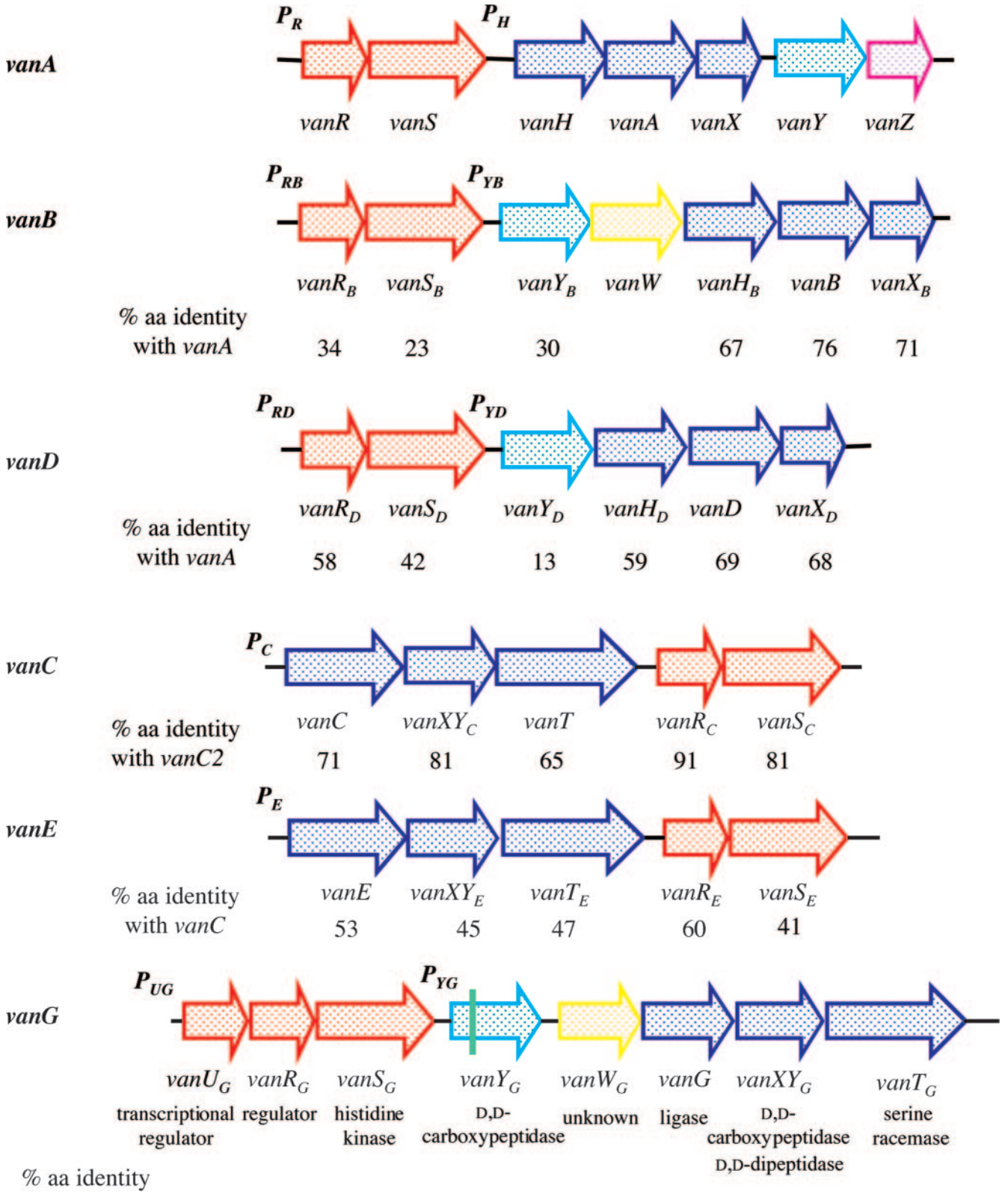
FIG. 1. Schematic representation of a two-component regulatory system. Structural features of sensor (top) and regulator (bottom) proteins. H, N, G1, F, and G2 refer to the motifs conserved in histidine protein kinases and are shown as hatched blue boxes. The phosphorylated histidine is nested in a highly conserved sequence termed the H box, close to the N-terminal border of the conserved kinase domain. The G1 and G2 domains are glycine rich and resemble nucleotide binding motifs seen in other proteins. The sequences of the remaining D and F boxes reveal little about their possible functions. In the regulator, the central aspartate is the site of phosphorylation, whereas the amino-terminal pair is probably important for catalysis. The conserved lysine may be involved in effecting the phosphorylation-induced conformational changes that regulate output activity. Asp, aspartate; His, histidine; P, phosphate; dotted blue box, sensor domain; blue box, transmembrane domain; white box, kinase domain; horizontally striped green box, receiver domain; checkerboard green box, effector domain. a.a., amino acids.

D,D-dipeptidase (VanX, $VanX_B$, or $VanX_D$) that hydrolyzes the dipeptide D-Ala–D-Ala synthesized by the host Ddl ligase and a membrane-bound D,D-carboxypeptidase (VanY, $VanY_B$, or $VanY_D$) that removes the C-terminal D-Ala residue of late peptidoglycan precursors when the elimination of D-Ala–D-Ala by VanX is incomplete (Fig. 2) (12, 217). In VanC-, VanE-, and VanG-type resistance, both activities are encoded by a single gene, $vanXY_C$, $vanXY_E$, or $vanXY_G$.

Classification of glycopeptide resistance is based on the primary sequence of the structural genes for the resistance-mediating ligases. VanA-type strains display high-level inducible resistance to both vancomycin and teicoplanin, whereas VanB-type strains have variable levels of inducible resistance to vancomycin only, since teicoplanin is not an inducer (15, 211). VanD-type strains are characterized by constitutive resistance to moderate levels of both glycopeptides (66, 67). VanC, VanE, and VanG are resistant to low levels of vancomycin but remain susceptible to teicoplanin (63, 80, 135). VanC- and VanE-type strains are inducibly or constitutively resistant (2, 187). In several constitutive strains of these types, various mutations in VanS could, as in VanB-type strains, account for constitutivity (26, 65).

Although all six types of resistance involve genes encoding related enzymatic functions, they can be distinguished by the location of the genes and by the various modes of regulation of gene expression (Fig. 2). The *vanA* and *vanB* operons are located on plasmids or in the chromosome (20), whereas the *vanD* (42, 66, 67), *vanG* (63), *vanE* (1), and *vanC* (10) operons have so far been found exclusively in the chromosome.

Two-component regulatory systems in Van-type enterococci. Among the ubiquitous two-component systems that constitute one of the largest families of transcriptional regulators in bacteria, the VanS/VanR-type systems are the only ones that control the expression of genes that mediate antibiotic resistance. Expression of VanA-, VanB-, VanD-, VanC-, VanE-, and VanG-type resistance is regulated by a VanS/VanR-type two-component signal transduction system composed of a membrane-bound histidine kinase (VanS, $VanS_B$, $VanS_D$, $VanS_C$,


vanA
P_R
P_H
vanR vanS vanH vanA vanX vanY vanZ
vanB
P_{RB}
P_{YB}
$vanR_B$ $vanS_B$ $vanY_B$ vanW $vanH_B$ vanB $vanX_B$
% aa identity with vanA
34 23 30 67 76 71
vanD
P_{RD}
P_{YD}
$vanR_D$ $vanS_D$ $vanY_D$ $vanH_D$ vanD $vanX_D$
% aa identity with vanA
58 42 13 59 69 68
vanC
P_C
vanC $vanXY_C$ vanT $vanR_C$ $vanS_C$
% aa identity with vanC2
71 81 65 91 81
vanE
P_E
vanE $vanXY_E$ $vanT_E$ $vanR_E$ $vanS_E$
% aa identity with vanC
53 45 47 60 41
vanG
P_{UG}
P_{YG}
$vanU_G$ $vanR_G$ $vanS_G$ $vanY_G$ $vanW_G$ vanG $vanXY_G$ $vanT_G$
transcriptional regulator
regulator
histidine kinase
D,D-carboxypeptidase
unknown
ligase
D,D-carboxypeptidase D,D-dipeptidase
serine racemase
% aa identity
with vanB 31 16 56 49 46 NA NA
with vanD 73 55 23 NA 44 NA NA
with vanC 62 40 NA NA 42 41 37
with vanE 55 29 NA NA 41 39 37

$VanS_E$, or $VanS_G$) and a cytoplasmic response regulator (VanR, $VanR_B$, $VanR_D$, $VanR_C$, $VanR_E$, or $VanR_G$) that acts as a transcriptional activator (Fig. 2) (1, 10, 18, 42, 63, 66, 67, 77). In the *vanA*, *vanB*, *vanD*, and *vanG* operons, the genes for the two-component regulatory system (*vanRS*, $vanR_BS_B$, $vanR_DS_D$, and $vanR_GS_G$) are present upstream from the structural genes for the resistance proteins (20, 42, 63, 67), whereas in the *vanC* and *vanE* clusters, $vanR_CS_C$ and $vanR_ES_E$ are located downstream (Fig. 2) (1, 10). The regulatory and resistance genes in the *vanA*, *vanB*, and *vanD* operons are transcribed from distinct promoters, P_R, P_{RB}, and P_{RD} and P_H, P_{YB}, and P_{YD}, respectively, that are coordinately regulated (13, 14, 43, 65, 77). The *vanC* and *vanE* clusters are cotranscribed from a single upstream promoter (Fig. 2) (1, 2, 187).

The $vanR_G$ and $vanS_G$ genes have the highest homology with $vanR_D$ and $vanS_D$, respectively (Fig. 2). Additionally, $vanU_G$ encodes a predicted transcriptional activator (63), and a protein of this type has not previously been associated with glycopeptide resistance. Thus, as opposed to the other *van* gene clusters, the *vanG* operon contains three genes, $vanU_G$, $vanR_G$, and $vanS_G$, for a putative regulatory system that are cotranscribed constitutively from the P_{UG} promoter, whereas inducible transcription of the $vanY_G$, $vanW_G$, *vanG*, $vanXY_G$, and $vanT_G$ resistance genes is initiated from the P_{YG} promoter (Fig. 2) (63).

Phosphotransfer reactions catalyzed by VanRS and $VanR_BS_B$ two-component systems. Despite the fact that the VanS/VanR and $VanS_B$/$VanR_B$ two-component systems are only distantly related, they catalyze similar reactions. The two response regulators are 34% identical, whereas the histidine kinases possess only 23% sequence identity, with unrelated amino-terminal sensing domains (Fig. 2). VanS-type sensors comprise an N-terminal sensor domain with two membrane-spanning segments and a C-terminal cytoplasmic kinase domain (Fig. 1) (18, 269). Following a signal related to the presence of a glycopeptide in the culture medium, the cytoplasmic domain of VanS or $VanS_B$ catalyzes ATP-dependent autophosphorylation of a specific histidine residue at positions 164 and 233, respectively, and transfers the phosphate group to an aspartate residue at position 53 of VanR or $VanR_B$ present in the effector domain (Fig. 3) (13, 18, 269).

Purified VanS and $VanS_B$ autophosphorylate in the presence of ATP and act as both a kinase and a phosphatase for VanR and $VanR_B$, respectively (65, 269). VanR and $VanR_B$ are phosphorylated following incubation either with the phosphorylated form of VanS or $VanS_B$, respectively, or with acetylphosphate. VanS and $VanS_B$ also stimulate the dephosphorylation of VanR and $VanR_B$ (65, 269). The VanS and $VanS_B$ sensors therefore respectively modulate the levels of phosphorylation of the VanR and $VanR_B$ regulators: they act primarily as a phosphatase under noninducing conditions and as a kinase in the presence of glycopeptides, leading to the phosphorylation of the response regulator and the activation of the resistance genes (Fig. 3) (13, 14, 64, 65, 112). The phosphorylation of VanR-type regulators enhances the affinity of the effector portion of the protein for the promoters and stimulates transcription of the regulatory and resistance genes of the *van* clusters (Fig. 3) (112). In contrast to VanR-VanS, the $VanR_B$-$VanS_B$ system mediates the activation of the P_{YB} promoter only in the presence of vancomycin, and the lack of activation by teicoplanin accounts for the susceptibility of VanB-type strains to this antibiotic (15, 65, 77). Spontaneous dephosphorylation of VanR and $VanR_B$ is slow in comparison with other response regulators, with half-lives of 10 h and 150 min, respectively, but VanS and $VanS_B$ stimulate the reaction (65, 269). The phosphatase activity of VanS and $VanS_B$ is required for the negative regulation of resistance genes in the absence of glycopeptides preventing the accumulation of VanR-phosphate (VanR-P) or $VanR_B$-phosphate ($VanR_B$-P) phosphorylated by acetylphosphate or by kinases encoded by the host chromosome (Fig. 3) (13).

In vitro binding of VanR and $VanR_B$ to promoter regulatory regions. There is sequence similarity between VanR and $VanR_B$ and response regulators of the OmpR/PhoB subclass in both the effector and DNA binding domains, with VanR being closer to OmpR (37% similarity) than to PhoB (35%), whereas $VanR_B$ is closer to PhoB (32% similarity) than to OmpR (26%). Phosphorylation of VanR and $VanR_B$ increases their DNA affinity, but VanR-P (112) appears to be more stable than $VanR_B$-P (64). The promoters in the *vanA* and *vanB* operons have common features, with a single binding site in the P_R and P_{RB} promoters and two sites in the P_H and P_{YB} promoters (64, 112). However, the positionings of these sites in the promoter regions differ: in the case of VanR, the binding site is upstream from the −35 region (112), whereas it overlaps the −35 region for $VanR_B$ (Fig. 4) (64). The binding site is centered at −54.5 for VanR in P_R and at −32.5 for $VanR_B$ in P_{RB}. In the P_H and P_{YB} promoter regions, the sites are centered at −53.5 and −86.5 for VanR (112) and at −33.5 and −55.5 for $VanR_B$ (64), respectively. The two copies of the binding sites at P_H and P_{YB} are 33 bp (112) and 22 bp (64) apart, respectively, suggesting that since these figures differ almost exactly by three or two helical turns of B-DNA (10.5 bp/turn), they both lie on the same face of the DNA helix. VanR and $VanR_B$ bind with higher affinity to the corresponding P_H and P_{YB} promoters controlling the resistance genes than to the P_R and P_{RB} promoters for the regulatory genes (Fig. 4) (64). Phosphorylation increases the affinity for P_H by 40-fold but increases the affinity for P_{YB} by only 10-fold, indicating that the cooperativity is higher at P_H than at P_{YB} (Fig. 4) (64, 112). A direct relationship between the binding cooperativity of $VanR_B$-P to its sites and the expression of the resistance genes may exist, since the levels of induction of the resistance genes are lower with $VanR_B$ than with VanR.

VanR and VanR-P bind to a similar 80-bp stretch of the

FIG. 2. Comparison of the *van* gene clusters. Open arrows represent coding sequences (red arrows, regulatory genes; purple arrows, genes required for resistance; blue arrows, accessory genes; pink and yellow arrows, genes of unknown function) and indicate the direction of transcription. The percentages of amino acid (aa) identity between the deduced proteins of reference strains BM4147 (VanA) (19), V583 (VanB) (77), BM4339 (VanD) (42), BM4174 (VanC) (10), BM4405 (VanE) (1), and BM4518 (VanG) (63) are indicated under the arrows. The vertical bar in $vanY_G$ indicates the frameshift mutation leading to a predicted truncated protein. NA, not applicable.

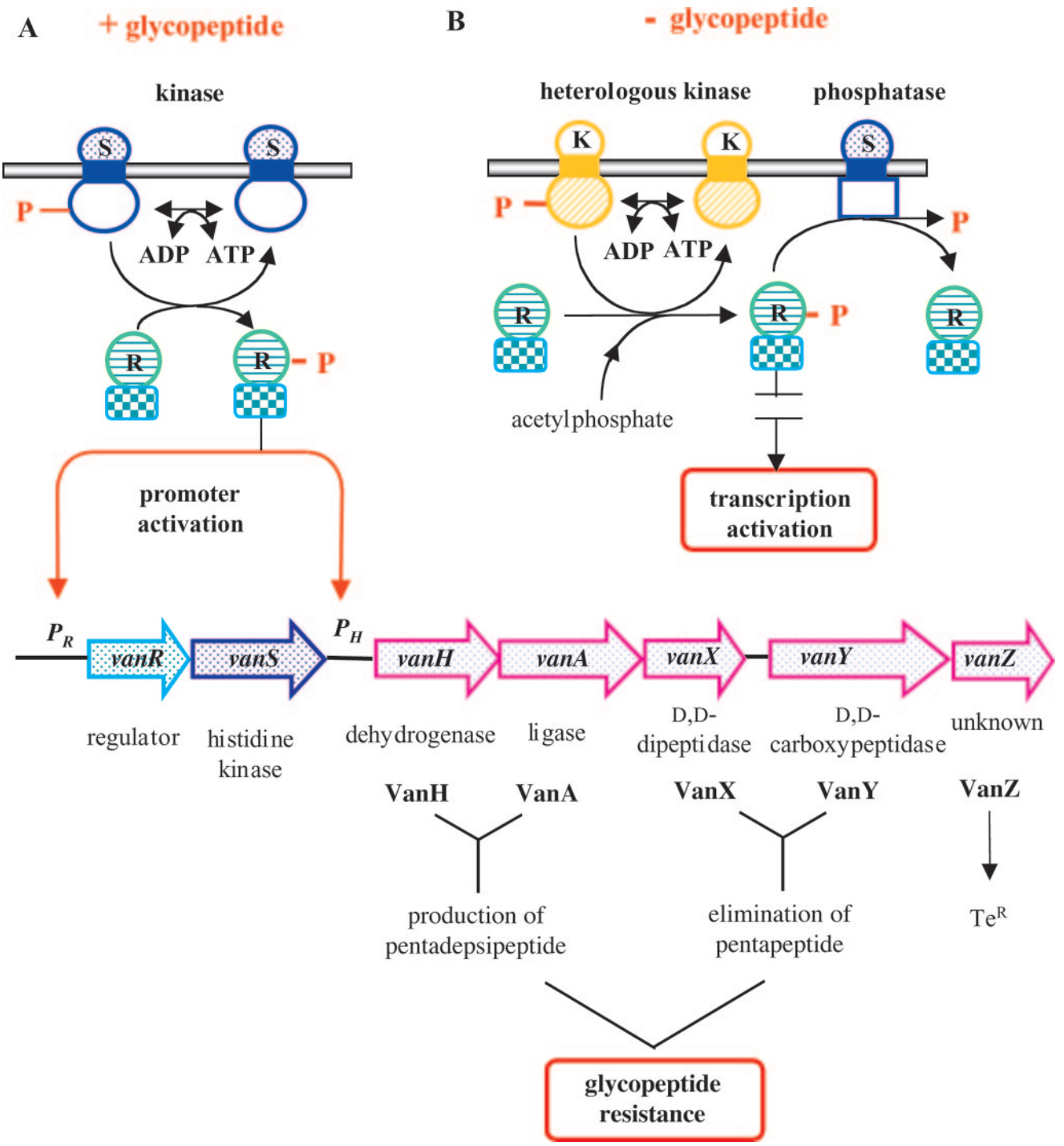
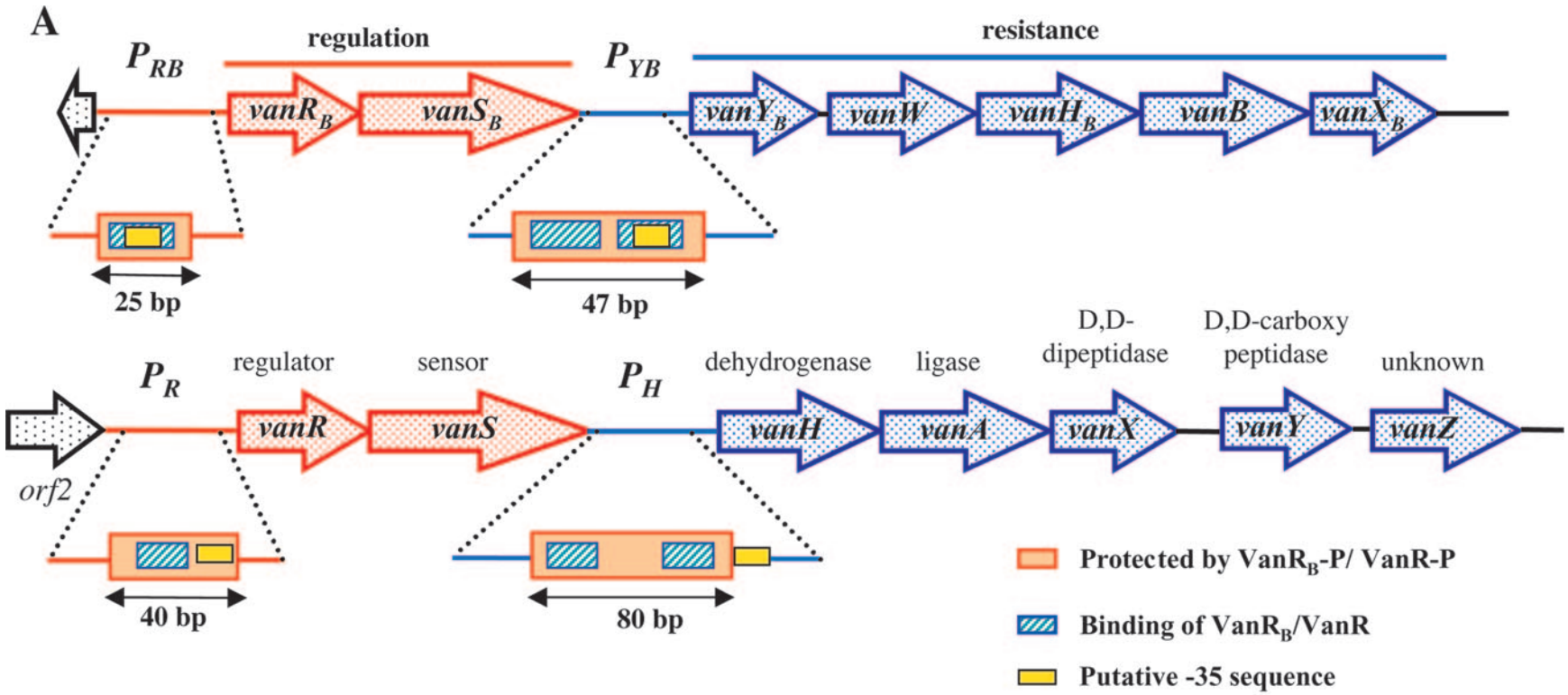


FIG. 3. Model for positive (phosphorylation) and negative (dephosphorylation) control of VanR by VanS and schematic representation of the synthesis of peptidoglycan precursors in VanA- or VanB-type strains. Kinase (A) and phosphatase (B) activities of VanS are depicted. K, heterologous kinase; R, regulator; S, sensor. Dotted blue circle, sensor domain; blue box, transmembrane domain; white circle, kinase domain; horizontally striped green circle, receiver domain; checkerboard green box, effector domain.

regulatory region of P_H that contains two putative 12-bp binding sites (Fig. 4) (112). The P_R promoter contains a single 12-bp binding site, and the phosphorylation of VanR increases the size of the protected region from 20 to 40 bp (Fig. 4) (112), whereas the phosphorylation of $VanR_B$ does not increase the size of the protected region in P_{RB} (64). After phosphorylation, VanR generates a more extensive footprint than $VanR_B$ (40 bp for P_R versus 25 bp for P_{RB} and 80 bp for P_H versus 47 bp for P_{YB}) due to higher cooperativity (Fig. 4).

A 21-bp consensus was identified within the binding regions



B

	$VanR_B$	VanR
-Affinity increase due to phosphorylation	6 X for P_{RB} 10 X for P_{YB}	60 X for P_R 500 X for P_H
- Relative affinity of the regulator	$P_{YB} > P_{RB}$ (6X)	$P_H > P_R$ (5X)
- Increase in affinity of the phosphorylated regulator	$P_{YB} > P_{RB}$ (10X)	$P_H > P_R$ (40 X)

FIG. 4. (A) Schematic representation of the binding of VanR-type regulators to the *vanA* and *vanB* promoters and (B) comparison of affinity of VanR or $VanR_B$ and VanR-P or $VanR_B$-P for DNA fragments carrying the P_R/P_{RB} and P_H/P_{YB} promoters. Open arrows represent coding sequences (red arrows, regulatory genes; purple arrows, genes required for resistance; black arrows, genes of unknown function).

of P_{RB} and P_{YB}, which consists of two and four direct repeats of the CTACAG(G/A) heptanucleotide, respectively (64). A similar organization has been observed in other response regulators such as CtsR (68) and PhoP (74, 270) from *Bacillus subtilis* and DcuR from *Escherichia coli* (3). The heptanucleotides, which correspond to the $VanR_B$ recognition sequence, are separated by four nucleotides, and at each site, the protected guanines are 10 bp apart and are thus positioned on the same face of the B-DNA helix (64). This tandem symmetry is consistent with the notion that $VanR_B$ binds to DNA as a head-to-tail dimer, as reported previously for PhoB (35). The consensus sequence of P_{RB} and P_{YB} is not present in the promoter regions of the other *van* operons. In contrast, sequence comparison of the P_{YG} promoter, controlling the resistance genes in the *vanG* operon; the P_{YD} promoter, controlling those of the *vanD* operon; and the P_H promoter revealed a 12-bp consensus sequence, (T/C)CGTAXGAAA(T/A)T, similar to T(T/C)GTA(G/A)GAAA(T/A)T, corresponding to the regions protected by VanR and VanR-P in the *vanA* operon (112) that is present three times in the P_{YG} region (63) and twice in the P_{YD} region.

$VanR_B$-P recruits the RNA polymerase to the regulatory and resistance gene promoters. As mentioned above, $VanR_B$ and $VanR_B$-P bind specifically to the same regions of the P_{RB} and P_{YB} promoters, and although not essential for binding, phosphorylation of the regulator significantly increases the affinity for the DNA targets (64). Treatment with acetylphosphate converts $VanR_B$ from a monomer with low affinity for its binding site into a homodimer with higher DNA affinity (64). Activation of gene expression in vivo most likely requires the phosphorylation and consequently the dimerization of $VanR_B$ to raise the binding affinity to physiologically relevant levels. In order to switch on the positive autoregulatory loop that leads to the expression of the vancomycin resistance genes, a VanB-type strain needs to synthesize a minimum number of $VanR_B$ and $VanS_B$ molecules even in the absence of antibiotic. $VanR_B$-P has a higher affinity for its targets than $VanR_B$ and appears to be more efficient than $VanR_B$ in promoting an open complex formation with P_{RB} and P_{YB} (64). The RNA polymerase is able to interact with the P_{RB} promoter region in the absence or presence of $VanR_B$ but is able to interact with P_{YB} only in the presence of $VanR_B$ and in both cases with an increased affinity when $VanR_B$ is phosphorylated. In vitro transcription assays showed that $VanR_B$-P activates P_{YB} more strongly than P_{RB} (64). The higher affinity of $VanR_B$ for P_{YB} relative to P_{RB} may result from P_{YB} having two heptanucleotide direct repeats, possibly resulting in the cooperative binding of the regulator to the two adjacent sites, which may serve as recognition sites for $VanR_B$ and $VanR_B$-P binding. Although the regions protected by $VanR_B$ and $VanR_B$-P encompass the −35 regions of the promoters, $VanR_B$-P is able to recruit the RNA polymerase at the promoters and allows efficient open complex formation. Unlike the situation with PhoB, the C-terminal domain of the RNA polymerase α subunit is required for transcription activation from the P_{RB} and P_{YB} promoters, possibly by making direct contact with the activator or by being mandatory for promoter binding (64).

In vivo activation of the P_R and P_H promoters in VanA-type strains. In VanA-type strains, the activation of the P_R and P_H promoters has been studied using various transcriptional fusions with reporter genes (13, 14). Determinations of D,D-dipeptidase activity and of the cytoplasmic pool of peptidoglycan precursors show that the expression of glycopeptide resistance is regulated at the level of transcriptional initiation at these promoters. The P_R and P_H promoters have similar strengths and are regulated similarly. They are not activated in the absence of VanR and VanS, are induced by glycopeptides when VanR and VanS are present, and are constitutively activated by VanR in the absence of VanS due, presumably, to phosphorylation of VanR by host kinases (13, 14). Consequently, VanR is a transcriptional activator required for initiation at both promoters, whereas VanS is not necessary for the full activation of the promoters since VanR can be phosphorylated independently of its partner sensor. However, VanS is required for negative control of the promoters in the absence of glycopeptides, acting as a phosphatase under noninducing conditions, thus preventing the accumulation of VanR-P. VanR-P binds to the P_R promoter and activates the transcription of the *vanR* and *vanS* genes. Regulation of the *vanA* gene cluster therefore involves not only a modulation of the relative amounts of VanR and VanR-P by the kinase and phosphatase activities of VanS but also a modulation of the concentration of the response regulator. An amplification loop results from the binding of VanR-P to the P_R promoter with a resultant increased expression of *vanR* and accumulation of VanR-P following phosphorylation. This may explain the high-level transcription of the resistance genes observed in *vanS* null mutants, since the amplification loop, in combination with the long half-life of VanR-P, may compensate for the inefficient phosphorylation of the response regulator by the putative host kinase.

Acquisition of teicoplanin resistance by VanB-type enterococci. As mentioned above, enterococci harboring clusters of the *vanB* class remain susceptible to teicoplanin since this antibiotic is not an inducer (15). However, mutations in the $vanS_B$ sensor gene have been obtained in vitro (26) and in vivo in animal models (21) following selection by teicoplanin, which have resulted in three phenotypic classes (constitutive, teicoplanin-inducible, or heterogeneous expression of the resistance genes) due to three types of alterations of $VanS_B$ function. Mutations leading to teicoplanin resistance also confer low-level resistance to the glycopeptide oritavancine (LY333328) (16). Derivatives of VanB-type strains that are resistant to teicoplanin have been isolated from two patients following treatment with vancomycin (103) or teicoplanin (125), but the isolates were not studied further.

(i) Inducible phenotype. Substitutions in the sensor domain of $VanS_B$ lead to inducible expression of resistance by vancomycin and teicoplanin (Fig. 5) (26). A minority of the mutations are located between the two putative transmembrane segments of $VanS_B$. This portion of the sensor is located at the outer surface of the membrane and may therefore interact directly with ligands, such as glycopeptides, which do not penetrate into the cytoplasm. The majority of the substitutions are located in the linker that connects the membrane-associated domain to the cytoplasmic catalytic domain. The N-terminal domain of $VanS_B$ is thus involved in signal recognition and is

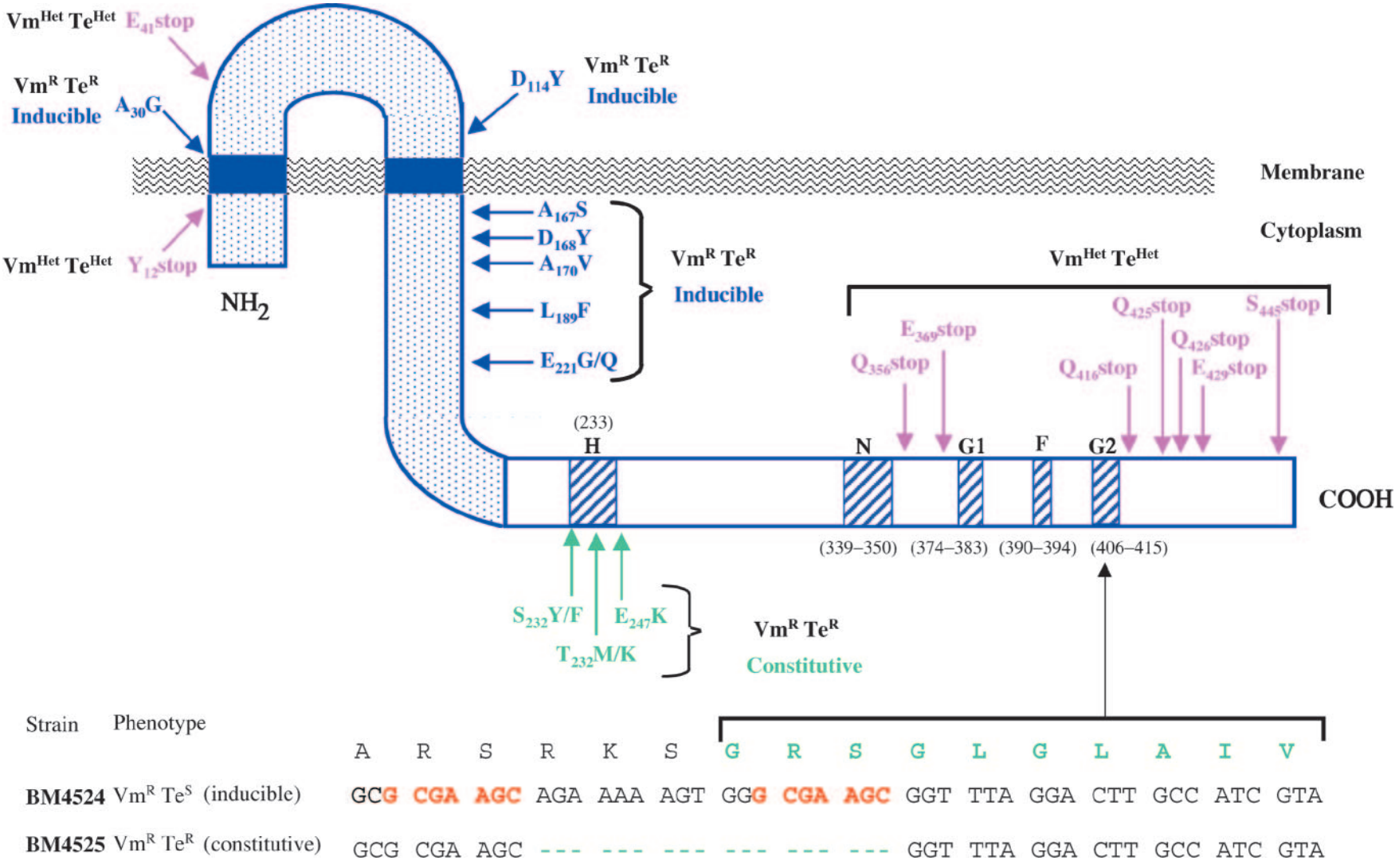


FIG. 5. Schematic representation of the $VanS_B$ sensor and location of amino acid substitutions in teicoplanin-resistant mutants. H, N, G1, F, and G2 refer to the motifs conserved in histidine protein kinases and are shown as hatched boxes. The putative membrane-associated sensor domain (dotted blue) containing transmembrane segments (blue) and the putative cytoplasmic kinase domain (white) are indicated. Het, heterogeneously resistant; R, resistant; S, sensitive; Te, teicoplanin; Vm, vancomycin.

associated with alterations of specificity that allow induction by teicoplanin but not by the nonglycopeptide moenomycin, which also inhibits the transglycosylation reaction (13, 25).

VanS and $VanS_B$ may sense the presence of glycopeptides by different mechanisms. VanA-type resistance is inducible by glycopeptides, moenomycin, and other antibiotics that inhibit the transglycosylation reaction but not by drugs that inhibit the reactions preceding (such as ramoplanin) or following (such as bacitracin and penicillin G) transglycosylation (25, 100). This narrow specificity suggests that the accumulation of lipid intermediate II, resulting from the inhibition of transglycosylation, may be the signal recognized by the VanS sensor. This would account for the induction by antibiotics that inhibit the same step of peptidoglycan synthesis but have different structures and modes of action. However, there are conflicting results in relation to antibiotics that can act as inducers, possibly resulting from using some of them at much higher concentrations than those inhibiting cell growth (260). In particular, bacitracin (6, 132) and ramoplanin (90) have been reported to induce vancomycin resistance, and although its mode of action remains somewhat controversial (260), it has recently been proposed that ramoplanin acts at the transglycosylation step (260). In contrast, the $VanS_B$ sensor may interact directly with vancomycin, since teicoplanin is not an inducer.

(ii) Constitutive phenotype. In the VanS-type sensors, five blocks (H, N, G1, F, and G2) of the kinase domain are highly conserved (Fig. 5). The H block is responsible for both autophosphorylation and kinase/phosphatase activities, and G1 and G2 correspond to ATP binding blocks. Mutations responsible for constitutive expression of the *vanB* cluster result from amino acid substitutions at two specific positions located on either side of the histidine at position 233, which is the putative autophosphorylation site in $VanS_B$ (Fig. 5) (26). Constitutive expression of glycopeptide resistance is most probably due to impaired dephosphorylation of $VanR_B$ by $VanS_B$, as similar substitutions affecting homologous residues of related sensor kinases impair the phosphatase but not the kinase activity of the proteins (26, 65). These observations confirm that dephosphorylation of $VanR_B$ is required to prevent the transcription of the resistance genes (13).

A VanB-type *Enterococcus faecium* strain that was resistant to vancomycin and susceptible to teicoplanin was isolated from a patient, and 2 weeks later, a derivative that was constitutively resistant to high levels of both glycopeptides was isolated from the same patient (65). Increased resistance in the derivative was shown to be due to the combination of a frameshift mutation leading to the loss of the Ddl ligase activity and the constitutive synthesis of pentadepsipeptide precursors by the loss of $VanS_B$ phosphatase activity following a six-amino-acid deletion, which partially overlaps the conserved G2 ATP-binding domain (Fig. 5) (65).

(iii) Heterogeneous phenotype. The heterogeneously resistant derivatives most probably harbor null alleles of $vanS_B$ since the mutations introduce translation termination codons at various positions in the gene (Fig. 5) (27). The antibiotic disk diffusion assay revealed the presence of inhibition zones containing scattered colonies of resistant bacteria that grew predominantly in 48 h (21, 27).

Resistance to Glycopeptides in *Staphylococcus aureus*

Some of the genes regulated by the VraSR two-component system in *S. aureus* are associated with cell wall biosynthesis, including *murZ*, for the production of murein monomer precursors, and *pbp2*, *sgtA*, and *sgtB*, for the polymerization of peptidoglycan (131). The production of VraSR is induced by the exposure of *S. aureus* to antibiotics that affect cell wall synthesis, such as glycopeptides, β-lactams, bacitracin, and D-cycloserine, suggesting that the VraS sensor kinase responds to damage or the inhibition of cell wall biosynthesis (131). Additionally, the *vraSR* null mutants derived from methicillin-resistant *S. aureus* isolates show reduced transcription of *murZ* and *pbp2*, which correlates with a significant decrease in resistance to teicoplanin, β-lactams, bacitracin, and fosfomycin but not to D-cycloserine and levofloxacin. Overexpression of the VraR response regulator confers a low level of resistance to vancomycin. These observations indicate that VraSR constitutes a positive regulator of peptidoglycan synthesis that is involved in the expression of resistance to certain cell wall inhibitors in *S. aureus*.

The overproduction of PBP2 significantly increases resistance to teicoplanin, whereas the reduction in teicoplanin resistance is observed in *vraSR* null mutants, which agrees well with a loss of PBP2 induction (97). PBP2 possesses transglycosylase activity that catalyzes the elongation of the nascent peptidoglycan chains (195). However, elongation of the chains is not completely abolished after the inactivation of the transglycosylase domain of PBP2, indicating that other transglycosylases also catalyze the elongation reaction. The VraSR system positively regulates the *sgtA* and *sgtB* glycosyltransferase genes. The deduced proteins show significant similarity with transglycosylase domains and, consequently, may be involved in glycopeptide resistance in *S. aureus* (109). It is considered that increased transglycosylase activity contributes to resistance either by competing with glycopeptides for the capture of the membrane-bound murein monomers or by increasing the production of nascent peptidoglycan chains to provide more D-Ala–D-Ala that serves as a false target for vancomycin. High copy numbers of the *vraSR* genes do not increase the transcription of *pbp2* and *sgtB* and require the presence of cell wall synthesis inhibitors to induce the expression of the genes (131). This indicates that the signal that activates the VraS sensor kinase could be generated by the inhibition of cell wall synthesis.

Resistance to β-Lactams in *Enterococcus faecalis*

E. faecalis produces a low-affinity penicillin-binding protein (PBP5) that mediates high-level resistance to cephalosporins. A regulatory system, designated CroRS for ceftriaxone resistance, is essential for this intrinsic resistance (56). Deletion of *croRS* leads to a 4,000-fold reduction in the MIC of expanded-spectrum cephalosporins such as ceftriaxone. The CroS kinase autophosphorylates and transfers its phosphate to the CroR response regulator. The *croR* and *croS* genes are cotranscribed from a promoter (*croRp*) located upstream from *croR*. CroRS is induced in response to β-lactams and inhibitors of early and late steps of peptidoglycan synthesis, indicating that this system does not respond to the inhibition of a specific biosynthetic

step (56). The *croRS* null mutant produces PBP5, and the expression of an additional copy of *pbp5* under the control of a heterologous promoter does not restore ceftriaxone resistance (56). Deletion of *croRS* is not associated with any defect in the synthesis of the UDP-MurNAc-pentapeptide precursor or of the D-Ala4→L-Ala–L-Ala–Lys3 peptidoglycan crossbridge. Thus, the CroRS two-component regulatory system is essential for β-lactam resistance mediated by PBP5 in enterococci. However, CroRS is not required for the production of low-affinity PBP5, suggesting that it controls other, as-yet-unidentified, factors essential for the activity of this low-affinity penicillin binding protein.

Recently, to gain a more comprehensive view of the role of two-component signal transduction pathways in the biology of *E. faecalis*, each of the 18 response regulators previously identified in *E. faecalis* V583 was targeted by insertion mutagenesis (99). An insertion in *croR* led to susceptibility to the cephalosporins, bacitracin, and vancomycin despite the presence of a functional *vanB* operon in strain V583. CroR is thus involved in resistance to a wide range of cell wall-active agents, indicating that this system may have a role in the regulation of cell wall synthesis.

Resistance by Efflux

Drug resistance among gram-negative bacilli such as *Escherichia coli* and *Pseudomonas aeruginosa* and gram-positive cocci such as *S. aureus*, *Staphylococcus epidermidis*, other coagulase-negative staphylococci, *E. faecalis*, *E. faecium*, and *Streptococcus pneumoniae* complicates the therapy of infections caused by these microorganisms. An important component of this resistance is the activity of membrane-based efflux proteins commonly referred to as "pumps" (205). The function of these efflux pumps is to export molecules through the bacterial envelope, thus limiting the intracellular accumulation of toxic compounds such as antibiotics. This pumping out is energized by ATP hydrolysis or by an ion antiport mechanism (144, 205). Efflux decreases the antibacterial efficacy of structurally unrelated drug classes and has been shown to be responsible for species- or genus-specific intrinsic or "natural" resistance to antibiotics. If the pump is overproduced, it can be responsible for extended cross-resistance, since it confers, by a single mechanism, resistance to various drug classes.

The envelope of gram-negative bacteria comprises two membranes, the inner or cytoplasmic membrane and the outer membrane, which are separated by the periplasmic space, whereas gram-positive bacteria possess a single membrane. The membrane-located transporters can be grouped into the following five families based on sequence homology, mechanisms, and molecular characteristics: the ATP binding cassette (ABC) family, the major facilitator superfamily (MFS), the multidrug and toxin extrusion family, the resistance-nodulation-division (RND) family, and the small multidrug resistance (SMR) family (Fig. 6). In gram-negative bacteria, the efflux machinery is complex, comprising a cytoplasmic membrane-located transporter, a periplasmic membrane adaptor protein, and an outer membrane channel protein. Genomes of gram-negative bacteria usually encode multiple members of each family of multidrug transporters (192). To date, only the ABC, MFS, and SMR families have been described in gram-positive organisms.

Generally, drug-specific efflux pumps tend to be encoded by plasmids and are thus transmissible, whereas MDR efflux pumps are usually specified by the chromosome (191, 210). The expression of plasmid-borne genes is often sufficient to confer resistance without the need for additional mutations owing to the multicopy state of these genetic elements. However, drug resistance due to chromosomally encoded MDR pump genes most often occurs because of increased gene expression, which can take place as a consequence of substrate-induced transcriptional activation, gene amplification, or the occurrence of regulatory mutations that, in certain instances, confer only low-level resistance to the host (91).

Resistance to quinolones in *Staphylococcus aureus*. NorA was the first chromosomally encoded *S. aureus* pump to be identified. Based on its sequence, the cloned *norA* gene of a fluoroquinolone-resistant clinical strain was predicted to encode a typical MFS-type protein with 12 membrane-spanning alpha helices. NorA has the highest degree of identity with the Bmr MFS pump of *Bacillus subtilis* (44%) and only 20 to 25% identity with several tetracycline-specific efflux proteins of gram-negative bacteria (121). Cloning of *norA* in a plasmid in either *S. aureus* or *E. coli* results in fluoroquinolone resistance, particularly to hydrophilic molecules. NorA has a broad substrate specificity, including hydrophilic fluoroquinolones, biocides, and dyes. In addition, the substrates of NorA are typical of those of MDR pumps, namely, amphipathic cations. NorA activity is inhibited by reserpine, a compound known to act as an inhibitor of the function of many MDR efflux proteins. Resistance associated with NorA occurs only when the structural gene for this protein is either amplified or overexpressed as a result of regulatory mutations (121).

Regulation of NorA expression depends on at least two systems, ArlRS and MgrA (formerly NorR) (83, 84, 255). MgrA is composed of 147 residues, has modest similarity with other regulatory proteins such as MarR in *E. coli* and SarR in *S. aureus*, and, when overexpressed, causes increased expression of *norA*. It binds upstream from the *norA* promoter, and experimental data suggest that repeats of the TTAATT consensus sequence may be involved in the binding of this protein (255). Four such hexamers are located upstream from the −35 motif of the *norA* promoter. MgrA is not a specific regulator of *norA* expression but, rather, is a global regulator, since it also regulates autolytic activity and the expression of several virulence factors, including alpha toxin, nuclease, and protein A (153). MgrA is transcribed from two promoters, positively regulates its own expression, and acts at the transcriptional level to enhance the expression of numerous genes. Recently, two novel efflux transporters, NorB and Tet38, that confer resistance to multiple drugs including quinolones and tetracycline, respectively, have been shown to be negatively regulated by MgrA (254).

The ArlR-ArlS two-component regulatory system is involved in adhesion, autolysis, and extracellular proteolytic activity of *S. aureus* (85). The binding of MgrA to the *norA* promoter is modified in a strain with a disrupted *arlS* such that increased *norA* expression is observed (83, 84, 255). Overexpression of *mgrA* in a strain producing the ArlS sensor results in increased transcription of *norA* and reduced susceptibility to various

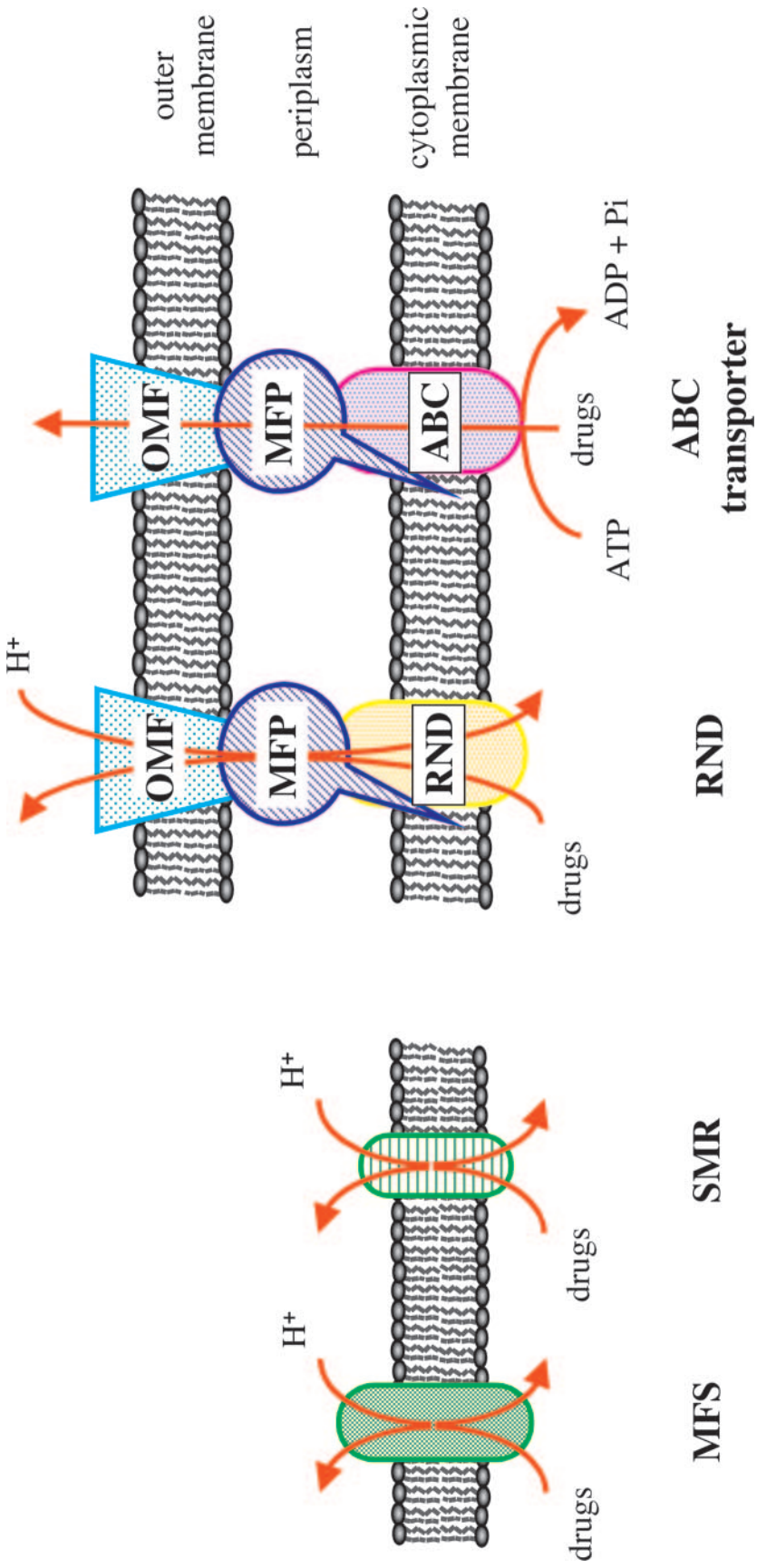


FIG. 6. Schematic representation of the cell membranes with examples of multidrug efflux systems. ABC, ATP binding cassette; MFP, membrane fusion protein; MFS, major facilitator superfamily; OM, outer membrane; OMF, outer membrane factor; RND, resistance nodulation cell division; SMR, small multidrug resistance.

NorA substrates. These data suggest that a mutation in *arlS* increases the effect of MgrA on the *norA* promoter and that wild-type levels of MgrA have little effect on *norA* expression. Highly fluoroquinolone-resistant strains of *S. aureus* in which *norA* expression is enhanced in the absence of any modification in *arlR-arlS* or change in *mgrA* expression have been reported, indicating that other loci must be involved in the regulation of *norA* expression.

Resistance to multiple drugs in gram-negative bacteria. The synthesis of the tripartite efflux systems of gram-negative bacteria (Fig. 6) depends on regulatory genes, implying individual control and thus distinct functions in the cell (180). Two-component systems are not commonly involved in the regulation of drug efflux transporters, although such systems have recently been associated with RND-type pumps, such as AdeABC in *Acinetobacter baumannii* (154), SmeABC in *Stenotrophomonas maltophilia* (145), and MdtABC in *E. coli* (28).

Intrinsic resistance of gram-negative bacteria is due to multidrug efflux by RND pumps that are widely distributed and act in synergy with the outer membrane barrier. The wide substrate range of these transporters often includes β-lactams and aminoglycosides, which are rarely subjected to efflux by other pump classes. RND transporters form a multiprotein complex with members of the outer membrane factor family and of the periplasmic linker membrane fusion protein family. These complexes allow the excretion of drugs directly into the medium. Chromosomally encoded multidrug RND efflux systems appear to be most important for resistance to antimicrobials in *P. aeruginosa* and other gram-negative pathogens.

(i) *Acinetobacter baumannii*. *A. baumannii* is one of the predominant bacteria associated with outbreaks of nosocomial infections that are often very difficult to treat because of the frequent resistance of this species to multiple antibiotics. Aminoglycosides can be used successfully in combination with a β-lactam, and combinations of a β-lactam with either a fluoroquinolone or rifampin have also been proposed. Partial resistance of *A. baumannii* to β-lactams is due to the synthesis of a species-specific cephalosporinase (258).

The chromosomally encoded three-component AdeABC pump in *A. baumannii* is composed of the membrane fusion homolog AdeA, the RND superfamily member AdeB with 12 transmembrane segments, and AdeC an outer membrane protein similar to OprM of *P. aeruginosa* (154). Insertional inactivation of *adeB* indicates that the corresponding protein is responsible for resistance not only to aminoglycosides but also to fluoroquinolones, tetracycline, chloramphenicol, erythromycin, and trimethoprim. Thus, this efflux pump recognizes a wide spectrum of substrates including hydrophobic, amphiphilic, and hydrophilic molecules, which can be either positively charged or neutral. When the *adeC* gene is inactivated, resistance to the various substrates of the AdeABC pump is unaltered (161), suggesting that AdeAB can utilize another outer membrane constituent, as already observed for MexXY from *P. aeruginosa* (see below).

The expression of multidrug transporters is commonly controlled by specific regulatory proteins. Their structural genes are most often adjacent to those encoding the efflux system. The *adeABC* genes are cotranscribed and adjacent to the *adeS* and *adeR* genes that are transcribed in the opposite direction and encode a sensor and a regulator, respectively (Fig. 7) (161). Inactivation of *adeS* leads to aminoglycoside susceptibility, indicating that this gene is required for the expression of the *adeABC* operon. Spontaneous aminoglycoside-resistant derivatives that have mutations in the AdeS sensor or in the AdeR regulator can be obtained in vitro. The $T_{153}M$ substitution in AdeS, downstream from histidine 149, the putative site of autophosphorylation, is presumably responsible for the loss of phosphatase activity of the sensor, as observed for EnvZ ($T_{247}R$), PhoR ($T_{220}N$), and $VanS_B$ ($T_{237}K$). In AdeR, the $P_{116}L$ mutation at the first residue of the α5 helix of the receiver domain is involved in interactions that control the output domain of response regulators. These mutations result in the constitutive expression of the AdeABC pump, which is otherwise cryptic in wild-type *A. baumannii* due to stringent control by AdeRS.

(ii) *Stenotrophomonas maltophilia*. *S. maltophilia* is an aerobic, nonfermentative, gram-negative bacterium, broadly distributed in nature, that has emerged as an important nosocomial pathogen. This species is characterized by high-level intrinsic resistance to a variety of structurally unrelated antimicrobials, which is partly attributable to limited outer membrane permeability combined with antibiotic efflux (145).

The SmeABC multidrug efflux system, a homolog of the *mexAB-oprM* efflux operon of *P. aeruginosa* (see below), is regulated by the SmeSR two-component system (Fig. 7) (145). A strain in which the *smeABC* genes are overexpressed displays resistance to aminoglycosides, β-lactams, and the fluoroquinolones. Deletions in *smeC* but not in *smeB* decrease resistance, suggesting that SmeC only, which possesses its own promoter, contributes to multidrug resistance. Thus, SmeABC does not function as a multidrug efflux system, but it rather appears that SmeC plays a role in antimicrobial resistance independently of SmeAB, possibly as the outer membrane factor component of another unidentified multidrug efflux system (145).

As has been observed for the AdeABC system of *A. baumannii*, two genes, *smeR* and *smeS*, upstream from the *smeABC* operon and transcribed in the opposite direction, encode a regulatory system composed of a sensor (SmeS) and a regulator (SmeR) (Fig. 7) (145). SmeR positively regulates both *smeABC* and its own *smeSR* operon.

(iii) *Pseudomonas aeruginosa*. *P. aeruginosa* is a ubiquitous aerobic gram-negative opportunistic pathogen and one of the most common causes of nosocomial infections. Treatment of *P. aeruginosa* infections is complicated by the intrinsic resistance of this organism to many antimicrobial agents, which results from the synergistic activity of the outer membrane barrier with that of various broad-substrate-range multidrug efflux systems. In addition to intrinsic resistance, multidrug efflux (Mex) systems promote acquired resistance by overexpression of the structural genes for the pumps following mutational events.

Six RND efflux systems in *P. aeruginosa* have been characterized (Table 1) (4, 5, 50, 111, 129, 206, 207). The efflux operons each encode an inner membrane RND transporter (MexB, MexD, MexF, MexX, MexK, or MexI), a periplasmic membrane fusion protein (MexA, MexC, MexE, MexY, MexJ, or MexH), and, in certain cases, an outer membrane channel protein (OprM, OprJ, OprN, or OpmD). All these RND operons are similar in their genetic organizations but not with respect to regulation, and the corresponding pumps differ in

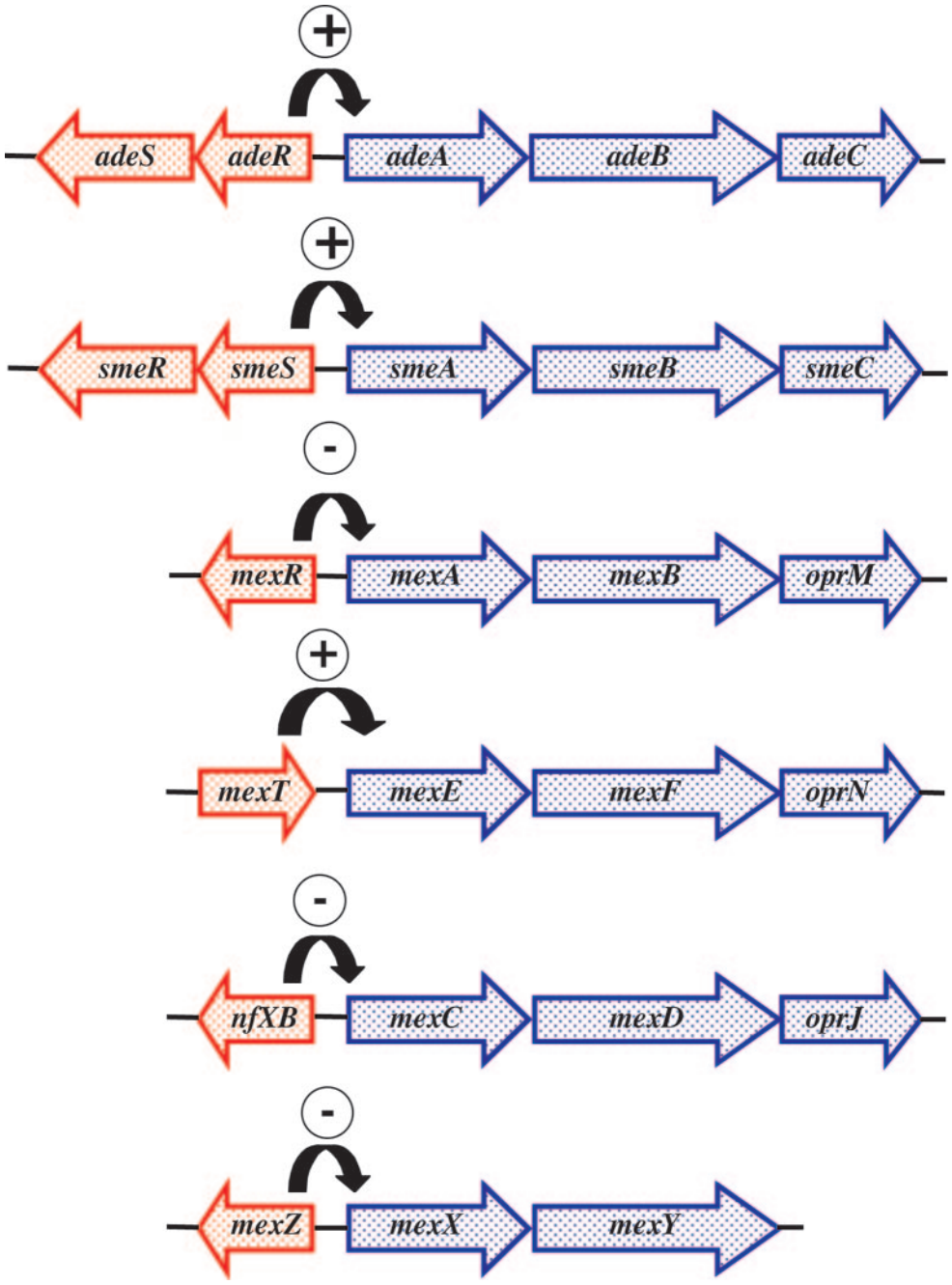


FIG. 7. Genetic organization of the *adeRS-adeABC* operon from *A. baumannii*, the *smeRS-smeABC* operon from *S. maltophilia*, and the *mexR-mexAB-oprM*, *mexT-mexEF-oprN*, *nfxB-mexCD-oprJ*, and *mexZ-mexXY* MDR operons from *P. aeruginosa*. Purple arrows, structural genes for drug efflux complexes; red arrows, regulatory genes that either repress (−) or activate (+) gene expression (this still has to be confirmed for *mexZ*).

their substrate specificities (Fig. 7 and Table 1). The antibiotic substrate spectrums of these systems are very wide (Table 1). MexAB-OprM, which exhibits an extraordinarily broad substrate range, is constitutively produced in wild-type bacteria and plays a major role in the intrinsic resistance of *P. aeruginosa* (Table 1) (128). The MexCD-OprJ, MexEF-OprN, and MexJK-OprM systems are not expressed in wild-type *P. aeruginosa* (50, 129, 206). Expression of many RND multidrug pumps is controlled by local regulators (Table 1), mostly repressors (Fig. 7). With the exception of MexAB-OprM, the expression of most of these efflux systems is tightly regulated.

The *mexR* and other regulatory genes, *nfxB* (206, 235), *mexZ* (166), and *mexL* (49), encode negative regulators (Table 1) (Fig. 7), and mutations in these genes lead to the overexpression of the *mexAB-oprM*, *mexCD-oprJ*, *mexXY*, and *mexJK* operons, respectively. MexR (76), NfxB (235), MexZ (166),

TABLE 1. Substrate profiles and regulatory components of *Pseudomonas aeruginosa* efflux pumps

Efflux pump	Regulator(s)	Substrates[a]	Reference(s)
MexAB-OprM	MexR	β-Lactams (except imipenem), fluoroquinolones, tetracycline, macrolides, chloramphenicol, novobiocin, trimethoprim	76, 207
MexCD-OprJ	NfxB	β-Lactams (except imipenem), fluoroquinolones, tetracycline, macrolides, chloramphenicol, novobiocin, trimethoprim	206, 235
MexEF-OprN	MexT	Fluoroquinolones, chloramphenicol, trimethoprim	127, 129
MexJK-OprM	MexL	Tetracycline, erythromycin	49, 50
MexXY-OprM	MexZ	Fluoroquinolones, aminoglycosides, tetracycline, macrolides	5, 111, 165
MexGHI-OpmD	LasR (?), RhlR (?)	Tetracycline, netilmicin, ticarcillin + clavulanic acid	4

[a] The list of substrates is limited to antibiotics.

and MexL (49) have been purified and shown to bind to DNA upstream from *mexA*, *mexC*, *mexX*, and *mexJ*, respectively. The *mexEF-oprN* operon is positively regulated by the *mexT* product, a transcriptional activator of the LysR family (127). Certain clinical isolates can broaden their drug resistance phenotypes by coexpressing MexAB-OprM and MexXY following mutations in multiple regulatory genes (151).

(iv) *Escherichia coli*. Certain multidrug efflux pumps in *E. coli* are regulated by two-component systems. BaeSR is involved in the expression of the RND transporter MdtABCD that pumps out novobiocin and deoxycholate (28, 178). The *baeS* and *baeR* genes are immediately downstream from the *mdtABCD* genes and together probably form an operon.

BaeR and BaeS exhibit in vitro phosphotransfer in the presence of ATP (28), but the nature of the stimulus recognized by the BaeS sensor is not known. The BaeR response regulator binds to the *mdtA* promoter, and its overexpression strongly stimulates the transcription of the *mdtABCD* gene cluster, leading to an increase in resistance to novobiocin and deoxycholate. The presence of the BaeS sensor kinase is not required for the full activity of overexpressed BaeR in intact cells. BaeR could be phosphorylated by other sensor kinases present in *E. coli*, since such cross talk occurs particularly when one of the noncognate partners is present in excess. Cross-regulation has been observed between the various two-component regulatory systems, BaeSR, PhoBR, which is implicated in phosphate metabolism, and CreBC, which is implicated in carbon and energy metabolism (181).

Many of the two-component signal transduction systems in *E. coli* control the expression of multiple target genes. BaeR modulates the expression of *mdtABCD* but also that of *acrD*, which encodes a multidrug exporter system conferring resistance to β-lactams and novobiocin (108).

ROLE OF IS ELEMENTS AND INTEGRONS IN THE MODULATION OF RESISTANCE GENE EXPRESSION

Besides the considerable impact that they have on the mobility and spread of antibiotic resistance genes when they make up composite transposons (31, 81, 146, 219), insertion sequences (ISs) as single elements may also exert noticeable effects on the expression of these genes either directly, by influencing the level of their transcription, or in various ways indirectly, by affecting genes involved in their regulation or in the modulation of resistance levels. Together with the integrons, which are natural expression vectors with the capacity to capture resistance genes (95, 227), they constitute two groups of genetic elements with the potential to contribute much to high-level and multiple-antibiotic resistance in clinical isolates.

Effects of IS Elements on the Expression of Resistance

General characteristics of IS elements. Insertion sequence elements are small transposable genetic elements, with a size generally between 0.8 and 2.5 kb and encoding only those functions required for their transposition. Currently, approximately 1,000 IS elements have been identified in some 200 gram-negative and gram-positive bacterial species and in archaea and are assigned to 19 families based on their structural and functional characteristics (31, 46) (http://www-IS.biotoul.fr).

IS elements may be present in one or several copies and localized on the chromosome, on plasmids, or on both and must reside on conjugative elements for intercellular transfer. Many transpose readily, and others, such as IS*200*, transpose rarely (32). There is great variability in the distribution of the IS elements of the different families among bacterial species, with some of them restricted to few hosts, such as IS*6110*, which has been found only in mycobacteria of the tuberculosis complex (156, 250).

IS elements are typically bounded by short repeat sequences of up to ca. 40 bp in an indirect orientation. These inverted repeats are specific for each element, and their presence and integrity are required for transposition, which may or may not be site specific. Upon insertion into the target DNA, a repeat sequence, 2 to 14 bp in length and characteristic for each element, is generated in a direct orientation (Fig. 8). Many elements carry a single, transposase-encoding open reading frame (ORF) covering most of the element, while others carry several ORFs, on a single strand or on both strands, the products of which may also play a role in the regulation of the transposition process. Of particular interest in the present context, IS elements may contain partial or complete promoters, often located at their extremities and in an outward orientation and capable of activating the expression of neighboring genes (Fig. 8) (46, 155).

IS-mediated effects on resistance-conferring and resistance-modulating genes. With respect to IS-mediated effects on antibiotic resistance genes in the strict sense, i.e., genes responsible for drug-specific resistance mechanisms such as antibiotic inactivation, drug target alteration, or specific efflux pump production, gene activation through promoter alteration is the rule. In contrast, insertional inactivation is the predominant effect of IS elements on genes involved in the modulation of

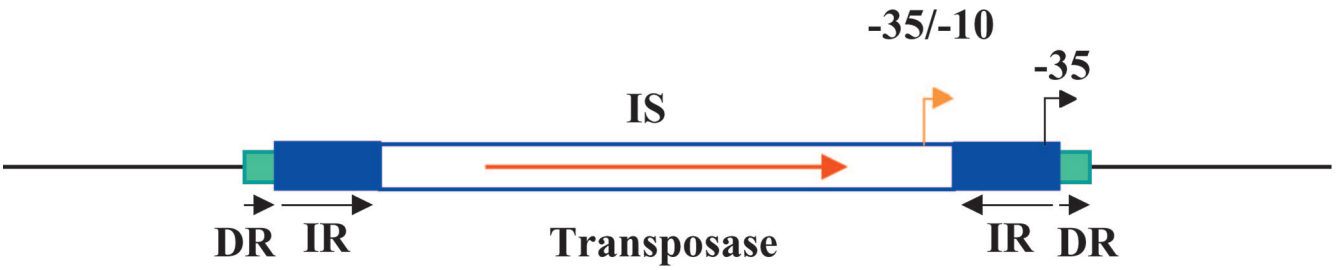


FIG. 8. Characteristics of IS elements. DR, direct repeat; IR, inverted repeat; −35/−10 and −35, approximate locations of promoter consensus sequences.

resistance levels (which may or may not encode resistance gene repressors), such as *ampD*, *mexR*, *acrR*, *nfxB*, *ompC*, *ompK36*, *oprD*, and *carO* in gram-negative bacteria and *mecR/I*, *tcaA*, and $vanS_D$ in gram-positive cocci (Table 2).

Altered expression of resistance-conferring or resistance-modulating genes, consisting in some cases of the activation of silent genes, has been described as a consequence of events mediated by over 20 distinct IS elements belonging to at least 10 families (Table 2) (http://www-IS.biotoul.fr). In one way or another, these elements may have a bearing on the efficiency of resistance mechanisms concerning antibiotics of most classes in clinical use, including the β-lactams, aminoglycosides, quinolones, glycopeptides, imidazoles, and tetracyclines, most often affording an increase in resistance levels. Events of this type have been described for members of many groups of bacteria encountered in the clinical setting, including the *Enterobacteriaceae*, strict aerobic and anaerobic gram-negative bacteria, staphylococci, and enterococci (Table 2).

(i) Activation of resistance genes by promoter alterations. The molecular mechanisms responsible for altered, IS-mediated expression are not specific for resistance genes. Transcriptional activation may result from IS insertion into a region carrying a weak, an incomplete, or no promoter. Therefore, a hybrid promoter with an alternative or new IS-borne −35 region may be generated, or a complete IS-borne promoter containing both the −35 and the −10 regions may be acquired (Fig. 8). With few exceptions (see below), these two regions conform to the canonical consensus sequences TTGACA and TATAAT, respectively, with a spacing distance of 17 bp for optimal promoter activity as determined for *E. coli* (149).

(a) Resistance gene activation by IS-mediated formation of hybrid promoters. An IS-mediated rearrangement of the promoter region of the *ampC* gene of *E. coli* was shown in an experimental setup (118) only shortly before the observation of similar events affecting resistance genes in clinical isolates. It was found that the insertion of IS*2*, of which *E. coli* carries five chromosomal copies, into the −10 region of the artificially plasmid-borne *ampC* gene resulted in concomitant, ca. 20-fold increases in *ampC* transcription, β-lactamase production, and ampicillin resistance levels. While the −10 region remained unaltered and the −35 region was changed to a sequence with less homology with the consensus sequence than that of the natural *ampC* promoter, the critical event was concluded to be the change of the spacer region from 16 to 17 bp. Despite the efficiency of this rearrangement in increasing the resistance level and although IS*2* belongs to the family that is most widely distributed among bacterial species (156), this element does not seem to have been involved similarly in clinical isolates. Another IS*2* insertion, with the creation of a putative hybrid promoter upstream from the efflux pump-encoding *acrEF* gene and its increased expression in an *E. coli* laboratory mutant, facilitated the determination of the substrate profile of the pump (119). Probably the first observation of an IS-mediated formation of a hybrid promoter for an antibiotic resistance gene in a clinical isolate was made by Bräu et al. (39) in *Salmonella*. They found the plasmid-borne *aac(3)-IV* and *aph(4)* genes, coding for gentamicin and hygromycin B resistance, respectively, in an operon-like arrangement downstream from IS*140* (IS*26*), which provided the −35 region.

IS-mediated rearrangements of promoters driving the transcription of genes encoding extended-spectrum β-lactamases belonging to several families of the class A or class D enzymes (117) have been observed (Table 2). The IS*26* element has been reported to contribute to the formation of a hybrid promoter for a chromosome-borne SHV-2A gene in *P. aeruginosa* and for a similar, plasmid-borne gene in a resistance operon (downstream from an aminoglycoside 3′-*O*-phosphotransferase gene) in *Klebsiella pneumoniae*, with the new −35 region in each case at the optimal distance of 17 bp from the respective resistance gene-specific −10 region (137, 177). The gene of TEM-6, as identified in a ceftazidime-resistant strain of *E. coli*, acquired a −35 region after the insertion of an IS*1*-like element into the spacer region of its "natural" promoter, P3, the strength of which was increased by a factor of 10 (89). It was speculated that this element, which was found to be widespread among β-lactamase-producing and non-β-lactamase-producing *Enterobacteriaceae*, had been derived from IS*1* through a substantial deletion of its central region as well as by point mutations in the remainder, which did not affect the −35 region. In a laboratory mutant, the replacement of the −35 region of the same P3 promoter of the TEM-1 gene carried on plasmid pBR322 by a similar IS*1*-borne region had previously been shown to result in decreased promoter strength, which was considered to be related to a lesser degree of homology between this region and the −35 consensus sequence (209).

In *Acinetobacter* species 13, aminoglycoside resistance is conferred by the species-specific 6′-*N*-acetyltransferase-encoding gene, *aac(6′)-Ij*, which may be expressed at various levels (133). The activation of silent copies of the *aac(6′)-Ij* gene in this species by the creation of a putative hybrid promoter with an IS*18*-borne −35 region appears to occur at a low frequency, at least as judged from the in vitro selection

TABLE 2. IS elements affecting genes conferring or modulating resistance to antibiotics

Mechanism	Element(s)	Gene(s) affected	Relevant R phenotype(s)[a] (fold increase)[b]	Occurrence[c]		Species	Reference(s)
				Natl.	Exptl.		
Resistance gene activation (hybrid promoter)	IS*1*	bla_{TEM-1}	Amp		+	*E. coli*	209
	IS*1*-like	bla_{TEM-6}	Caz, Azt (10 P)	+		*Enterobacteriaceae*	89
	IS*2*	*ampC*	Amp (20 P)		+	*E. coli*	118
		acrEF	FQ (~10)		+	*E. coli*	119
	IS*18*	*aac(6′)-Ij*	Ami	+		*Acinetobacter*	229
	IS*26*	*aphA7*, bla_{S2A}	Kan, Ctx	+		*K. pneumoniae*	137
		bla_{SHV-2a}	Caz, Ctx, Azt	+		*P. aeruginosa*	177
	IS*140* (IS*26*)	*aac(3)-IV–aph(4)*[d]	Gen, Hyg	+		*Salmonella* sp.	39
	IS*256*	*mecA*	Met (8–100)	+	+	*S. sciuri*	59
		llm	Met (4–16)		+	*S. aureus*	158
	IS*257*	*dfrA*	Tmp	+		*S. aureus*	138
		tetA(K)	Tet	+		*S. aureus*	237
	IS*1224*	*cepA*	Amp	+		*B. fragilis*	222
Resistance gene activation (complete promoter)	IS*257*	*tetA*(K)	Tet	+		*S. aureus*	236
	IS*612*, IS*613*, IS*614*, IS*615*, IS*942*, IS*943*, IS*1186*, IS*1187*, IS*1188*, IS*4351*	*cfiA*	Imi, Mer	+	+	*B. fragilis*	124, 197, 198, 199, 239, 262
	IS*642*, IS*1168*, IS*1169*, IS*1170*	*nimA*, *nimB*, *nimC*, *nimD*, *nimE*	Mtz	+		*B. fragilis*	94, 240, 253
	IS*1999*	bla_{VEB-1}	Caz, Ctx, Azt (1.6 SA)	+		*P. aeruginosa*	22
		oxa-48	Imi (~30)	+		*K. pneumoniae*	202
	IS*4351*	*ermF/S*	Ery	+		*B. fragilis*	213
	IS*Aba1*	*ampC*	Caz, Tic	+		*A. baumannii*	57, 105, 234
	IS*Ecp1*	$bla_{CTX-M-15}$	Ctx, Atz	+		*Enterobacteriaceae*	122
		$bla_{CTX-M-17}$	Ctx, Atz	+		*K. pneumoniae*	41
	IS*Ecp1B*	$bla_{CTX-M-19}$	Ctx, Atz	+		*K. pneumoniae*	201
	IS*Pa12*	bla_{PER-1}	Caz, Ctx, Azt	+		*S. enterica* serovar Typhimurium *P. aeruginosa*	200
Gene disruption	IS*1*	*ampD*	Pen		+	*E. coli*	148
	IS*1*, IS*5*, IS*26*, IS*903*	*ompK36*	Cfx		+	*K. pneumoniae*	107
	IS*5*-like, IS*102*	*ompK36*	Cfx	+		*K. pneumoniae*	107
	IS*26*	*ompK36*	Imi, Mer	+		*K. pneumoniae*	172
	IS*17*	*aac(6′)-Ig*	AG S[f]	+		*Acinetobacter*	228
	IS*21*	*mexR*	Tic, Azt	+		*P. aeruginosa*	37
	IS*186*	*acrR*	FQ (~30)		+	*E. coli*	119
	IS*256*	*tcaA*	Tei (5–8) Van (2)	+		*S. aureus*	157
	IS*431*	*mecI*, *mecRI*	Met (8–32)		+	*S. haemolyticus*	123
	IS*1669*	*ampD*	Caz (64–400)	+		*P. aeruginosa*	24
	IS*6110*	*pncA*	Pyr	+		*M. tuberculosis*	139
	IS*Aba825*, IS*Aba125*	*carO*	Imi, Mer (16)	+		*A. baumannii*	175
	IS*Efa4*	$vanS_D$	Van constitutive	+		*E. faecium*	67
	IS*Efm1*/IS*19*	*ddl*	Van constitutive	+		*E. faecium*	38, 193
	ISPa*1328*, ISPa*1635*	*oprD*	Imi, Mer	+		*P. aeruginosa*	268
	IS NN[e]	*nfxB*	Tet, Tig		+	*P. aeruginosa*	61
	IS NN	*cmlA*, *oxa-10*	Cmp, Tic	+		*K. pneumoniae*	257

[a] Abbreviations: R, resistance; Ami, amikacin; Amp, ampicillin; Azt, aztreonam; Caz, ceftazidime; Cfx, cefoxitine; Cmp, chloramphenicol; Ctx, cefotaxime; Ery, erythromycin; FQ, fluoroquinolones; Gen, gentamicin; Hyg, hygromycin; Imi, imipenem; Kan, kanamycin; Mer, meropenem; Met, methicillin; Mtz, metronidazole; Pen, penicillin; Pyr, pyrazinamide; Tei, teicoplanin; Tet, tetracycline; Tic, ticarcillin; Tig, tigecycline; Tmp, trimethoprim; Van, vancomycin.

[b] Numbers in parentheses refer to the increase in MIC (*n*-fold); numbers followed by "P" indicate changes in promoter strength; numbers followed by "SA" indicate changes in specific β-lactamase activity.

[c] Natl., insertion observed in clinical isolates; Exptl., spontaneous insertion observed under experimental conditions.

[d] The two genes are organized in a transcriptional unit.

[e] NN, IS element not named.

[f] AG S, susceptibility to aminoglycosides.

of tobramycin-resistant mutants of a susceptible clinical isolate (229).

The IS*256* and IS*257* elements have a proven role in the activation of resistance gene transcription in staphylococci. IS*256* belongs to a large family with members in gram-negative and gram-positive bacteria (http://www-IS.biotoul.fr). It flanks the composite aminoglycoside resistance transposon Tn*4001* and related elements and is involved in their dissemination in

staphylococci, enterococci, and streptococci (158). IS*256* is infrequently observed in the animal commensal species *Staphylococcus sciuri*, in which two-thirds of the isolates are susceptible to β-lactam antibiotics including methicillin, although they carry a close homolog of the *mecA* gene, the primary drug resistance determinant in methicillin-resistant *S. aureus* (58). Analysis of a heterogeneously methicillin-resistant human clinical isolate of *S. sciuri* (with MICs of methicillin of between 25 and 800 μg/ml as opposed to between 3 and 6 μg/ml for a *mecA*-positive, IS*256*-negative control strain) revealed the insertion of an IS*256* copy into the upstream region of *mecA* with the creation of a powerful hybrid promoter. This led to the speculation that the *S. sciuri* isolate had acquired IS*256* in a clinical environment where the activation of *mecA* had then been selected under drug pressure (59). In that same study, *mecA* activation in *S. sciuri* was obtained in vitro, and the −35 region of the hybrid promoter was the same as that previously identified as being responsible for the transcriptional activation of *llm*, a gene of *S. aureus* encoding a putatively membrane-associated protein that contributes to methicillin heteroresistance in this species in an as-yet-unknown manner (159).

A variation on the theme of hybrid promoter formation has been found in *S. aureus* in connection with the IS*257*-dependent effects on the levels of trimethoprim resistance resulting from the association of a constant, IS-borne −35 region with variable −10 regions upstream from the resistance gene. Trimethoprim resistance in *S. aureus* occurs at low or high levels (with MICs of 50 to 300 μg/ml or ≥1,000 μg/ml, respectively) and is mediated by the dihydrofolate reductase gene *dfrA*, which resides in the center of a three-gene operon carried by Tn*4003* (or Tn*4003*-like elements), a composite transposon flanked by three copies of IS*257* (138, 225). The promoter of this operon overlaps the right end of the left copy of the element IS*257*L, with its −10 sequence located in the central region of the transposon and the −35 sequence in the right terminus of IS*257*L. Low-level resistance was found to be associated with various deletions that extend ca. 10 to 300 bp away from the right end of IS*257*L and unmask alternative −10 regions differing in sequence or distance to the −35 box, or both, from the corresponding region in the high-level resistance-conferring form of the transposon. Such deletion variants exist in *S. aureus* as well as in coagulase-negative staphylococci. It is believed that IS*257* itself is involved in the generation of the flanking deletions and that the transposon variants that carry them may have established themselves by imposing less strain on the fitness of their hosts while conferring levels of resistance that are still advantageous (138). IS*257* has also been found to affect the level of *tetA*(K)-dependent efflux-mediated tetracycline resistance in *S. aureus* (237). Analysis of an IS*257*-flanked cointegrated copy of a *tetA*(K)-carrying, pT181-like plasmid in the *mec* region of a methicillin-resistant strain of *S. aureus* revealed the replacement of the −35 region of *tetA*(K), in the nonintegrated form of the plasmid, by the more efficient IS-borne counterpart (the same as in IS*257*L of Tn*4003*); in addition, the existence of a complete promoter was detected in the right extremity of IS*257*, which was, however, less powerful than the hybrid promoter. The combined strength of the complete and the hybrid promoter in the cointegrate, compared to that of the single promoter in the autonomous plasmid, was determined to lead to substantially higher levels of tetracycline resistance as well as relative fitness in the presence of tetracycline at low concentrations (237). Apart from affecting the levels of resistance to trimethoprim and tetracycline, as well as methicillin (see below), IS*257* has been found to be associated with genes conferring resistance to antibiotics of five additional classes and is suspected to provide hybrid promoters for the aminoglycoside and mupirocin resistance genes *aadA* and *mupA*, respectively. In light of the involvement of IS*257* in the capture and expression of resistance genes in staphylococci, its impact on the assembly of multiresistance gene clusters has been likened to that of the integrons in gram-negative bacteria (81).

(b) Resistance gene activation by IS-mediated formation of complete promoters. Many IS elements provide complete promoters for resistance genes (Table 2). The contribution of such a promoter by IS*Ecp1* to the expression of CTX-M-type extended-spectrum β-lactamase genes has been reported in several instances. The suggested promoter for $bla_{\text{CTX-M-15}}$ on the right end of IS*Ecp1* (122) as well as the suggested mode of IS*Ecp1*-supported gene mobilization by one-ended transposition (241a) have been validated experimentally for $bla_{\text{CTX-M-17}}$ (41) and for $bla_{\text{CTX-M-19}}$ (201, 203). Considering that IS*Ecp1* or IS*Ecp1*-like elements are present upstream from genes of multiple other CTX-M- and also CMY-type enzymes in various species of *Enterobacteriaceae* (see references 36, 73, 150, and 203 and references therein), this element may be among those most largely involved in the expression of extended-spectrum β-lactamase genes.

A complete promoter on the left end of IS*1999* was suggested to drive the transcription of *oxa-48* in an isolate of *K. pneumoniae* in which the corresponding extended-spectrum class D enzyme, OXA-48, contributed to carbapenem resistance (202). Also, in *P. aeruginosa*, this same promoter was present upstream of the experimentally determined site of the initiation of transcription of $bla_{\text{VEB-1}}$ (22). In this case, IS*1999* (which was found to coexist with $bla_{\text{VEB-1}}$ frequently in *P. aeruginosa* but rarely in *Enterobacteriaceae*) and the adjacent β-lactamase gene were located inside a chromosome-borne integron. The IS-borne promoter, which matches the −35 consensus sequence only poorly (at one out of six positions), was shown to slightly increase the efficiency of the integron-specific promoter Pc (see below) by a factor of 1.6. There was no such increase when a second element, IS*2000*, was inserted between IS*1999* and $bla_{\text{VEB-1}}$, an arrangement observed in some ceftazidime-resistant, VEB-1-producing clinical isolates of *P. aeruginosa* (22).

Two distinct promoters, one complete and one almost complete and with different spacing, have been found on the left end of IS*Pa12* upstream from the transcriptional start sites of the extended-spectrum β-lactamase gene $bla_{\text{PER-1}}$ in strains of *P. aeruginosa* and *Salmonella enterica* serovar Typhimurium, respectively. In the case of the *S. enterica* serovar Typhimurium strain, the −10 region overlapped the left inverted repeat and the direct repeat of the element (200). This observation would suggest that, depending upon the nucleotide sequence at the site of its insertion, IS*Pa12* may have the capacity to promote the expression of resistance genes with variable efficiencies.

The expression of the AmpC gene in *Acinetobacter baumannii* has been found to vary with the absence or presence of IS*Aba1*, or closely related elements, immediately upstream

from the gene (57, 234). An outward-directed promoter was identified after mapping of the transcription initiation site (234), and its strength was determined to be approximately 10-fold greater than that found in the absence of the IS element (105). The IS*Aba1*-borne promoter notably accounted for high-level resistance to ceftazidime (57, 105).

In the gram-negative anaerobe *Bacteroides fragilis*, the species-specific, endogenous cephalosporinase gene *cepA* is present in over 90% of the members of the species but is expressed at either low or high levels, with ca. 15- to 100-fold differences in the MICs of ampicillin for the two categories (197, 222). These differences have largely been explained by increased levels of *cepA* transcription in the highly resistant strains due to a modified promoter structure resulting from the insertion, ca. 50 nucleotides upstream from the translational start codon, of IS*1224*, an IS*21*-like element (222). In *Bacteroides*, the consensus sequences of the two promoter regions (−33, TTTG; −7, TANNTTTG) do not conform to those of the corresponding −35 and −10 regions recognized by typical σ^{70} factors and do not appear to require as strict a spacing (29), a situation in keeping with the existence of a particular primary sigma factor in the *Bacteroidetes phylum* (259). In all high-level but not in the low-level ampicillin-resistant *B. fragilis* strains analyzed, a TTTG sequence was present at the *cepA*-proximal extremity of a remnant of the IS*21*-like element and observed with appropriate spacing with respect to the TAcc TTTG (c, nonconsensus nucleotide) region, thus contributing to the formation of a hybrid promoter (222). With the exception of *cepA* and the *tet* genes, most other resistance genes in *B. fragilis* are efficiently expressed when transcription is driven by complete, IS-borne promoters. This is the case for *cfiA*, the *nim* genes, and *ermF*/*S*, conferring resistance to the β-lactam antibiotics including the carbapenems, the nitroimidazoles, and the macrolides, lincosamides, and streptogramins B (MLS_B), respectively. The activation of a silent *cfiA* gene by the *Bacteroides*-type promoter of IS*1186* was first demonstrated in vitro (198) and was also later reported to occur similarly (with an IS*942*/IS*1170*-related element) in vivo during imipenem therapy (75). In virtually all *B. fragilis* strains with MICs of imipenem of ≥16 μg/ml, IS insertions of great diversity have been found in a region of less than 100 bp upstream from *cfiA* involving over a dozen elements, or isoforms thereof, belonging to at least four families (Table 2) (http://www-IS.biotoul.fr). Curiously, these elements may also carry, next to the *Bacteroides*-type promoter regions, typical −10, −35 sequences (199), which, in the case of IS*942* and IS*1187*, have been shown to drive reporter gene expression in *E. coli* (D. Vingadassalom, unpublished data). A similar array of IS elements has been found upstream from the *nim* genes, and, by analogy but without experimental verification, it is assumed that they also contribute to their expression. IS*4351* (which may also activate *cfiA*) provides the promoter for *ermF*/*S* carried by the composite transposon Tn*4351*, but not all strains with *ermF*/*S*-mediated macrolide resistance harbor this IS element (197, 213).

(ii) Disruption of resistance-modulating genes. There is a variety of examples of insertional inactivation by IS elements of genes encoding proteins that modulate, in one way or another, the efficiency of a given resistance mechanism. These proteins include negative regulators of resistance genes in the strict sense or of multidrug efflux pump genes mediating nonspecific resistance. Other resistance-modulating proteins that may be affected are porins, which condition antibiotic influx across the outer membrane in gram-negative bacteria, or rare proteins without a clearly established function.

In gram-negative bacteria, inducible β-lactam resistance due to the production of the cephalosporinase AmpC is controlled by a complex regulatory circuit involving (next to the transcriptional regulator AmpR and the permease AmpG) AmpD, an amidase affecting the intracellular levels of the muropeptide that conditions the regulatory status of AmpR (116). It had been shown previously that impaired AmpD function leads to the derepression of *ampC* expression. High semiconstitutive *ampC* expression resulted from the spontaneous insertion of IS*1* into *ampD* of a strain of *E. coli* into which the *ampR* and *ampC* genes from *Citrobacter freundii* had been introduced (148). A comparable insertion event occurred in ceftazidime-resistant clinical isolates of *P. aeruginosa* with stably derepressed AmpC production in which IS*1669* had disrupted the AmpD gene (24).

Expression of the *mecA* gene, encoding the low-affinity PBP2a responsible for methicillin resistance in staphylococci, may be connected to the presence of a regulator region upstream that contains *mecR1* and *mecI*, the divergently transcribed genes of a sensor-transducer and a *mecA* repressor, respectively (171). IS-mediated rearrangements of the regulator region involving IS*1272* or IS*431* have resulted in the deletion of *mecI* and various sections of *mecR1*. As shown in spontaneous mutants selected in the laboratory, these rearrangements may lead to heterogeneous methicillin resistance (123). The particular deletion configurations characterize three of the five classes of the so-called *mecA* gene complex, which occur with different frequencies in *S. aureus* or coagulase-negative staphylococci (123, 126).

In *P. aeruginosa*, expression of the three-component efflux pumps of the RND family is negatively controlled. Repressor gene disruption leads to resistance phenotypes that depend on the substrate specificity of the corresponding pump. Disruption by IS*21* of *mexR*, which controls the expression of the *mexAB-oprM* operon (241), was found in a clinical ticarcillin- and aztreonam-resistant *P. aeruginosa* isolate in which eight- and fourfold-higher MICs of the respective drugs were associated with a threefold-higher level of *mexA* transcripts in comparison with a control strain containing intact *mexR* (37). The *mexCD-oprJ* operon, controlled by *nfxB*, does not appear to be substantially expressed under normal growth conditions. Its expression was triggered, in a *mexB*/*mexXY*-deficient mutant subjected to growth in the presence of tigecycline, by the disruption of *nfxB* by an unnamed IS element of *P. aeruginosa*, demonstrating the capability of MexCD-OprJ to pump out the minocycline analog and to afford, in this particular genetic background, a 16- to 32-fold increase in the MIC of the compound (61).

Expression of the multidrug efflux pump AcrAB in *E. coli* is negatively regulated by *acrR* and is also controlled by the *marRAB* locus (184). A discrete, ca. 1.5-fold increase in *acrB* transcription was accompanied by a similar increase in the MICs of fluoroquinolones and β-lactams in a *mar* deletion mutant in which an insertion of IS*186* into *acrR* had occurred after exposure to ofloxacin (119).

Bacterial susceptibility to antibiotics, notably to β-lactams,

can be altered directly by gene disruption when IS elements insert into the structural genes of porins. This was first shown in *K. pneumoniae* isolates with a disrupted *ompK36*, the OmpC gene homolog of the species (106). The IS*1*, IS*5*, IS*26*, or IS*903* element was found, in nine randomly in vitro-selected cefoxitin-resistant derivatives, at various positions within the plasmid-borne porin gene that had been introduced into an OmpK36-deficient host, while in a collection of cefoxitin-resistant clinical isolates (some of them from patients undergoing therapy), *ompK36* was inactivated by an IS*5*-like element in three isolates and by IS*102* in one isolate (107). A similar gene disruption caused by IS*26* in a CTX-M-1-producing *K. pneumoniae* isolate led to carbapenem resistance (172). Porin gene inactivations were previously described as being responsible for carbapenem resistance in clinical isolates of *P. aeruginosa* and *A. baumannii*. In *P. aeruginosa*, carbapenem diffusion through the outer membrane is facilitated by OprD2 (252). In several multiple-drug-resistant isolates of this species, with MICs of imipenem of 16 to 32 μg/ml, the corresponding gene was disrupted at various sites by IS*Pa1328* or IS*Pa1635*, leading to the absence of OprD production and also to the down-regulation of *oprD* transcription (268). In *A. baumannii*, carbapenem resistance may be associated with the loss of an outer membrane protein termed CarO, which has been suggested to be a functional analog of OprD (147, 175). Among a collection of CarO-deficient isolates, the corresponding gene was found to be disrupted in two isolates by IS*Aba825* or IS*Aba125*, giving credence to the suggested role of CarO in the diffusion of carbapenems (175).

In strains of *Enterococcus faecium* and *S. aureus*, IS elements have been shown to influence glycopeptide resistance. In enterococci, acquired resistance of the VanA, VanB, and VanD type depends on the production of peptidoglycan precursors with a D-Ala–D-Lac instead of the D-Ala–D-Ala terminus, which forms a complex with the glycopeptides in susceptible strains (194). While VanA- and VanB-type resistance is inducible, critically requiring the chromosome-encoded ligase Ddl (for D-Ala–D-Ala synthesis) and the resistance operon-encoded regulatory proteins VanR and VanS, VanD-type resistance to vancomycin and teicoplanin in *E. faecium* is constitutively expressed. In strains with this resistance phenotype, both *ddl* and *vanS* have been found to be mutationally altered, entailing the absence of all D-Ala–D-Ala incorporation into the membrane-associated peptidoglycan precursor along with sustained activation of the resistance genes ($vanH_DDX_D$) by VanR, which is only slowly dephosphorylated in the absence of VanS. Either gene has been found to be disrupted by an insertion element: *ddl* by IS*19*, also called IS*Efm1* (38, 193), and $vanS_D$ by IS*Efa4* (67).

In intermediately glycopeptide-resistant strains of *S. aureus*, resistance is not specified by a defined set of acquired genes but, rather, is due to the accumulation of mutations in an array of genes controlling mainly cell wall metabolism and composition (33). One of these genes, *tcaA*, encodes a putative transmembrane protein that might act as a sensor or a signal transducer. Although up-regulated in the presence of teicoplanin, it is the absence of the gene that causes a decrease in glycopeptide susceptibility. Disruption of *tcaA* by IS*256*, accompanied by increased glycopeptide resistance, was found in a spontaneous derivative of a glycopeptide-intermediate *S. aureus* isolate (157).

IS-mediated gene disruption leading to pyrazinamide resistance in *Mycobacterium tuberculosis* has been reported in one case. In this species, the susceptibility to pyrazinamide is linked to the production of the *pncA*-encoded enzyme pyrazinamidase, which transforms the drug into a bactericidal derivative (104). Analysis of 19 pyrazinamide-resistant isolates revealed that the absence of pyrazinamide activity in one of them was due to the insertion of IS*6110* into *pncA* and that this insertion had occurred into the preferential 10-bp target site of the element that is present in the gene (139).

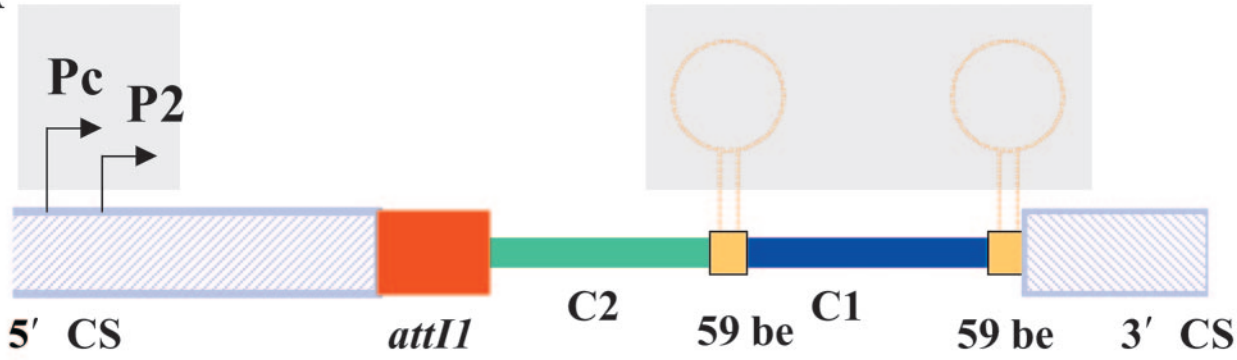
To a large extent, the instances of IS effects on resistance gene expression and on resistance levels reviewed here represent observations of individual cases. Few molecular epidemiological studies seem to have been attempted to determine the frequencies at which the identified elements are involved in the resistance process and to quantify their true impact in the clinical setting [which potentially also includes the abolition of resistance, as observed in an aminoglycoside-susceptible strain of *Acinetobacter haemolyticus* with its species-specific *aac(6′)-Ig* gene disrupted by IS*17* (228) or as suspected in a strain of *K. pneumoniae*, with its integron cassette-borne *cmlA* and *oxa-10* genes disrupted by two putative IS elements (257)]. Considering the multiplicity of IS elements and the diversity of resistance-related targets into which they have been found to insert spontaneously, under natural or experimental conditions, a larger involvement than is obvious from the individual descriptions would not be surprising, nor would the future description of known or novel elements as being capable of rendering existing mechanisms of resistance more efficient.

Modulation of Resistance Gene Expression in Class 1 Integrons

General characteristics of integrons. Integrons are genetic elements that are able to capture genes on small mobile elements, called cassettes, in a process of site-specific recombination (95, 214). These elements comprise, as their characteristic features, a recombinase gene (*intI*), a recombination site (*attI*), and a promoter (Pc) that drives the expression of the generally promoterless cassette-associated genes (Fig. 9A) (54, 96, 141). The integrase catalyzes the recombination between *attI* and the cassette-associated recombination site, called the 59-base element (59-be) or *attC*, at the recombination point that lies between the G and the first T of the core site sequence GTT RRRY (190, 214, 244).

Integrons have been assigned to several classes depending upon their *intI* sequences and subdivided into two categories, the mobilized integrons and the chromosomal integrons (53, 95, 96, 169, 226). Cassettes that mediate antibiotic resistance are typically found in the mobilized integrons, but chromosomal integrons may also provide or capture such cassettes (53, 226). Integrons of class 1 are the most abundant and exist in a great number of gram-negative genera (82, 168). They reside, although not exclusively, on transposons and conjugative plasmids, accounting for their wide distribution and their significant association with a multiresistance phenotype in *Enterobacteriaceae* (82, 140). The cassettes in this integron class encode a variety of enzymes, aminoglycoside-modifying enzymes, dihydrofolate reductases, β-lactamases, and chloram-

A



B

Promoter variant	Relative strength[a]	*aadA*-mediated streptomycin resistance[b]
Pc strong	6.5	1,000
Pc weak	0.2	65
Pc hybrid	0.7	-
Pc weak + P2	3.2	360

C

Cassette order[a]	IC_{50} (μg/ml)[b]		
	Sm	Gm	Km
aadA2-*aacC1*-orfE	**1,120**	11.3	-
aacC1-***aadA2***	**580**	55	-
aacC1-orfE-***aadA2***	**60**	45	-
aacA4-*aadA2*	310	-	**170**
aadA2-***aacA4***	1,220	-	**115**
aadA2-orfE-***aacA4***	1,250	-	**50**

FIG. 9. Transcriptional control in class 1 integrons. (A) Schematic representation of the integron platform. 5′ CS and 3′ CS, 5′- and 3′-conserved segments, respectively; Pc and P2, promoter regions (see the text); *attI1*, recombination site; C1 and C2, gene cassettes; 59 be, 59-base-pair elements with possible stem-loop structures. (B) Relative strength of integron-borne promoter variants. [a]Determined relative to the strength of the *tac* promoter, set at 1 (data are from reference 141). [b]Streptomycin concentration at which 50% of cells plated formed colonies (data are from reference 54). (C) Effects of cassette order on resistance levels. [a]Resistance is conferred to streptomycin (Sm) by *aadA2*, to gentamicin (Gm) by *aacC1*, and to kanamycin (Km) by *aacA4* (the genes for which a position effect is observed and the corresponding antibiotic concentrations at which 50% of cells plated formed colonies [IC_{50}s] are shown in boldface type). [b]IC_{50} data are from reference 54.

phenicol acetyltransferases; they may also encode nonenzymatic chloramphenicol resistance. More recently identified cassettes are associated with genes that mediate resistance to rifampin and quinolones and increasingly also with genes encoding extended-spectrum β-lactamases and carbapenemases (82, 88, 136, 200, 203, 208, 261, 264).

Transcriptional control of resistance gene expression in class 1 integrons. Transcription of cassette-associated resistance genes is controlled twofold. While the transcription of probably all promoterless genes in a cassette array is driven by the Pc promoter, four variants of which exist, the expression of genes in the second position and further downstream is additionally conditioned by putative 59-be-encoded transcription terminators with probably low efficiency (Fig. 9A) (54).

(i) Impact of the integron-borne promoter region. The strengths of three variants of Pc (which was initially called P1 or Pant) and of a secondary promoter, P2, located 252 to 223 bp and 133 to 107 bp, respectively, from the recombination point, have been measured relative to the strength of the *tac* promoter or as reflected in antibiotic resistance levels (54, 141). The three variants of Pc, with the sequences TTGACA/TAAACT, TGGACA/TAAGCT, or TGGACA/TAAACT in their −35/−10 regions (separated by a 17-bp spacer), were categorized as "strong," "weak," or "hybrid," respectively, and vary in strength by a factor of approximately 30 (141). The weak form may be associated with the secondary promoter, P2 (TTGTTA/TAAGCT), which is active when carrying a 3-bp insertion in its otherwise 14-bp spacer. When the transcription of *aadA2* (or *aadA1*) was driven by the strong or the weak form of Pc, or by the combination of Pc[weak] and P2, there was good agreement between the relative promoter strengths and the resistance levels (Fig. 9B). Primer extension mapping of the transcription start sites revealed that when the combination of Pc[weak] and P2 is functional, the majority of the transcripts originates at P2, confirming the finding that, in this configuration, P2 contributes 90% of the total promoter activity (54, 141). The combination of Pc[strong] and P2 promoters has been observed recently, but its strength has not been determined (208).

When a BLAST search was carried out for the class 1 integron sequences that cover the distance between the −35 region of Pc and the inner boundary of the 5′-conserved segment and that are identical with the nucleotide sequences specifying any one of the four promoter variants Pc[strong], Pc[weak], Pc[hybrid], and Pc[weak] plus P2, it appears that among more than 100 retrieved sequences, there are quite similar numbers of each variant. Although this information cannot be taken as true molecular epidemiological data, it would suggest that neither promoter variant may be of singular advantage for resistance gene expression. The same search failed to reveal clearly preferential associations between any of the promoter variants and individual cassette-associated genes inserted at *attI1*, although there may be a tendency for carbapenemase genes of the bla_{IMP} type to occur more frequently downstream from Pc[strong] or Pc[weak] and of the bla_{VIM} type to occur more frequently downstream from Pc[hybrid].

(ii) Impact of the cassette-borne 59-be. The expression of promoterless, cassette-associated resistance genes is markedly influenced by their position in a cassette array. Using a series of plasmid constructs in which the transcription of the cassette genes is driven from the same promoter (in this case, Pc[strong]), and as exemplified in Fig. 9C, Collis and Hall showed that the resistance level conferred by a given gene is highest when it is promoter proximal and that this level is reduced, by factors of generally between 2 and 5, when a cassette is present upstream (54). It was suggested that this modulation of resistance gene expression occurs essentially at the level of transcription and that it is linked to properties of the 59-be-containing 3′ ends of the cassettes, since these ends were found to coincide roughly with the 3′ ends of the major mRNA transcripts of the cassettes. The possibility was put forward that the 59-base elements, which generally contain inverted repeats, function as transcription terminators (54). Substantial silencing of a downstream gene, as of *aac(6′)-Ib* downstream from bla_{IMP-1}, may be observed when there is the potential for the formation of a stable stem-loop structure, although in this case, silencing might alternatively, or in addition, be due to poor translation if one considers the presence of a ribosomal binding site of only three nucleotides (8). Full silencing has also been reported, as for *oxa-9* downstream from the *cmlA-2* cassette in In40 (196).

(iii) Transcription independent of integron-specific sequences. The expression of some cassette-associated resistance genes is driven by promoters other than Pc. This was first shown for the *cmlA* cassette of In4, specifying nonenzymatic chloramphenicol resistance, which contains a cluster of three overlapping promoter sequences (34), and later also for several *cmlA* cassette variants (176, 196, 204). (The control of *cmlA* expression at the translational level is described below in the section on translational attenuation.) A promoter region upstream from, but apparently not part of, the resistance gene cassette has been reported for the fused *oxa-10–aadA1* cassette of In53 (176). In complex class 1 integrons, various resistance genes are found downstream from the orf513-containing common region, CR1, an atypical class of insertion sequence now called IS*CR1* (189, 251). An involvement of this region in gene expression could be suspected, e.g., from the observation that $bla_{CTX-M-2}$ downstream from IS*CR1* confers high levels of β-lactam resistance to *Enterobacteriaceae* (9), while the closely related, chromosome-encoded class A β-lactamase gene, bla_{KLUA}, from which $bla_{CTX-M-2}$ is speculated to have been derived, confers only low levels of resistance to its host, *Kluyvera ascorbata* (115). IS*CR1*-borne promoter sequences have been identified recently for *qnrA*, $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, and *dfrA10* in various gram-negative bacteria (160, 220). A comprehensive compilation of the genes associated with IS*CR1* has been published in a recent review (251).

Translational control of gene expression in class 1 integrons. Efficient gene translation normally requires the presence of a translation initiation region (TIR) consisting of the initiation codon, a Shine-Dalgarno (SD) sequence, and an adequate spacer between them. Typically, the initiation codon ATG (or, less frequently, GTG or, rarely, TTG) is separated by ca. 5 to 15 nucleotides from an SD sequence, made up of any four or more nucleotides (but maybe as few as three) within the sequence AAGGAGG (48, 93, 130). While a canonical TIR is found in the majority of the resistance gene cassettes, examination of published sequences reveals that their presence is not the rule, and in some cases, the mechanism

of translation initiation remains obscure.

The absence of a TIR in cassettes inserted at *attI* of class 1 integrons can be compensated for by the coincidence of two circumstances. The first is the (constant) presence of a short ORF, overlapping the inner boundary of the 5′-conserved segment of the integron, which has an SD sequence (GGAG) eight nucleotides upstream from its initiation codon; the second is the (frequent) occurrence of a stop codon, TAG more often than TAA, at positions 3 to 5 (underlined) in the GTT RRRY core site of the cassette-associated 59-base element. Under these circumstances, insertion of the cassette with the formation of the composite *attI1* site (190) results in the placement of a stop codon in phase with the reading frame of the short ORF, which then has a coding capacity for a peptide of 11 amino acids and which, for that reason, has been termed ORF-11 (Fig. 10A) (98).

A role has been assigned to ORF-11 in the initiation of translation of a TIR-deficient, cassette-associated *aac(6′)-Ib*-type gene conferring resistance to aminoglycosides (98). It was shown that the deletion of ORF-11, with or without maintenance of the 20-bp region connecting its stop codon to the initiation codon of the resistance gene, reduced the efficiency of translation by over 80%, while the deletion of the connecting region alone had no effect (Fig. 11). Considering also that replacement of either the SD sequence or the initiation codon of ORF-11 with noncanonical sequences reduced the translation efficiency by two-thirds, these results were taken to support the role of ORF-11 as a strong enhancer of the initiation of translation of TIR-deficient genes. Whether the residual translational activity of close to 20% that was observed under experimental conditions in the absence of ORF-11 and of any recognizable TIR feature is of relevance in vivo is unclear. Since there was no evidence for a specific function of the ORF-encoded peptide, the translation of the resistance gene would be dependent on the translation of ORF-11 per se and, as such, should be considered as coupled. However, the precise coupling mechanism that operates in this context has not been determined.

Inspection of recently published sequences supports the assumption made previously that the expression of at least one-quarter, and maybe more, of the cassette-associated genes inserted at *attI1* profits from the presence of ORF-11 via translational coupling. This process has been found not to be impeded substantially by an artificial increase in distance to up to ca. 50 nucleotides between the stop codon of ORF-11 and the translation initiation codon of the resistance gene, a distance that is rarely exceeded in naturally occurring cassettes (98).

Cassette integration at *attI1* has also resulted in the generation of an alternative ORF with its coding capacity extended to 18 amino acids (ORF-18), as first evidenced in the nucleotide sequence of the *oxa-1* cassette (186). In this case, the TIR and the first 11 amino acids are identical to those of ORF-11, but the stop codon, separated from the translation initiation codon of the oxacillinase gene by a stretch of 45 nucleotides, is apparently not provided by *attC*. Not surprisingly, this configuration is the same in a cassette named *oxa-30* (*oxa-1* and *oxa-30* are identical) (245), in an *oxa-31* cassette, and in an *aac(6′)-Ib* cassette, which encodes an acetyltransferase fused to the N-terminal amino acids of OXA-1 (23, 44, 238). ORF-18 promotes translation of the downstream gene in a way similar to that of ORF-11. Its deletion abolished the translation of *oxa-1* fully, as measured in terms of β-lactamase activity and ticarcillin resistance, while the introduction of an SD sequence (GGAG) in the deletion mutant upstream from the oxacillinase gene restored enzyme production and β-lactam resistance to their original levels (B. Berçot, unpublished data).

Several cassettes have borrowed the TIR of ORF-11 directly after an in-frame fusion between the resistance gene and the ORF brought about by small sequence duplications or deletions. Such events have occurred in cassettes with associated aminoglycoside 3-*N*- or 6′-*N*-acetyltransferase genes in gram-negative bacteria and dihydropteroate synthase genes in *Mycobacterium fortuitum* and *Corynebacterium striatum* (Fig. 10B) (163, 173, 267; M. C. Ploy, unpublished data) (GenBank accession number AJ294721). Whether the common presence of ORF-11 and its TIR has any impact on the efficiency of translation of the genes that have their own canonical SD sequence is not clear (Fig. 10C), but the possibility that it augments the recruitment of ribosomes in the vicinity of the initiation codons of these genes could be imagined.

Since the integrons of classes 1, 2, and 3, i.e., those predominantly bearing resistance gene cassettes, possess similar Pc promoters (there is an 18-bp spacer in class 2) at equivalent positions and since the cassettes are considered to be exchangeable between classes (55, 101), it would seem likely that resistance gene transcription in all classes is similarly subject to the mechanisms of control described for class 1 integrons (54, 141). Whether the potential for enhanced translation of cassette-associated genes in class 1 integrons is a trait that has contributed to their predominance over integrons of the other two classes remains a matter of speculation (53, 98).

POSTTRANSCRIPTIONAL (TRANSLATIONAL) ATTENUATION

Control of mRNA translation is a widespread mechanism of regulation in both prokaryotes and eukaryotes that often complements transcriptional regulation. Posttranscriptional (or translational) attenuation controls the inducible expression of several resistance genes, and this mechanism has been particularly well studied for the *erm* and *cat* genes, which are responsible for resistance to macrolides by ribosome methylation and inactivation of chloramphenicol by production of a chloramphenicol acetyltransferase, respectively (72, 263). A similar mechanism regulates the inducible *cmlA* gene that controls permeability to chloramphenicol and is part of class 1 integrons (see above) (243). The regulation of expression of antimicrobial resistance results from an interplay between the ribosome, a short peptide sequence encoded by the mRNA, and the antibiotic. The following paragraphs will focus on the regulation of macrolide resistance by ribosome methylation because of its major clinical significance.

Inducible Expression of Macrolide Resistance

The *erm*(C) paradigm. Resistance to macrolides emerged in staphylococci in 1956 soon after the introduction of erythromycin into therapy (45, 87). Biochemical studies indicated that resistance was due to the methylation of the ribosomal target of the antibiotics, which yielded a broad-spectrum cross-resis-

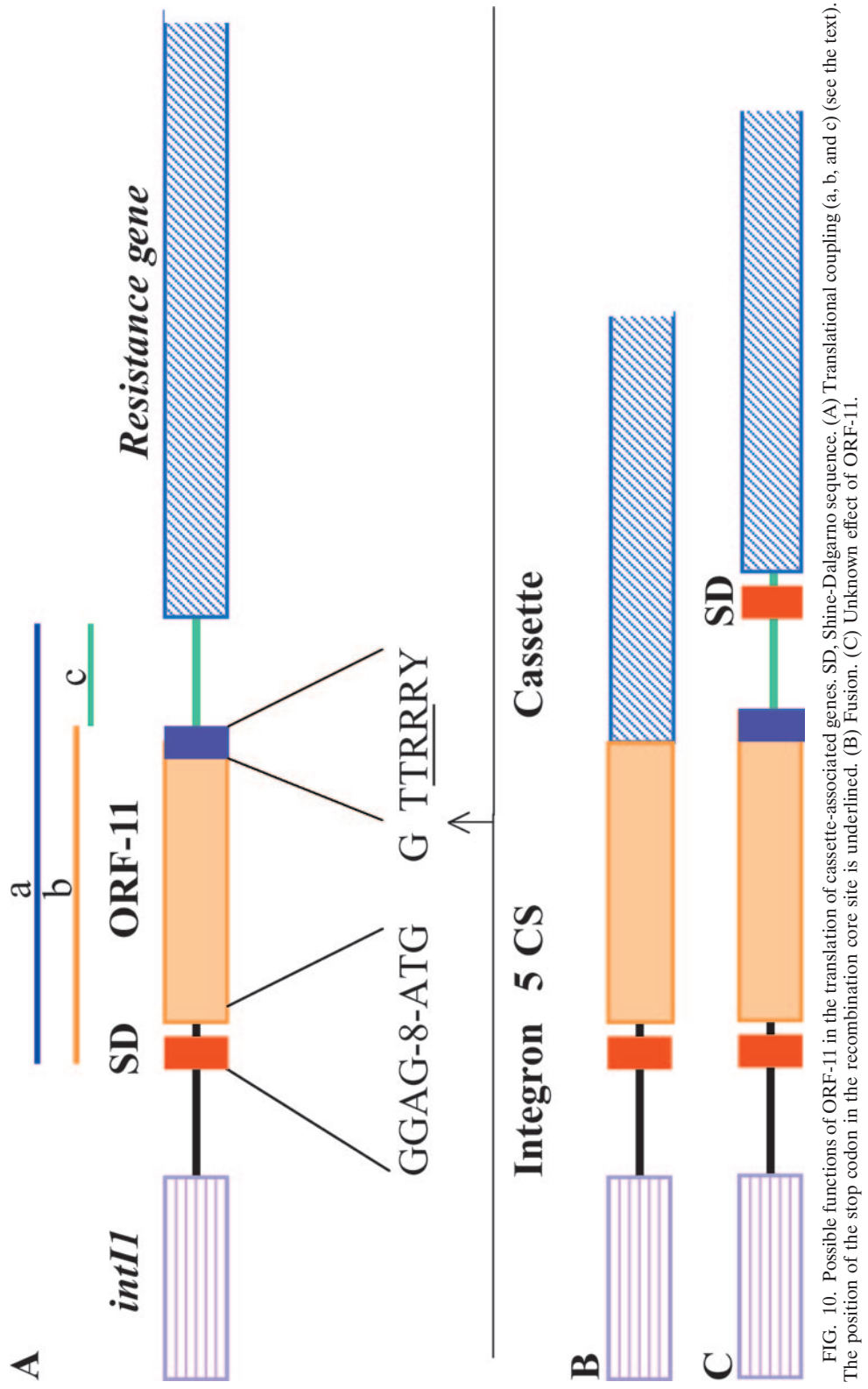


FIG. 10. Possible functions of ORF-11 in the translation of cassette-associated genes. SD, Shine-Dalgarno sequence. (A) Translational coupling (a, b, and c) (see the text). The position of the stop codon in the recombination core site is underlined. (B) Fusion. (C) Unknown effect of ORF-11.

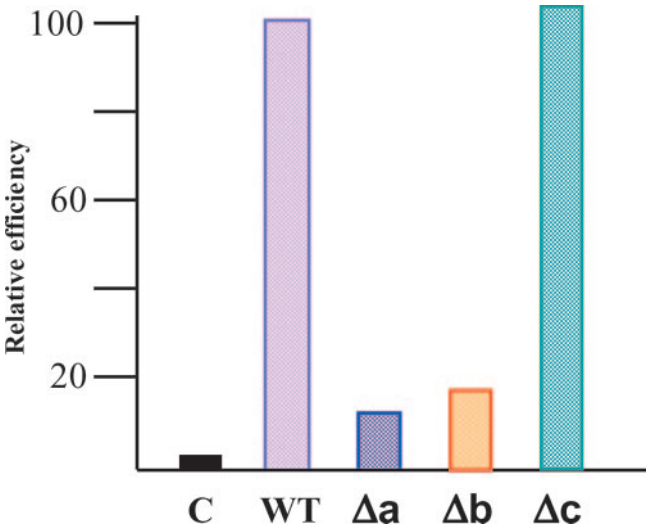


FIG. 11. Relative ORF-11-mediated translation efficiencies. C, control; WT, configuration depicted in Fig. 10A (wild type); Δa, Δb, and Δc, mutants with deletion of fragments a, b, and c as shown in Fig. 10A (data are from reference 98).

tance to macrolides, lincosamides, and streptogramins B, the so-called MLS_B phenotype. Subsequently, the MLS_B phenotype was reported in a large number of microorganisms and was found to be encoded by a variety of *erm* (erythromycin ribosome methylase) genes. Erm methylases make up a family of highly related proteins that use *S*-adenosylmethionine as a methyl donor to mono- or dimethylate a single adenine residue (A2058 [*E. coli* numbering]) in nascent 23S rRNA. The A2058 residue is located within a conserved region of domain V of 23S rRNA and plays a key role in the binding of MLS_B antibiotics. As a consequence of 23S rRNA methylation, the binding of erythromycin to its target is impaired. Cross-resistance between all macrolides, lincosamides, and streptogramins B (pristinamycin I and quinupristin) occurs because of overlapping binding sites in 23S rRNA (263).

It rapidly appeared that resistance to macrolides occurred with two major phenotypes. Acquisition of an *erm*(A) or *erm*(C) determinant in staphylococci yields either dissociated resistance to macrolides with susceptibility to lincosamides or cross-resistance to macrolides and lincosamides, corresponding to inducible and constitutive expression of MLS_B resistance, respectively. Inducible expression yields dissociated resistance to macrolides due to differences in the inducing ability of the antibiotics. The genetic basis for induction has been studied in detail in the case of the *erm*(C) gene of staphylococcal plasmid pE194 (92, 114). Strains harboring *erm*(C) are resistant to inducer macrolides such as erythromycin and its derivatives (azithromycin, clarithromycin, dirithromycin, and roxithromycin). In contrast, noninducer macrolides such as spiramycin (a 16-membered macrolide), lincosamides (lincomycin and clindamycin), and streptogramins B (pristinamycin I and quinupristin) remain active.

Early studies in the laboratories of B. Weisblum and D. Dubnau showed that induction arises posttranscriptionally according to the model of translation attenuation (92, 114, 263). *erm* mRNA is synthesized but in an inactive conformation and becomes active only in the presence of inducer macrolides. The inactivity of the mRNA is due to the structure of its 5′ end, which has a set of four inverted repeats that sequester the initiation sequences (ribosome binding site and initiation codon) for the methylase by base pairing in the absence of erythromycin (Fig. 12, conformation A). Thus, the methylase cannot be produced, since the initiation motifs for the translation of the enzyme are not accessible to the ribosome. Induction is related to the presence of an open reading frame, encoding a short 14-amino-acid peptide, upstream from the *erm*(C) structural gene. In the presence of low concentrations of erythromycin, the binding of the antibiotic to a ribosome translating the leader peptide causes the ribosome to stall. Ribosome stalling probably induces the destabilization of the two stem-loop structures of configuration A and other conformational rearrangements in the mRNA. In particular, the formation of the alternative stem-loop structure (Fig. 12, conformation B) would unmask the initiation sequences for the methylase, allowing synthesis to proceed by ribosomes that are not complexed with erythromycin or by those that are methylated. Methylation of some ribosomes might occur through transient rearrangements of the stem-loop structures, which would lead to the synthesis of a basal level of methylase. A third alternate mRNA conformation has been predicted, which could occur at the end of the induction process when the concentration of the inducer macrolide has decreased and the majority of ribosomes are methylated (263).

Three interrelated mechanisms contribute to the regulation of methylase production. The most important mechanism is mRNA stabilization that occurs during the induction process. This stabilization, a consequence of ribosome stalling, protects the transcripts from degradation by RNases (30, 230), leading to a spectacular increase in mRNA half-life that enhances enzyme synthesis. A feedback mechanism negatively regulates methylase synthesis: as the pool of methylated ribosomes increases during induction, fewer ribosomes are able to stall, and therefore, transcripts return to the inactive conformation. Finally, it has been shown in *Bacillus subtilis* that the Erm(C) methylase binds to its own mRNA at a site with structural similarity to the site of methylation in 23S rRNA. As a consequence, the methylase might block its own production when synthesized in excess (62).

The mechanism responsible for the specificity of induction remains poorly understood. It is not related to the class of *erm* gene but depends on the structure of the specific attenuator, which controls the expression of the *erm* gene, and, for a given attenuator, the structure of the MLS_B antibiotic determines whether a particular macrolide is an inducer or not (167). Probably, the interactions between the macrolide, the leader peptide, and the ribosome are critical for proper ribosome stalling, which is required for induction. In the *erm*(C) leader peptide, four amino acids, IFVI, are critical for induction (263). A similar sequence is found in specific small peptides encoded in *Escherichia coli* 23S rRNA by five-codon minigenes (248). These peptides can render cells resistant to low levels of a variety of macrolide antibiotics (247, 249). A "bottle brush" model of action for these macrolide resistance peptides, in which newly translated peptides interact with the macrolide molecule on the ribosome and actively displace it from its binding site, has been proposed (247). Probably, a similar type

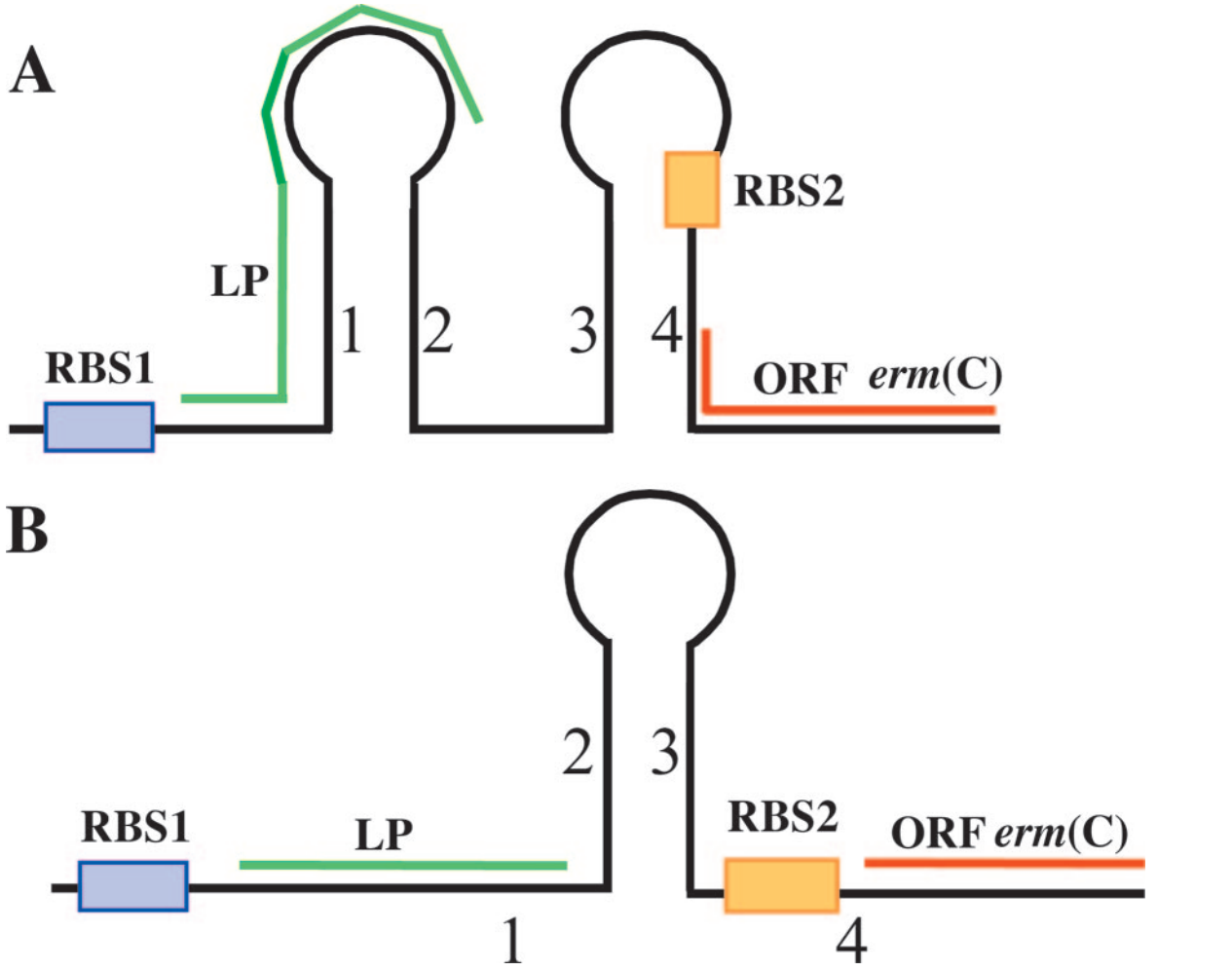


FIG. 12. Alternative conformations of the mRNA from the inducible *erm*(C) gene of pE194. Shown is the secondary structure of the mRNA in the absence (A) or presence (B) of erythromycin. RBS, ribosome binding site; LP, leader peptide; ORF, open reading frame; 1, 2, 3, and 4, inverted repeat. Green and red lines indicate the coding sequence.

of interaction between the leader peptide and macrolides can occur. It seems that the leader peptide could be the selector of the site of ribosome stalling in leader mRNA by *cis* interference with translation, as previously demonstrated for the leader peptides controlling the inducible expression of *cat* genes (221).

Considering the importance of the leader peptide sequence for specificity of induction, it is not surprising that certain mutations in this sequence lead to changes in the induction patterns. For instance, changes in the relative activity of erythromycin and lincosamides as inducers of *erm*(C) have been observed (167). These changes, obtained in the laboratory, are not common in clinical isolates.

Control of expression of other *erm* genes. Many other *erm* genes, including those detected in pathogenic bacteria, are also inducibly expressed. A model of posttranscriptional regulation similar to that described for *erm*(C) has been proposed for the regulation of *erm*(A) (mostly found in staphylococci) and *erm*(B) (mostly found in streptococci and enterococci). However, the regulatory regions of these determinants are more complex than that of *erm*(C). The attenuator of *erm*(A) contains two short control peptides, and induction may involve a series of rearrangements of the inverted repeats (174). The subset of the *erm*(A) class genes previously called *erm*(TR), which is mostly present in beta-hemolytic streptococci, has a similar attenuator structure. The 5′ end of *erm*(B) also presents a series of inverted repeats that are responsible for the lack of methylase synthesis in the absence of erythromycin (113). Fourteen pairs of repeats that could form alternative stem-loop structures by base pairing have been identified, and one of them might sequester the ribosome binding site and the initiation codon of the methylase gene. Induction would be related to the presence of sequences coding for a small leader peptide of 36 amino acids upstream from the gene.

The specificity of induction relies, as mentioned above, on the structure of the attenuator and on the precise mode of action of specific MLS compounds. Since the structure of the attenuator differs in each class or subclass of *erm* gene, different patterns of inducible MLS_B resistance are observed. For instance, spiramycin is a common inducer, like erythromycin, of *erm*(B) expression [whereas it is not an inducer for *erm*(C)

or *erm*(A)] (51). The genetic background and the bacterial host may also have roles in the specificity of induction, possibly in relation to differences in ribosomal structure or in methylase expression (164).

Again, changes in the sequence of the leader peptide lead to changes in the pattern of inducibility. For instance, a clinical isolate of *Enterococcus faecalis* that contains an R7C mutation in the putative leader peptide of its *erm*(B) gene is more strongly induced by tylosin, a 16-membered macrolide, than by erythromycin (183). Similarly, a clinical isolate of *S. aureus* with an unusual inducible cross-resistance to erythromycin, clindamycin, lincomycin, and quinupristin had mutations in the attenuator of the *erm*(A) gene (52).

Phenotypes of inducible MLS_B resistance. Due to the diversity of the *erm* genes and the specificity of the corresponding regulators, inducible expression of these genes gives rise to a large variety of phenotypes. However, in practice, since each class of *erm* gene is preferentially distributed in certain bacterial species, a few major phenotypes of inducible MLS_B resistance are observed in staphylococci and streptococci/enterococci.

In staphylococci, inducible expression of *erm*(A) or *erm*(C) leads to a similar dissociated phenotype of resistance. The strains are resistant to 14-membered (clarithromycin, dirithromycin, erythromycin, and roxithromycin) and 15-membered (azithromycin) macrolides, which are inducers. In contrast, the noninducer ketolide (telithromycin), 16-membered macrolides available in certain countries (josamycin, midecamycin, miocamycin, rokitamycin, and spiramycin) or in veterinary practice (tylosin), the lincosamides (lincomycin and clindamycin), and streptogramins B (pristinamycin I and quinupristin) remain active. In disk diffusion tests, the blunting of the clindamycin (or any noninducer macrolide) inhibition zone, as in a D-shaped zone, can be observed, provided that a disk of erythromycin is placed nearby (Fig. 13).

In streptococci, the inducible phenotypes are more diverse and complex. Several members of the MLS_B group, including erythromycin and its derivatives, and spiramycin are inducers at various degrees of ErmB methylase production (51). In addition, recent studies have demonstrated that in *Streptococcus pyogenes* and *S. pneumoniae* containing an inducible *erm*(B) gene, the ribosomes are partly methylated in the absence of induction (69). The variety of resistance phenotypes in inducibly resistant streptococci might thus be explained by the complex pattern of inducibility combined with enzyme production at various basal levels, probably in relation to a relaxed control of methylase synthesis by the *erm*(B) attenuator (223, 224). It has been shown, by induction studies including fusions of attenuators with a reporter gene, that the MLS_B phenotype characterized by high-level cross-resistance to macrolides and lincosamides, which is commonly detected in pneumococci, is frequently inducible (223).

Constitutive Expression of *erm* Genes

In the laboratory, constitutive expression of MLS_B resistance can be obtained in initially inducible strains of staphylococci by selection on agar plates containing inhibitory concentrations of a noninducer macrolide, lincosamide, or streptogramin B at frequencies varying between 10^{-6} and 10^{-8}, depending on the strain and the selector antibiotic. Clinical isolates that are constitutively resistant to erythromycin are widespread, especially in methicillin-resistant staphylococci. Whether in laboratory strains or in clinical isolates, deletion of the entire attenuator yields constitutive resistance; also, point mutations or tandem duplications in the attenuator lead to constitutive resistance by decreasing the stability of the hairpin structure sequestering the initiation sequences for the methylase or by duplicating the initiation signal-containing sequences, which are thus available for translation (231, 232, 233).

Similarly, in vitro selection of constitutive resistance at a frequency of 10^{-7} with clindamycin has been reported for a clinical isolate of *Streptococcus pyogenes* UCN1 that is inducibly resistant to erythromycin and that harbors an *erm*(A) [*erm*(TR) subset] gene. Clindamycin resistance was associated with deletions of 163 and 6 bp, which is probably explained by illegitimate recombination between different parts of the regulatory region, as well as a tandem duplication of 101 bp in the regulatory sequence of the *erm*(TR) gene (79).

Deletion of the attenuator has been found in constitutively resistant clinical isolates of *Staphylococcus epidermidis* and *S. aureus* containing *erm*(C) or *erm*(A) (134, 232, 265) and *Enterococcus faecalis*, *Streptococcus agalactiae*, and *S. pneumoniae* containing *erm*(B) (162, 224). In addition, point mutations in the attenuators of *erm*(T) of *Lactobacillus reuteri* (246) or of *erm*(A) of *S. aureus* (265) or tandem duplications in the attenuators of *erm*(C) of *S. aureus*, *Staphylococcus saprophyticus*, and *Staphylococcus equorum* and of *erm*(A) of *S. aureus* (102, 152, 185, 232, 266) have been reported.

Probably, constitutive MLS_B-resistant isolates have evolved from the inducibly resistant isolates under selective pressure by noninducer macrolide/lincosamide antibiotics. Constitutive production of a methylase confers a characteristic phenotype with cross-resistance to the MLS_B drugs, regardless of the nature of the *erm* gene (Fig. 14). However, the level of resistance may vary according to the degree of methylation of the ribosome. Although all members of the Erm family methylate the adenine of 23S rRNA located at position A2058, they differ in their capacities to monomethylate or dimethylate the nucleotide at this position. The major Erm methylases detected in pathogens, Erm(A), Erm(B), and Erm(C), generally function as dimethylases that confer high-level cross-resistance to MLS_B drugs (including telithromycin). However, it has recently been shown, using mass spectrometry to analyze the methylated DNA, that Erm(B) in a *Streptococcus pneumoniae* background monomethylates the 23S rRNA, which renders cells resistant to erythromycin and clindamycin but not to telithromycin (69). This explains, at least in part, why telithromycin is active against nearly all *S. pneumoniae* isolates containing *erm*(B) but is active against only a few *S. pyogenes* isolates containing that gene (69, 159).

Clinical Implications of Inducible MLS_B Resistance

What is the clinical evidence for failure of clindamycin treatment? As mentioned above, constitutive mutants can be readily selected in vitro from inducibly MLS_B-resistant strains in the presence of clindamycin. The frequency of mutation may be as high as 10^{-6} to 10^{-7} per parent cell. The notion that bacterial inocula exceeding 10^7 CFU can be found in mediastinitis and in certain lower respiratory tract

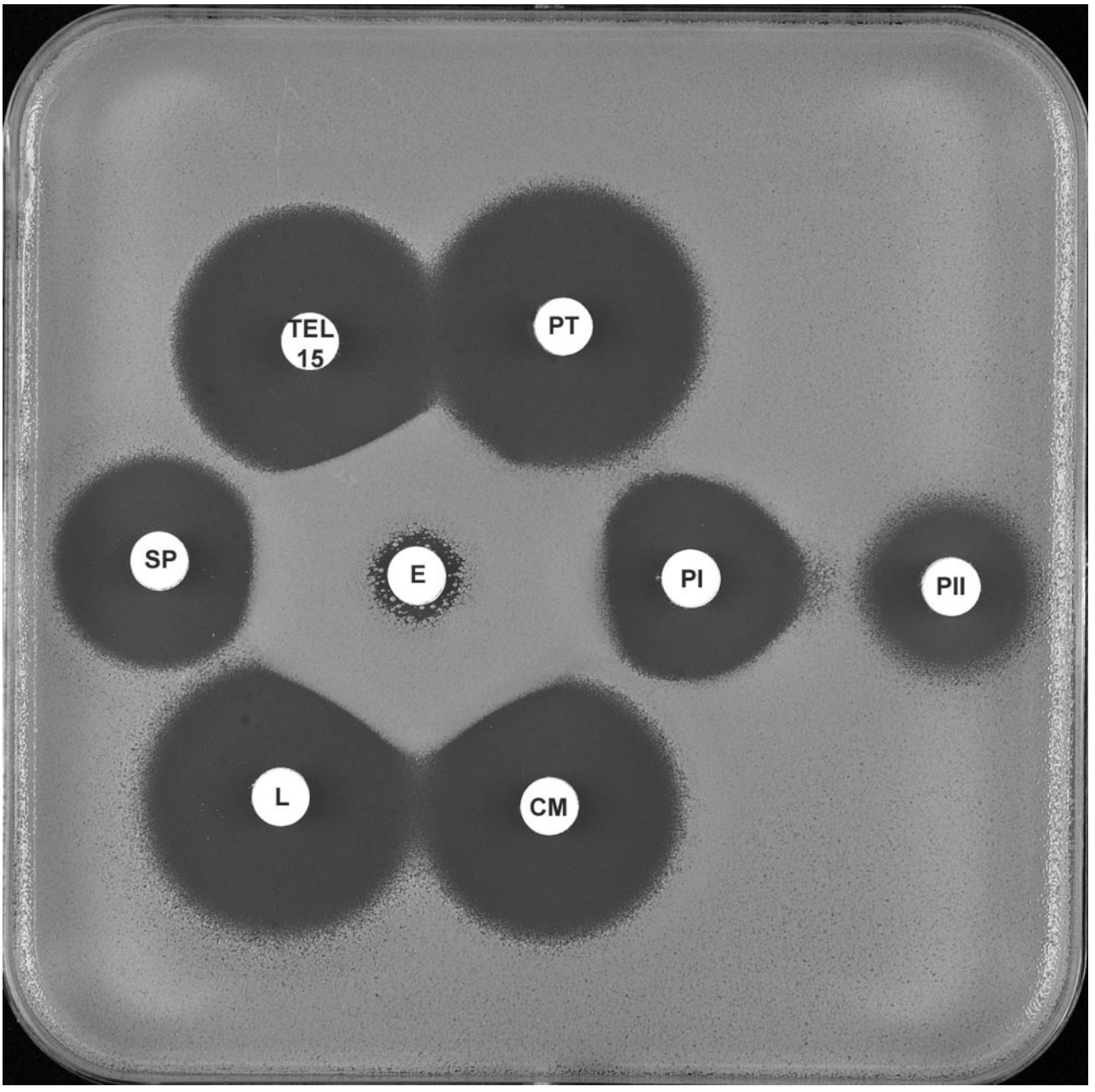


FIG. 13. *S. aureus* containing an *erm*(C) gene that is inducibly expressed. CM, clindamycin; E, erythromycin; L, lincomycin; SP, spiramycin; PI, pristinamycin IA (streptogramin factor B); PII, pristinamycin IIA (streptogramin factor A); PT, pristinamycin; TEL, telithromycin. A D-shaped zone can be observed for the clindamycin (and noninducer macrolides) zone of inhibition on the edge closest to the erythromycin zone of inhibition.

infections implies that the use of clindamycin for the treatment of an infection due to an inducibly resistant strain of *S. aureus* is not devoid of risk. In fact, recent reports indicate that the observation made primarily in the laboratory holds true for clinical isolates (70, 86, 142, 143, 170, 212, 236). However, clinical evidence of the risk of emergence of clindamycin resistance is based on only a few case reports. Also, the limited number of cases may bias the situation, since the reports have been justified by the observation of clinical failures, and successes are usually not reported. Much of the data come from pediatric patients. Infections due to community-acquired methicillin-resistant *S. aureus* are increasing in this category of patient, and therapeutic options are limited since β-lactams (because of resistance) and quinolones (in children) cannot be used. Therefore, since clindamycin represents an interesting alternative to vancomycin, the proportion of community-acquired methicillin-resistant *S. aureus* isolates with an inducible MLS_B phenotype should be carefully surveyed. In fact, the incidence of isolates that are resistant to erythromycin but susceptible to clindamycin

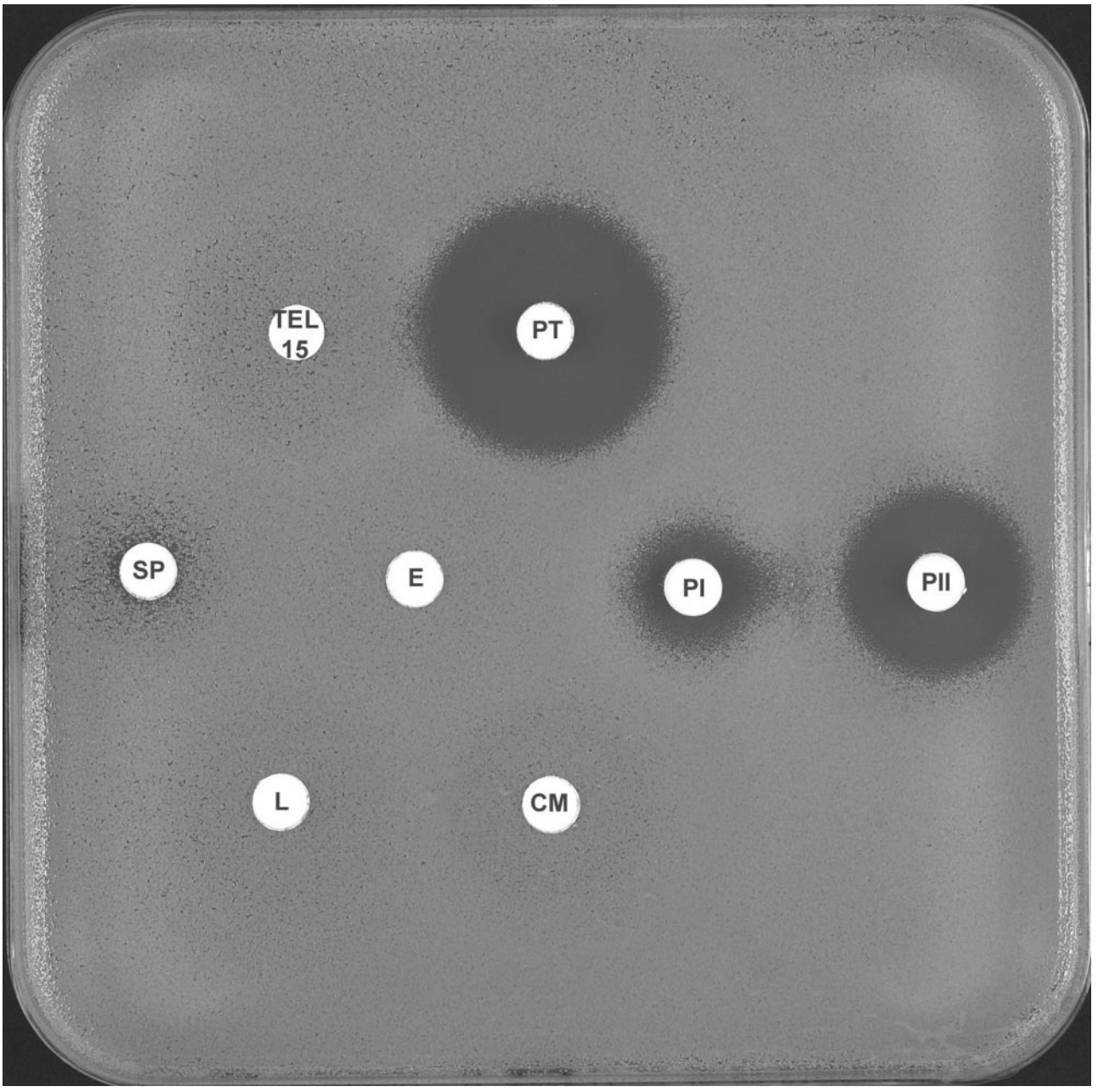


FIG. 14. *S. aureus* containing an *erm*(C) gene that is constitutively expressed. CM, clindamycin; E, erythromycin; L, lincomycin; SP, spiramycin; PI, pristinamycin IA (streptogramin factor B); PII, pristinamycin IIA (streptogramin factor A); PT, pristinamycin; TEL, telithromycin.

varies worldwide (256). In the United States, the incidence of the inducible MLS_B phenotype also varies widely by hospital, by geographical area, and temporally, ranging from 2.6% to 27.9% (7, 40, 47, 182).

For streptococci, similar doubts concerning the activity of clindamycin against isolates susceptible to this antibiotic but with an inducible MLS_B phenotype can be raised. Although no clinical failure has been reported, the use of clindamycin does not appear to be safe.

Implications for the clinical microbiology laboratory. The reporting of clindamycin susceptibility raises problems when the isolate is resistant to erythromycin. In that case, the detection of inducible MLS_B resistance (which is possible only by revealing induction of clindamycin resistance) is required. Disk diffusion is an easy method to detect this phenotype, by placing an erythromycin disk in close proximity to a clindamycin disk on an agar plate (78). A D-shaped zone is specific for the inducible MLS_B phenotype. The 2004 CLSI (formerly NCCLS) susceptibility testing standards recommend this approach to detect inducible MLS_B resistance in staphylococci. When staphylococci are tested using a broth-based method, particularly when using automated instruments, the CLSI recommends placing erythromycin and clindamycin disks 15 mm apart on the blood agar plate that is routinely used to verify the

purity of the bacterial inoculum (120).

Isolates displaying a D-shaped zone should be reported as clindamycin resistant by the laboratory (179). However, the CLSI suggests the possibility of including the comment, "This isolate is presumed to be resistant based on detection of inducible clindamycin resistance. Clindamycin may still be effective in some patients." The final decision, to treat or not to treat the patient with clindamycin, should be based on an analysis of every specific case, and if clindamycin therapy is started, it requires close follow-up of the patient for signs of failure. So far, although it seems reasonable to discourage the use of clindamycin in deep-seated infections or in infections with high bacterial densities that increase the risk of selection of constitutive mutants, there are no criteria to confidently predict the success or the failure of clindamycin therapy in infections due to staphylococci with inducible MLS_B resistance. Although the CLSI recommendation is limited to clindamycin, the same reasoning should be applied to telithromycin and 16-membered macrolides in countries where they are available for staphylococci with inducible MLS_B resistance.

The inducible MLS_B phenotype should be distinguished from another phenotype of dissociated resistance to erythromycin and susceptibility to clindamycin, which is due to the acquisition of *msr*(A). This gene encodes an inducibly produced efflux pump belonging to the ABC transporters. Erythromycin and related 14- and 15-membered macrolides are inducers and substrates for the pump. In contrast, clindamycin is neither an inducer nor a substrate, and thus, *msr*(A)-carrying strains are fully susceptible to this compound. Constitutive mutants are resistant to erythromycin but remain fully susceptible to clindamycin. Therefore, the microorganisms that are resistant to erythromycin but susceptible to clindamycin and that do not display a D-shaped zone are presumably resistant to erythromycin by efflux and can be safely reported as being susceptible to clindamycin. The case of telithromycin is different, since this antimicrobial is not an inducer but is a substrate for the MsrA pump and may select for constitutive resistant mutants. However, there have been no reports of clinical failure of telithromycin therapy for patients with infections caused by telithromycin-susceptible, erythromycin-resistant isolates (60).

Although the issue of detection and reporting of inducible MLS_B resistance in streptococci has still not been fully addressed, recommendations similar to those for staphylococci should be made for clindamycin and telithromycin. Pratically, this applies to beta-hemolytic streptococci with the *erm*(A) gene. Isolates that are resistant to erythromycin but susceptible to clindamycin and that do not exhibit a D-shaped zone may be safely reported as being susceptible to clindamycin and telithromycin. In this case, resistance is due to an efflux pump encoded by a *mef*(A) gene for which neither clindamycin nor telithromycin are substrates. This is in contrast with the MsrA pump for which, as mentioned above, telithromycin is a substrate.

Finally, we need more prospective studies of cases of infections due to staphylococci or hemolytic streptococci treated with clindamycin to more definitively define the place of this antimicrobial compound in the treatment of infections due to microorganisms with various macrolide resistance phenotypes.

CONCLUSION

The plethora of interactions between antibiotics and bacteria testifies to the remarkable adaptability of living organisms to changing and hostile environments. It provides privileged systems for the study of gene regulation and dissemination in that the manifestation of the genetic information, resistance, is easy to trace and that bacteria are subjected to accelerated evolution driven by the massive use of antibiotics. Study of the mode of action of, and of bacterial resistance to, antibiotics in the past decades has helped to elucidate numerous aspects of bacterial physiology and, not least, those of gene expression and its regulation.

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