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

Bruno Périchon and Patrice Courvalin
Institut Pasteur, Unité des Agents Antibactériens, Paris, France

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, IS1546, at the same locus in the vanX-vanY intergenic region of Tn1546 and an S_{83}A 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, P_{216}T, 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 pentapeptide-pentapeptide 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 (NARS). 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-resistant COL.Δmeca (9) were used as positive and negative controls, respectively, for detection of the penicillin-binding protein PB2’. Escherichia coli Neb5ox 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’Antiibiogramme 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 Mueller-Hinton (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).
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 *dld* 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/PtsSA1 (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* Neb5α with recombinant plasmid DNA were performed by standard methods (16).

**D,D-Dipeptidase activity.** *Van* *X* 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 μg/ml) were harvested and lysed by treatment with lysoptastin (2 mg/ml) at 37°C, followed by sonication, and membrane fractions were pelleted (100,000 × g, 45 min). Cytoplasmic fractions (S100) were collected and assayed for D,D-dipeptidase (*Van* *X*) 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 D,D-dipeptidase activity.

**RESULTS AND DISCUSSION.**

**Characterization of strains.** The clinical isolates were highly resistant to both vancomycin (MICs > 256 μg/ml) and teicoplanin (MICs = 128 μg/ml). VRSA-10 was resistant to oxacillin (MIC = 32 μg/ml), whereas VRSA-11A and -11B were susceptible (MIC = 0.094 μg/ml and 0.125 μg/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. PB2’, 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 mecA (Fig. 1). Furthermore, the level of PB2’ 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 PB2’.

**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 IS*efj* 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 drug. For VRSA-11A, 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 dld gene.** Partial vancomycin dependence of VRSA-11A was likely to be due to a mutation in the host Ddl, as
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 E16-S183-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 murA (4), which is explained by the following: (i) partial inactivation of the Ddl responsible for diminished production of peptidoglycan precursors ending in D-Ala-D-Lac that are responsible for oxacillin susceptibility.

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-Dipeptidase activities.** D,D-Dipeptidase 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 µg/ml) in the culture medium and in the absence of inducer in VRSA-11B, confirming constitutiveness 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 (C<sub>646A</sub>), leading to a proline-to-threonine substitution (P<sub>216T</sub>).

VanR belongs to the same family of transcriptional activators as PhoB and OmpR from <i>E. coli</i>. 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 P<sub>216</sub> 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 ISE<sub>Fl</sub> inserted at the same site in Tn<sub>1546</sub>. 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 <i>E. faecalis</i> strains that harbored identical vanD operons but distinct mutations in their d-Ala<sub>2</sub>-d-Ala ligases (6) and of a VanD-type <i>E. faecium</i> 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.

ACKNOWLEDGMENTS

We thank S. Mobashery and M. Page for the gift of purified PBP2’ and of the anti-PBP2’ monoclonal antibody, respectively. <i>S. aureus</i> strains were obtained through the Network on Antimicrobial Resistance in <i>Staphylococcus aureus</i>. This work was supported in part by an unrestricted grant from Novartis.

REFERENCES


