Staphylococcus aureus VRSA-11B Is a Constitutive Vancomycin-Resistant Mutant of Vancomycin-Dependent VRSA-11A

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Vancomycin-resistant *Staphylococcus aureus* VRSA-10 was isolated in 2009, whereas VRSA-11A and VRSA-11B were isolated from the same patient in 2010. Growth curves and determination of the nature of the peptidoglycan precursors and of the VanX D,D-dipeptidase activity in the absence and in the presence of vancomycin indicated that vancomycin resistance was inducible in VRSA-10, that VRSA-11A was partially dependent on glycopeptide for growth, and that VRSA-11B was constitutively resistant. Both VRSA-11A and -11B harbored an insertion sequence, *ISEf1*, at the same locus in the *vanX-vanY* intergenic region of Tn1546 and an S₁₈₃A mutation in the chromosomal D-alanyl:D-alanine ligase (Ddl). This substitution has been shown to be responsible for a drastic diminution of the affinity of the enzyme for D-Ala at subsite 1 in *Escherichia coli* DdlB. VRSA-11B exhibited an additional mutation, P₂₁₆T, in the transcriptional regulator VanR, most probably associated with constitutive expression of vancomycin resistance. It is thus likely that VRSA-11B is a constitutive derivative of VRSA-11A selected during prolonged vancomycin therapy. Synthesis of peptidoglycan precursors ending in D-Ala-D-lactate was responsible for oxacillin susceptibility of VRSA-11A and VRSA-11B despite the presence of a wild-type *mecA* gene in both strains.

Staphylococcus aureus, in particular methicillin-resistant strains (MRSA), is responsible for severe infections at the hospital and in the community. Extensive use of vancomycin to treat infections caused by MRSA led to the emergence of vancomycin- and methicillin-resistant *S. aureus* (VRSA). To date, 11 VRSA strains, which have acquired the *vanA* operon from glycopeptide-resistant enterococci (21), have been isolated in the United States (<http://www.narsa.net/>). VanA-type resistance, which is a high level of resistance to vancomycin and teicoplanin, is mediated by transposon Tn1546, which encodes proteins involved in transposition, regulation of resistance expression, synthesis of modified peptidoglycan precursors ending in D-alanine-D-lactate (D-Ala-D-Lac), and elimination of normal precursors ending in D-Ala-D-Ala (3). Synthesis of pentadepsipeptide precursors is responsible for diminished binding affinity of glycopeptides for their target. In all the VRSA strains studied, Tn1546 is plasmid borne (14, 21), whether Tn1546 was transposed from the incoming enterococcal plasmid into a resident plasmid or, in certain instances, the enterococcal plasmid was maintained in the *S. aureus* recipient (20). Two strains, VRSA-7 and VRSA-9, are partially dependent on vancomycin for growth (11, 12). As observed for vancomycin-dependent enterococci, these isolates possess an impaired chromosomal D-Ala:D-Ala ligase (Ddl), with mutations responsible for 1,000- and 200-fold decreases in enzyme activity, respectively. Growth of these strains is enhanced in the presence of vancomycin or teicoplanin in the culture medium, which allows induction of the *vanA* resistance pathway.

We report the genetic study of the last three strains, VRSA-10, VRSA-11A, and VRSA-11B, isolated in the United States in 2009 and 2010, the latter two isolated from the same patient. We describe a mutation in VRSA-11A Ddl responsible for partial vancomycin dependence for growth and demonstrate that dependence is alleviated in derivative VRSA-11B by constitutive expression of the *vanA* operon following a mutation in the VanR transcriptional regulator.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. *S. aureus* strains were obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA). *S. aureus* VRSA-10 was isolated in Michigan in 2009 from skin and soft tissue of a 53-year-old patient, and VRSA-11A and VRSA-11B were coisolated in Delaware in 2010 from a prosthetic knee drainage of a 63-year-old patient unsuccessfully treated continuously for 3 months with vancomycin. VRSA-10 was used as a control for VanA-type vancomycin-inducible resistance. VRSA-11A and -11B had indistinguishable pulsed-field gel electrophoresis (PFGE) patterns (B. Limbago, personal communication). Methicillin-resistant *S. aureus* COL (8) and methicillin-susceptible COLΔ*mecA* (9) were used as positive and negative controls, respectively, for detection of the penicillin-binding protein PBP2'. *Escherichia coli* Neb5α was used as a host for cloning experiments. All strains were grown in brain heart infusion (BHI) broth or on BHI agar (Difco Laboratories, Detroit, MI) at 37°C. Kanamycin (50 μg/ml) was used as a selective agent for cloning PCR products into the pCR-Blunt vector (Invitrogen, San Diego, CA).

Susceptibility testing. Antibiotic susceptibility was tested by disk diffusion on Mueller-Hinton (MH) agar according to the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie (<http://www.sfm-microbiologie.org>). MICs of antimicrobial agents were determined by the Etest procedure (bioMérieux, Marcy l'Etoile, France) on MH agar.

Growth rate studies. Inducibility of glycopeptide resistance by vancomycin was studied by determination of growth rates under various conditions. The VRSA strains were grown overnight at 37°C in BHI broth without or with 1:20 of the vancomycin MIC (16 μg/ml). The cultures were diluted 1:20 in 20 ml of BHI without or with vancomycin (16 μg/ml)

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and grown at 37°C with shaking, and the optical density at 600 nm was monitored.

PCR analysis and sequencing. Glycopeptide resistance genotyping and species identification of the VRSA strains were achieved by multiplex PCR as described previously (6). PCR mapping of the *vanA* operon was performed by using primers specific for every gene of the cluster (2). The primers MecAStaph1 and MecAStaph2 were used to amplify the *mecA* gene and its promoter region (12). Amplification was performed by using Phusion *Taq* DNA polymerase (Finnzymes, Espoo, Finland) in a 9700 thermal cycler (Perkin Elmer Cetus, Norwalk, CT). The sequences of the *ddl* gene from VRSA-10, -11A, and -11B and of *vanR-vanS* from VRSA-11A and -11B were determined after amplification of total DNA with the oligodeoxynucleotides MurSA1/FtsSA1 (12) and P9/P10-P11/P12, respectively (2). The PCR fragments, with expected sizes of 1,930 bp, 1,284 bp, and 1,671 bp, respectively, were purified using the QIAquick PCR purification kit (Qiagen, Inc., Chatsworth, CA) and cloned into PCR-blunt. Plasmid DNA was labeled using a dye-labeled dideoxynucleoside triphosphate (ddNTP) terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA) and sequenced with a CEQ 8000 automated sequencer (Beckman Coulter).

Recombinant DNA techniques. Plasmid DNA isolation, digestion with restriction endonucleases (New England BioLabs, Ipswich, MA), ligation with T4 DNA ligase (Amersham), and transformation of *E. coli* Neb5 α with recombinant plasmid DNA were performed by standard methods (16).

D,D-Dipeptidase activity. VanX enzymatic activity was assayed as described previously (1). Bacteria grown in BHI broth until the optical density at 600 nm reached 0.7 in the absence or in the presence of vancomycin (16 μ g/ml) were harvested and lysed by treatment with lysostaphin (2 mg/ml) at 37°C, followed by sonication, and membrane fractions were pelleted (100,000 $\times$ g, 45 min). Cytoplasmic fractions (S100) were collected and assayed for D,D-dipeptidase (VanX) activity by measuring the D-Ala released from the dipeptide D-Ala-D-Ala (6.56 mM) by using D-amino acid oxidase, horseradish peroxidase, and O-dianisidine as a chromogen. Specific activities were defined as the number of nanomoles of product formed at 37°C per minute per milligram of protein contained in the extracts.

Analysis of peptidoglycan precursors. Extraction and analysis of the peptidoglycan precursors of VRSA strains were performed as described previously (10, 15). Strains were grown in BHI with or without vancomycin (16 μ g/ml) to mid-exponential phase (A_{600} = 1). Ramoplanin was added, and incubation continued for 20 min. Bacteria were harvested by centrifugation (12,000 $\times$ g, 2 min, 4°C), and the cytoplasmic precursors which had accumulated were extracted with 8% trichloroacetic acid (15 min, 4°C), desalted, and analyzed by high-performance liquid chromatography.

Results were expressed as the percentages of total late peptidoglycan precursors represented by tetrapeptides, pentapeptides, and pentadepsipeptides that were determined from the integrated peak areas.

Detection of penicillin-binding protein 2'. Membrane preparations of strains VRSA-10, -11A, -11B, COL, and COL Δ *mecA* were obtained as follows. Overnight cultures were harvested, and the pellets were washed in 1 $\times$ phosphate-buffered saline. Proteins were solubilized by heating in buffer containing 10% SDS, separated by SDS-polyacrylamide gel electrophoresis, and transferred electrophoretically to a Hybond-P membrane (polyvinylidene difluoride [PVDF]; Amersham Biosciences, Buckinghamshire, United Kingdom) by blotting at 80 mA for 45 min. Detection of PBP2' was performed with primary mouse anti-PBP2' 14A9C9-C6 monoclonal antibody used at a 1:20,000 dilution, and sheep anti-mouse IgG antibodies conjugated to horseradish peroxidase (ECL Plus Western blotting; Amersham Biosciences) were used as the secondary reagent at a 1:20,000 dilution.

Nucleotide sequence accession number. The sequence of the VanR gene of VRSA-11B was submitted to GenBank and was assigned the accession number JX041893.

FIG 1 Expression of PBP2'. Total proteins of methicillin-resistant COL and VRSA-10 and of methicillin-susceptible COL Δ *mecA*, VRSA-11A, and VRSA-11B were isolated, subjected to SDS-PAGE, and probed with 14A9C9-C6 monoclonal antibody against PBP2'. Purified PBP2' was used as a size marker.

RESULTS AND DISCUSSION

Characterization of strains. The clinical isolates were highly resistant to both vancomycin (MICs > 256 μ g/ml) and teicoplanin (MICs = 128 μ g/ml). VRSA-10 was resistant to oxacillin (MIC = 32 μ g/ml), whereas VRSA-11A and -11B were susceptible (MIC = 0.094 μ g/ml and 0.125 μ g/ml, respectively). Identification of staphylococci at the species level and the *vanA* genotype were confirmed by multiplex PCR (6).

Sequence determination of *mecA* and production of MecA. Both VRSA-11A and -11B are susceptible to oxacillin despite the fact that they harbor the *mecA* gene (data not shown). The sequence of *mecA* was determined, and no mutations were detected compared to the functional *mecA* gene of *S. aureus* VRSA-10. PBP2', with the expected size of 76 kDa, was detected by Western blotting in *S. aureus* VRSA-10, -11A, -11B, and COL using an anti-PBP2' monoclonal antibody but not in COL Δ *mecA* (Fig. 1). Furthermore, the level of PBP2' synthesis appeared similar in VRSA-10, -11A, and -11B. These results demonstrate that methicillin susceptibility in VRSA-11A and -11B was not due to a functional alteration of PBP2'.

***vanA* gene cluster.** The organization of the *vanA* gene cluster in the three strains was determined by PCR mapping (6). For VRSA-10, all the PCR fragments had the expected sizes, indicating that the nine genes in the two operons were present and in the same order as in Tn1546. For VRSA-11A and -11B, similar results were obtained except for the PCR with primers P17/P18, which gave a larger amplification product (ca. 3,000 bp instead of 1,584 bp). Sequencing of this fragment revealed the presence in both strains of an ISEf1 copy (1,338 bp) in the *vanX-vanY* intergenic region just upstream from the start codon of *vanY* (position 9051 of Tn1546) in the same orientation as *vanY* and flanked by a GACTGAAA 8-bp duplication of target DNA.

Growth with and without vancomycin. As observed for VRSA-7 and VRSA-9 (11, 12), determination of antibiotic susceptibilities of the three strains by disc diffusion or by Etest showed that for VRSA-11A, the culture density was higher around the vancomycin and teicoplanin discs or strips. Analysis of the effect of vancomycin (16 μ g/ml) on growth of VRSA-10, -11A, and -11B was studied in liquid culture (Fig. 2). For VRSA-10, growth was much more efficient in the absence of drug. For VRSA-11A, growth was slightly better in the presence of vancomycin, whereas VRSA-11B grew similarly in the absence or in the presence of the drug. These results suggest that vancomycin resistance was inducible in VRSA-10, VRSA-11A was partially dependent on glycopeptide for growth, and vancomycin resistance was constitutively expressed in VRSA-11B.

Sequence of the *ddl* gene. Partial vancomycin dependence of VRSA-11A was likely to be due to a mutation in the host Ddl, as

FIG 2 Growth of *S. aureus* VRSA-10, VRSA-11A, and VRSA-11B in the absence (N.I., not induced) or the presence (Vm, 16 μ g/ml) of vancomycin.

previously shown in enterococci (18, 19) and staphylococci (11, 12). Sequence determination of the *ddl* gene of VRSA-11A revealed a G₅₄₇C mutation responsible for a serine-to-alanine substitution (S₁₈₃A). The same mutation was also present in VRSA-11B but not in VRSA-10. S₁₈₃ corresponds to S₁₅₀ of DdlB of *E. coli*. It has been demonstrated that the S₁₅₀ residue of DdlB is located in the first D-Ala subsite and that the E₁₅-S₁₅₀-Y₂₁₆ H-bonded triad, which is also present in *S. aureus* Ddl (E₁₆-S₁₈₃-Y₂₅₂), could make interaction with α -NH₃⁺ of the first D-Ala (17). Interestingly, it has been demonstrated that the S₁₅₀A mutation is responsible for an approximately 600-fold drop in affinity of *E. coli* DdlB for D-Ala at subsite 1 (17). Thus, it is likely that the S₁₈₃A mutation alters the activity of the Ddl of *S. aureus* VRSA-11A and -11B.

Nature of peptidoglycan precursors. The nature of the cytoplasmic peptidoglycan precursors of strains VRSA-10, -11A, and -11B was studied after growth in the presence (16 μ g/ml) or in the absence of vancomycin (Table 1). In noninduced VRSA-10, UDP-

TABLE 1 Cytoplasmic peptidoglycan precursors in extracts from strains VRSA-10, -11A, and -11B^a

Strain and growth condition	% of peptidoglycan precursor		
	UDP-MurNac-tetrapeptide	UDP-MurNac-pentapeptide (D-Ala)	UDP-MurNac-pentapeptide (D-Lac)
VRSA-10			
Uninduced	3	97	ND
VAN (16 μ g/ml)	7	4	89
VRSA-11A			
Uninduced	5	30	65
VAN (16 μ g/ml)	10	3	87
VRSA-11B			
Uninduced	13	4	83
VAN (16 μ g/ml)	10	ND	90

^a The cytoplasmic peptidoglycan precursors of VRSA-10, -11A, and -11B, grown with or without glycopeptides, were analyzed as described previously (10). VAN, vancomycin; ND, not detectable.

Nanomoles of D-Ala released/min/mg

FIG 3 D,D-Di-peptidase (VanX) activity in cytoplasmic extracts from *S. aureus* VRSA-10, -11A, and -11B. Results are the means from three independent experiments. N.I., not induced; Vm, induced by growth in the presence of 16 μ g/ml of vancomycin.

Mur-Nac-pentapeptide was the main late peptidoglycan precursor identified (97%), whereas UDP-Mur-Nac-pentapeptide ending in D-Ala-D-Lac was highly dominant (89%) after induction. These data confirm inducible glycopeptide resistance in this strain. In VRSA-11A, in the absence of vancomycin, UDP-Mur-Nac-pentapeptide and UDP-Mur-Nac-pentapeptide represented, respectively, 65% and 30% of the late precursors, indicating that the strain relies heavily on the resistance pathway for peptidoglycan synthesis, even in the absence of inducer. In the presence of vancomycin, a higher proportion (87%) of UDP-Mur-Nac-pentapeptide was detected in the cells due to inducibility of resistance. For VRSA-11B, peptidoglycan precursors ending in D-Ala-D-Lac were mainly detected under both noninduced (83%) and induced (90%) conditions, demonstrating that the *vanA* operon is constitutively expressed in this strain.

D,D-Di-peptidase activities. D,D-Di-peptidase activity, determined as the amount of D-Ala released from hydrolysis of the D-Ala-D-Ala dipeptide, was measured in the cytoplasmic fractions of the VRSA-11A and -11B strains (Fig. 3). For VRSA-11A, a weaker VanX activity was found in the absence of induction than in the presence of vancomycin, compatible with inducible resistance. Similar and high VanX activities were found in the presence of vancomycin (16 μ g/ml) in the culture medium and in the absence of inducer in VRSA-11B, confirming constitutivity of *vanA* expression.

Methicillin susceptibility in VRSA-11A and -11B. For vancomycin-dependent VRSA-11A, as previously demonstrated (11, 12), susceptibility to oxacillin in the absence of vancomycin is explained by the following: (i) partial inactivation of the Ddl responsible for diminished production of peptidoglycan precursors ending in D-Ala-D-Ala and (ii) the basal level of expression of the *vanA* operon, which allows synthesis of modified precursors ending in D-Ala-D-Lac that cannot be processed by PBP2' (4), which is the only transpeptidase that remains active in the presence of β -lactams in the medium. VRSA-11B, in which the *vanA* operon is constitutively expressed, mainly synthesizes, under both induced and noninduced conditions, peptidoglycan precursors ending in D-Ala-D-Lac that are responsible for oxacillin susceptibility.

Sequence of *vanR-vanS*. It has been demonstrated that mutations in the genes for the VanRS two-component system are re-

sponsible for constitutive expression of the *vanA* operon (5, 7). We have determined the sequences of the *vanR-vanS* genes in both VRSA-11A and -11B. No differences were found in *vanS*, but a point mutation was detected at position 646 in *vanR* of VRSA-11B, responsible for the replacement of a cytosine by an adenine (C₆₄₆A), leading to a proline-to-threonine substitution (P₂₁₆T).

VanR belongs to the same family of transcriptional activators as PhoB and OmpR from *E. coli*. Structure analysis revealed that the two regulators are composed of two functional domains: an N-terminal receiver and a C-terminal effector domain, the latter having DNA binding and transactivation functions (13). Although P₂₁₆ has not yet been demonstrated to be essential, we can hypothesize that replacement of this residue could alter the function of this type of regulators.

VRSA-11A and -11B exhibit the same mutation in the *ddl* gene and harbor the same *ISEf1* inserted at the same site in Tn1546. Thus, it is most likely that the mutation in *vanR* of VRSA-11B occurred after acquisition of the *ddl* mutation and that VRSA-11B is a constitutive derivative, obtained under vancomycin therapy, of the partially vancomycin-dependent VRSA-11A. It has been demonstrated that all but one of the VanD-type enterococci isolated so far possess an impaired Ddl, responsible for the lack of UDP-Mur-Nac-pentapeptide precursors, and express constitutive vancomycin resistance secondary to a mutation in the VanR-VanS two-component system (6). These observations suggest that the regulatory mutations were acquired before those in the Ddl, since otherwise the strains would have been transiently glycopeptide dependent. This hypothesis was confirmed by the study of two VanD-type *E. faecalis* strains that harbored identical *vanD* operons but distinct mutations in their D-Ala:D-Ala ligases (6) and of a VanD-type *E. faecium* strain harboring a mutation in VanS but not in the Ddl (5). On the contrary, in VRSA-11A, the Ddl activity is not totally abolished, allowing synthesis of a low proportion of UDP-Mur-Nac-pentapeptide precursors (30%), which permits growth in the absence of vancomycin. Survival provides the time period necessary for a second, regulatory mutation to occur, which relieves the host from glycopeptide dependence, leading to strain VRSA-11B.

To date, 11 VRSA strains have been isolated in the United States. Surprisingly, among those, VRSA-7, VRSA-9, and VRSA-11A are partially vancomycin dependent for growth due to impaired activity of the chromosomal D-Ala:D-Ala ligase. The reason why one-fourth of the VRSA have an impaired Ddl remains unknown. VRSA-11B is the first VRSA strain that constitutively expresses the *vanA* operon. It is likely that vancomycin therapy for a prolonged period of time was responsible for selection of the constitutively resistant VRSA-11B from the partially vancomycin-dependent VRSA-11A. Thus, early detection of vancomycin-dependent VRSA is of importance, since these strains, despite the fact that they produce a functional MecA (12), are susceptible to β -lactams. Thus, patients infected by vancomycin-dependent or constitutively resistant VRSA could conceivably be treated with a β -lactam.

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REFERENCES

- Arthur M, Depardieu F, Reynolds P, Courvalin P. 1996. Quantitative analysis of the metabolism of soluble cytoplasmic peptidoglycan precursors of glycopeptide-resistant enterococci. *Mol. Microbiol.* 21:33–44.
- Arthur M, Molinas C, Depardieu F, Courvalin P. 1993. Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J. Bacteriol.* 175:117–127.
- Arthur M, et al. 1996. Mechanisms of glycopeptide resistance in enterococci. *J. Infect.* 32:11–16.
- De Jonge BLM, Chang YS, Gage D, Tomasz A. 1992. Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. *J. Biol. Chem.* 267:11248–11254.
- Depardieu F, Kolbert M, Pruihl H, Bell J, Courvalin P. 2004. VanD-type vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*. *Antimicrob. Agents Chemother.* 48:3892–3904.
- Depardieu F, Perichon B, Courvalin P. 2004. Detection of the *van* alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. *J. Clin. Microbiol.* 42:5857–5860.
- Depardieu F, Podglajen I, Leclercq R, Collatz E, Courvalin P. 2007. Modes and modulations of antibiotic resistance gene expression. *Clin. Microbiol. Rev.* 20:79–114.
- Dyke KG, Jevons MP, Parker MT. 1966. Penicillinase production and intrinsic resistance to penicillins in *Staphylococcus aureus*. *Lancet* i:835–838.
- Finan JE, Rosato AE, Dickinson TM, Ko D, Archer GL. 2002. Conversion of oxacillin-resistant staphylococci from heterotypic to homotypic resistance expression. *Antimicrob. Agents Chemother.* 46:24–30.
- Messer J, Reynolds PE. 1992. Modified peptidoglycan precursors produced by glycopeptide-resistant enterococci. *FEMS Microbiol. Lett.* 73:195–200.
- Meziane-Cherif D, et al. 2010. Molecular basis of vancomycin dependence in VanA-type *Staphylococcus aureus* VRSA-9. *J. Bacteriol.* 192:5465–5471.
- Moubareck C, Meziane-Cherif D, Courvalin P, Perichon B. 2009. VanA-type *Staphylococcus aureus* strain VRSA-7 is partially dependent on vancomycin for growth. *Antimicrob. Agents Chemother.* 53:3657–3663.
- Okamura H, Hanaoka S, Nagadoi A, Makino K, Nishimura Y. 2000. Structural comparison of the PhoB and OmpR DNA-binding/transactivation domains and the arrangement of PhoB molecules on the phosphate box. *J. Mol. Biol.* 295:1225–1236.
- Périchon B, Courvalin P. 2009. VanA-type vancomycin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 53:4580–4587.
- Reynolds PE, Depardieu F, Dutka-Malen S, Arthur M, Courvalin P. 1994. Glycopeptide resistance mediated by enterococcal transposon Tn1546 requires production of VanX for hydrolysis of D-alanyl-D-alanine. *Mol. Microbiol.* 13:1065–1070.
- Sambrook J, Russell DW. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Shi Y, Walsh CT. 1995. Active site mapping of *Escherichia coli* D-Ala-D-Ala ligase by structure-based mutagenesis. *Biochemistry* 34:2768–2776.
- Sifaoui F, Gutmann L. 1997. Vancomycin dependence in a VanA-producing *Enterococcus avium* strain with a nonsense mutation in the natural D-Ala-D-Ala ligase gene. *Antimicrob. Agents Chemother.* 41:1409.
- Van Bambeke F, Chauvel M, Reynolds PE, Fraimow HS, Courvalin P. 1999. Vancomycin-dependent *Enterococcus faecalis* clinical isolates and revertant mutants. *Antimicrob. Agents Chemother.* 43:41–47.
- Zhu W, et al. 2008. Vancomycin-resistant *Staphylococcus aureus* isolates associated with Inc18-like *vanA* plasmids in Michigan. *Antimicrob. Agents Chemother.* 52:452–457.
- Zhu W, et al. 2010. Dissemination of an *Enterococcus* Inc18-like *vanA* plasmid associated with vancomycin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 54:4314–4320.