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VanA-Type *Staphylococcus aureus* Strain VRSA-7 Is Partially Dependent on Vancomycin for Growth▽

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VanA-type *Staphylococcus aureus* strain VRSA-7 was partially dependent on glycopeptides for growth. The *vanA* gene cluster, together with the *erm*(A) and the *ant(9)-Ia* resistance genes, was carried by the ca. 35- to 40-kb conjugative plasmid pIP848 present at five copies per cell. The chromosomal *ddl* gene had a mutation that led to a $N_{308}K$ substitution in the D-Ala:D-Ala ligase that resulted in a 1,000-fold decrease in activity relative to that of strain VRSA-6. Strain VRSA-7 grown in the absence or in the presence of vancomycin mainly synthesized precursors ending in D-Ala–D-Lac, indicating that the strain relied on the vancomycin resistance pathway for peptidoglycan synthesis. Greatly enhanced growth in the presence of glycopeptides and the absence of mutations in the genes for VanR and VanS indicated the inducible expression of resistance. Thus, a combination of loose regulation of the *vanA* operon by the two-component system and a gene dosage effect accounts for the partial glycopeptide dependence of VRSA-7. Since peptidoglycan precursors ending in D-Ala–D-Lac are not processed by PBP 2′, the strain was fully susceptible to oxacillin, despite the production of a wild-type PBP 2′.

Methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA) strains that have acquired the *vanA* operon from glycopeptide-resistant enterococci are designated vancomycin-resistant *S. aureus* (VRSA). The first two VRSA isolates were recovered in the United States in 2002 (25, 36). Since then, an additional seven MRSA isolates carrying the *vanA* gene cluster have been detected in the United States (one in New York and six in Michigan), and two tentative identifications of VRSA have been reported in India (32) and Iran (1). Vancomycin acts by binding to the C-terminal acyl-D-alanyl–D-alanine (acyl-D-Ala–D-Ala) of pentapeptide peptidoglycan precursors and inhibits transglycosylation and transpeptidation reactions, thus preventing cell wall formation (6) and with the acyl-D-Ala–D-Ala residues being incorporated into peptidoglycan precursors as dipeptides synthesized by the host D-Ala:D-Ala ligase (Ddl). VanA-type resistance is characterized by high-level inducible resistance to vancomycin and teicoplanin due to synthesis of peptidoglycan pentadepsipeptide precursors ending in D-Ala–D-lactate (D-Ala–D-Lac). This alteration is responsible for the diminished binding affinity of glycopeptides for their target. VanA-type resistance is mediated by a gene cluster often carried by Tn*1546* encoding nine proteins. Two are implicated in the movement of the element: open reading frame 1 (ORF1), a transposase, and ORF2, a resolvase. Two are essential for the ultimate production of pentadepsipeptides: VanH, a dehydrogenase that reduces pyruvate to D-Lac, and VanA, a ligase that catalyzes the formation of an ester bond between D-Ala and D-Lac. Two other proteins are responsible for the elimination of pentapeptide precursors: VanX, a D,D-dipeptidase that cleaves the D-Ala–D-Ala dipeptide synthesized by the host Ddl, and VanY, a D,D-carboxypeptidase that removes the ultimate D-Ala of the pentapeptide precursors. Expression of the *vanA* gene cluster is regulated by the two-component regulatory system VanR-VanS, which is responsible for recognition of the presence of glycopeptides in the culture medium and transcriptional activation of the resistance and regulatory genes. The remaining protein, VanZ, whose function is unknown, is implicated in teicoplanin resistance (2).

Enterococci that require the presence of vancomycin in the culture medium for growth have been isolated from patients treated with this antibiotic (14, 39). Although many of these vancomycin-dependent strains are VanB-type *Enterococcus faecium*, vancomycin-dependent *Enterococcus faecalis* and *Enterococcus avium* containing the *vanA* operon have also been isolated (16, 38). To the best of our knowledge, no VRSA strain with this phenotype has been reported. Here we describe the mechanism of vancomycin dependence in VanA-type *S. aureus* strain VRSA-7, isolated from a patient treated with vancomycin for prolonged periods of time. The activity of the host Ddl and the expression of the *vanA* gene cluster of VRSA-7 were compared with those of strain VRSA-6, another highly vancomycin-resistant *S. aureus* of the VanA type (37, 41).

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MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The origins and characteristics of the bacterial strains and plasmids used in this study are described in Table 1. *S. aureus* strains were obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus*. *S. aureus* strains VRSA-6 and VRSA-7 were isolated in Michigan in 2005 from a surgical site wound and a nonhealing plantar ulcer, respectively (37). *Escherichia coli* Top10 was used as the host in cloning experiments. *S. aureus* BM4602 (from a laboratory collection), *E. faecalis* JH2-2 (19), and *E. faecium* 64/3 (40) were used as recipient strains in the transfer experi-

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli		
Top10	F$^-$ *mcrA* Δ(*mrr hsdRMS mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen
Bl21	BL21(DE3)pLys	Novagen
S. aureus		
VRSA-6	*vanRSHAXYZ*, *erm*(B), *ant(9)-Ia*, *ant(4′)-Ia*, *mecA*	37
VRSA-7	*vanRSHAXYZ*, *erm*(A), *erm*(C), *ant(9)-Ia*, *ant(4′)-Ia*, *mecA*	37
COL	*mecA* Vm^s Te^s	15
BM4602	Rif^r Fu^r	Laboratory collection
RN4220	Oxa^s	21
E. faecium		
BM4339	$vanR_DS_DH_DDX_DY_D$	30
64/3	Rif^r Fu^r	40
E. faecalis JH2-2	Rif^r Fu^r	19
Plasmid		
pCR-Blunt	Km^r Zeocin^r *oriR* from ColE1 *lacZ*α *ccdB*	Invitrogen
pET28a	Km^r, vector for expression of recombinant proteins	Novagen
pAT79	*oriR* from pAMβ1 *oriR* from pUC *oriT* from RK2 Sp^r *lacZ*α P_2*cat*	4
pAT515	1,128-bp SacI-XbaI PCR fragment (ddl_{VRSA-6}) of *S. aureus* VRSA-6 cloned into pAT79	This study
pAT516	1,128-bp SacI-XbaI PCR fragment (ddl_{VRSA-7}) of *S. aureus* VRSA-7 cloned into pAT79	This study
pAT518	1,087-bp BsaI-XhoI PCR fragment (ddl_{COL}) of *S. aureus* COL cloned into pET28a(+)	This study
pAT519	1,087-bp BsaI-XhoI PCR fragment (ddl_{VRSA-7}) of *S. aureus* VRSA-7 cloned into pET28a(+)	This study

[a] Fu, fusidic acid; Km, kanamycin; Oxa, oxacillin; Rif, rifampin; r, resistant; s, susceptible; Sp, spectinomycin.

ments. MRSA COL (15) and methicillin-susceptible *S. aureus* RN4220 (21) were used as positive and negative controls, respectively, for detection of penicillin-binding protein (PBP) PBP 2′. *E. coli* BL21(DE3)pLys (Novagen, Madison, WI) was used with the pET28a(+) expression vector (Novagen). 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 of the PCR products into the pCR-Blunt vector (Invitrogen, Leek, The Netherlands). Spectinomycin (60 μg/ml) was used to prevent the loss of plasmids derived from pAT79 (4).

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 (9). The MICs of antimicrobial agents were determined by Etest (AB Biodisk, Combourg, France) on MH agar.

Growth rate studies. The induction of resistance by vancomycin in strain VRSA-7 was studied by the determination of growth rates under various conditions. The isolate was grown overnight at 37°C in BHI broth without or with vancomycin (8 μg/ml). The cultures were diluted 1:20 into 20 ml of BHI without or with vancomycin (8 μg/ml) and were then 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 VRSA-6 and VRSA-7 were performed by a multiplex PCR assay, as described previously (13). PCR mapping of the *vanA* operon was performed by using primers specific for every gene of the cluster (5). The strains were screened by PCR for the spectinomycin resistance gene *ant(9)-Ia* (20); the kanamycin and tobramycin resistance gene *ant(4′)-Ia* (34); and the macrolide-lincosamide-streptogramin B (MLS_B) resistance genes *erm*(A), *erm*(B), and *erm*(C) with the following primers: ermA(+) (5′-CTTCGATAGTTTATTAATATTAGT-3′), ermA(−) (5′-TCTAAAAAGCATGTAAAAGAA-3′), ermB(+) (5′-GAAAAGGTACTCAACCAAATA-3′), ermB(−) (5′-AGTAACGGTACTTAAATTGTTTAC-3′), ermC(+) (5′-TCAAAACATAATATAGATAAA-3′), and ermC(−) (5′-GCTAATATTGTTTAAATCGTCAAT-3′). Primers MecAStaph1 (5′-TAACGTGGAGACGAGCAC-3′) and MecAStaph2 (5′-AACAGTGAAGCAACCATCGT-3′) were used to amplify the *mecA* gene and its promoter region. PCRs were performed with a 50-μl volume containing 20 pmol of each pair of oligodeoxynucleotide primers, 5 nmol of each 2′-deoxynucleoside 5′-triphosphate, reaction buffer, 100 ng of DNA, and 5 U of DNA *Pfu* polymerase (Stratagene, La Jolla, CA). PCR elongation times and temperatures were adjusted according to the expected size of the PCR product and the nucleotide sequences of the primers, respectively. Amplification was in a model 9700 thermal cycler (Perkin Elmer Cetus, Norwalk, CT). The sequences of the *ddl* genes from VRSA-6 and VRSA-7 and of the *vanR-vanS* genes from VRSA-7 were determined after the amplification of total DNA with oligodeoxynucleotides MurSA1 (5′-CCCCAGCACCATCTTGTAAT-3′) and FtsSA1 (5′-GGCCAAACGTGTACCAACTT-3′) and with oligodeoxynucleotides P9/P10 and P11/P12, respectively (5). The PCR fragments, which had expected sizes of 1,930 bp, 1,284 bp, and 1,671 bp, respectively, were purified with the PCR purification kit (Qiagen, Inc., Chatsworth, CA) and cloned into PCR-Blunt. Plasmid DNA was labeled by using a dye-labeled dideoxynucleoside triphosphate Terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA) and sequenced with a model CEQ 2000 automated sequencer (Beckman Coulter). The sequences were analyzed with the GCG sequence analysis software package (version 10.1; Genetics Computer Group, Madison, WI).

Contour-clamped homogeneous electric field gel electrophoresis. Pulsed-field gel electrophoresis of genomic DNA embedded in agarose plugs digested with I-CeuI or SmaI was performed as described previously (12). The fragments generated by I-CeuI were hybridized successively to a α-^{32}P-labeled 16S rRNA (*rrs*) probe obtained by amplification of an internal portion of the *rrs* gene (18) and to a *vanA*-specific probe obtained by PCR with primers EA1 and EA2 (13).

Recombinant DNA techniques. Plasmid DNA isolation, digestion with restriction endonucleases (Amersham Pharmacia Biotech, Uppsala, Sweden), ligation with T4 DNA ligase (Amersham), and transformation of *E. coli* Top10 with recombinant plasmid DNA were performed by standard methods (33). For the isolation of plasmid DNA from *S. aureus*, lysostaphin (Sigma-Aldrich, St. Louis, MO) was added to a Tris-HCl (10 mM)–EDTA (1 mM)–glucose (9 g/liter) buffer at a final concentration of 2 mg/ml, and the bacteria were incubated in the buffer at 37°C for 1 h before the lysis step.

Plasmid analysis. The attempted transfer of vancomycin resistance from strain VRSA-7 to *S. aureus* BM4602, *E. faecalis* JH2-2, and *E. faecium* 64/3 was performed by filter mating. Plasmid DNA extracted from VRSA-7 was electrotransformed into *S. aureus* BM4602. Transformants were selected on agar containing rifampin (rifampicin; 20 μg/ml), fusidic acid (10 μg/ml), and vancomycin (10 μg/ml) or erythromycin (8 μg/ml). Plasmid DNA that had been digested with

EcoRI was transferred to a nylon membrane and hybridized separately to α-^{32}P-labeled probes for *vanA*, *erm*(A), *erm*(C), and *ant(9)-Ia*.

qPCR. Quantitative PCR (qPCR) was performed in a LightCycler instrument with version 4.1 of the LightCycler software (Roche Diagnostics) and a Fast Start DNA Master Plus SYBR green I kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. The *ddl*, *rpoB*, and *rrs* chromosomal genes, which are present in *S. aureus* in one, one, and five copies, respectively, were selected as controls. The copy numbers of the *vanA* and *vanH* genes relative to those of *ddl*, *rpoB*, and *rrs* were estimated from three independent experiments with five sets of primers specific for each gene: primers qPCRrrs1 (5′AGGTAACGGCTTACCAAGGCA3′) and qPCRrrs2 (5′ACGATCCGAAGACCTTCA3′), primers qPCRddl1 (5′CCCATTATTACATGGTCCTA3′) and qPCRddl2 (5′ GGTAACTGTGGTAACCCT3′), primers qPCRrpoB1 (5′GTACAGTGCTTGATCGTCGT3′) and qPCRrpoB2 (5′CAGTTGTCGTACGACCTTCA3′), primers qPCRvanA1 (5′TTCAGCTTTGCATGGCAAGT3′) and qPCRvanA2 (5′ACCCAAAAGGCGGGAGTA3′), and primers qPCR vanH1 (5′CGGATAGCGTTGCCGATTAT3′) and qPCRvanH2 (5′GCTCAATAACCGCTTTGCCT3′). The data were analyzed by the method of Peirson et al. (28).

Plasmid construction. The chromosomal *ddl* genes from *S. aureus* VRSA-6 ($ddl_{VRSA\text{-}6}$) and VRSA-7 ($ddl_{VRSA\text{-}7}$) with their ribosome-binding sites were amplified by PCR from total DNA with oligodeoxynucleotides DdlSASacI (5′-CGCTGCAGAGCTCATATATCATTGGAGGAA-3′) and DdlSAXbaI (5′-TTTGGGATCTAGATTTAATGTAACATTAAT-3′) containing a SacI site and a XbaI site (underlined), respectively. The PCR fragments were cloned under the control of the constitutive P_2 promoter and upstream from the *cat* reporter gene of the shuttle vector pAT79, leading to plasmids pAT515 (pAT79ΩP_2-$ddl_{VRSA\text{-}6}$-*cat*) and pAT516 (pAT79ΩP_2-$ddl_{VRSA\text{-}7}$-*cat*), which were electrotransformed into VanD-type *E. faecium* BM4339 with selection on spectinomycin (120 μg/ml), followed by screening for resistance to chloramphenicol. The nucleotide sequence of the amplified fragments was redetermined.

Production and purification of Ddls of *S. aureus* VRSA-7 and VRSA-6 strains. Oligodeoxynucleotides DdlSABsaI (5′-CGTGGTCTCCCATGACAAAAGAAAATATTT-3′) and DdlSAXhoI (5′-CTCCTCGAGGTCAATTTTGTATTTATTTTT-3′) containing a BsaI and a XhoI restriction site (underlined), respectively, were used to amplify the *ddl* gene from strains VRSA-6 and VRSA-7 with *Pfu* DNA polymerase. The PCR products were cloned in the PCR-Blunt vector, sequenced, and subcloned under the control of the T7 promoter in pET28a(+) previously digested with NcoI and XhoI, producing pAT518 [pET28a(+) Ω$ddl_{VRSA\text{-}6}$] and pAT519 [pET28a(+)Ω$ddl_{VRSA\text{-}7}$]. Protein production and purification were performed as described previously (22), with slight modifications. Briefly, Ddl enzymes engineered to have a C-terminal His_6 tag were produced from *E. coli* BL21(DE3)pLys harboring plasmid pAT518 or pAT519 in 1 liter of LB medium containing 25 μg/ml kanamycin and 100 μg/ml ampicillin. Extraction buffer was supplemented with 10% (vol/vol) BugBuster 10× protein extraction reagent and Benzonase (25 U; Sigma-Aldrich). Recombinant His_6 proteins were purified by using 5 ml HisTrap fast-flow columns (GE Healthcare, Uppsala, Sweden). The purified proteins were stored at −80°C in buffer containing 50 mM HEPES, pH 7.5, 150 mM KCl, and 1 mM EDTA.

Size-exclusion chromatography. Size-exclusion chromatography was performed with a Hiload 16/60 Superdex 200 column (GE Healthcare) equilibrated with 50 mM HEPES, pH 7.5, 150 mM KCl, and 1 mM EDTA and eluted in the same buffer at a flow rate of 0.8 ml/min. The column was calibrated according to the manufacturer's instructions.

Enzyme kinetics. Steady-state kinetic constants for the Ddl activities were determined by a spectrophotometric assay at 340 nm, in which the production of ADP is coupled to the oxidation of NADH through pyruvate kinase and L-lactate dehydrogenase (10). The assays were performed at 37°C in 100 mM HEPES, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 2.5 mM phosphoenolpyruvate, 0.2 mM NADH, 50 U/ml L-lactate dehydrogenase, 50 U/ml pyruvate kinase, and D-Ala in a total volume of 0.5 ml. The initial rates of hydrolysis were determined with a Uvikon UV931 spectrophotometer (Kontron Instruments, Saint-Quentin-en-Yvelines, France). The steady-state kinetic parameters were determined by using the equations described previously (26). To obtain the k_{cat} and K_{m2} values for the Ddl activities, the enzymes were incubated in the presence of increasing concentrations of D-Ala (lowest concentration, 20 mM). K_{m1} was calculated from the activity measured in the presence of low concentrations of D-Ala (0.5–3 mM). The K_m for ATP was measured with fixed concentrations of D-Ala of 10 and 80 mM for the VRSA-6 Ddl and the VRSA-7 Ddl, respectively.

Analysis of peptidoglycan precursors. Extraction and analysis of peptidoglycan precursors were performed with strains VRSA-6 and VRSA-7 after growth without or with 8 μg/ml of vancomycin, as described previously (24). The results were expressed as the percentages of the total late peptidoglycan precursors, represented by tetrapeptides, pentapeptides, and pentadepsipeptides, that were determined from the integrated peak areas.

Detection of PBP 2′. Membrane preparations of strains VRSA-7, RN4220, and COL were obtained as described previously (27). Briefly, exponentially growing bacteria were harvested, washed in 50 mM Tris-HCl–145 mM NaCl, pH 7.5, and resuspended at 30 mg (dry weight)/ml in the same buffer containing lysostaphin (20 mg/liter). The suspension was incubated at 37°C for 15 min, and after addition of 5 mM $MgCl_2$ and DNase (30 mg/liter) the suspension was incubated for an additional 5 min. Membranes were collected by centrifugation at 45,000 × g for 20 min at 4°C. The proteins were solubilized by heating the suspension in buffer containing 10% sodium dodecyl sulfate (SDS), separated by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred electrophoretically to a Hybond-P (polyvinylidene difluoride) membrane (Amersham Biosciences, Buckinghamshire, United Kingdom) by blotting at 80 mA for 45 min. The detection of PBP 2′ was performed with a primary mouse anti-PBP 2′ monoclonal antibody (monoclonal antibody 14A9C9-C6), which was used at a 1:20,000 dilution, and sheep anti-mouse immunoglobulin G antibodies conjugated to horseradish peroxidase (ECL Plus Western blotting; Amersham Biosciences) was used as the secondary reagent at a 1:20,000 dilution.

RESULTS AND DISCUSSION

Characterization of strains VRSA-6 and VRSA-7. Both VRSA-6 and VRSA-7 were resistant to vancomycin (MICs > 256 μg/ml) and teicoplanin (MICs = 16 μg/ml). Multiplex PCR for identification of the *van* genotype and of staphylococci at the species level (13) confirmed that VRSA-6 and VRSA-7 were *S. aureus* and that the glycopeptide resistance was of the VanA type. SmaI-digested genomic DNA of the two strains separated by pulsed-field gel electrophoresis revealed two patterns indicating that the isolates were distinct (data not shown). The stability of the vancomycin resistance was tested by replica plating. In three independent experiments, no derivatives susceptible to vancomycin were obtained. The organization of the *vanA* gene cluster was determined by PCR mapping; and the fragments had sizes indicating that the *vanR*, *vanS*, *vanH*, *vanA*, *vanX*, *vanY*, and *vanZ* genes constituting the *vanA* operon were present and were present in the same order as they are in Tn*1546*. Both strains were constitutively resistant to MLS_B antibiotics, and PCR with specific primers indicated the presence of *erm*(B) in VRSA-6 and of *erm*(A) along with *erm*(C) in VRSA-7. The two strains were also resistant to spectinomycin due to the presence of the *ant(9)-Ia* gene and to kanamycin and tobramycin due to the *ant(4′)-Ia* gene.

Strain VRSA-7 grew slowly on plates without antibiotic as small colonies with various morphologies but grew normally on plates supplemented with vancomycin (8 μg/ml). Determination of the antimicrobial susceptibility of VRSA-7 by Etest showed a high-density culture surrounding the vancomycin strip or the edge of the teicoplanin inhibition zone (Fig. 1), suggesting that the strain was partially dependent on a glycopeptide for growth. The effect of exposure to vancomycin (8 μg/ml) on the growth of VRSA-7 was determined in liquid medium, which better revealed the growth of the strain in the presence of vancomycin (Fig. 2).

Transfer of antibiotic resistance from VRSA-7. Attempts to transfer VanA-type resistance from strain VRSA-7 to *E. faecalis* JH2-2 and *E. faecium* 64/3 by conjugation were unsuccessful. However, vancomycin, erythromycin, and spectinomycin resistance was cotransferred to *S. aureus* BM4602 at a frequency of ca. 1. 10^{-5} per donor cell. The transconjugants acquired, in addition to the *vanA* gene cluster, the *erm*(A) and *ant(9)-Ia* genes but not *erm*(C) or *ant(4′)-Ia*. The SmaI-gener-

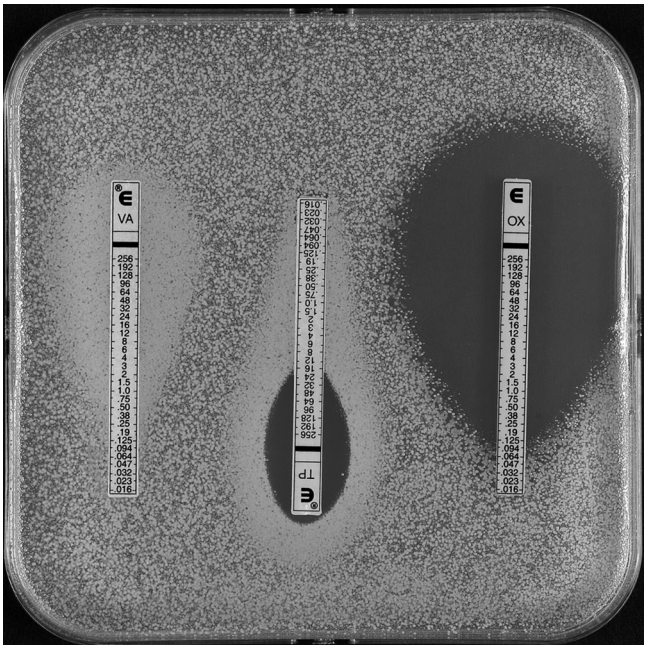

FIG. 1. Determination of MICs for VRSA-7 by Etest. VA, vancomycin; TP, teicoplanin; OX, oxacillin.

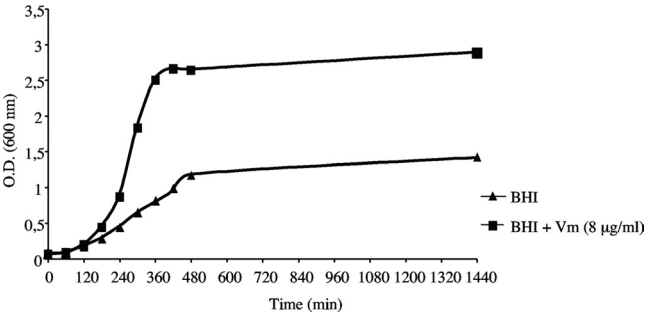

FIG. 2. Effect of exposure to vancomycin (Vm; 8 μg/ml) on growth of VRSA-7. O.D., optical density.

ated patterns of four randomly selected transconjugants were indistinguishable and differed from that of the recipient by a fragment of ca. 35–40 kb that was also present in the donor and that hybridized with the *vanA* probe (data not shown).

Plasmid analysis and localization of the resistance genes in VRSA-7. The *vanA* gene cluster in strain VRSA-7 was assigned to plasmid pIP848 by contour-clamped homogeneous electric field gel electrophoresis of total DNA digested with I-CeuI, followed by successive hybridization with 16S rRNA (*rrs*) and *vanA* probes (data not shown). The copy numbers of the *vanA* and *vanH* genes relative to those of the *ddl*, *rpoB*, and *rrs* chromosomal genes were estimated by qPCR from three independent experiments to be 4.6 ± 1.2 and 5.2 ± 1.9, respectively. Electrotransformation of the VRSA-7 plasmid DNA content into BM4602 with selection on erythromycin (8 μg/ml) gave rise to transformants that were resistant only to MLS_B antibiotics. The plasmid DNA EcoRI restriction patterns of the transconjugants and of the transformants differed from each other: the former harbored only pIP848, whereas two smaller plasmids were present in the transformants. Hybridization experiments indicated that the *vanA*, *erm*(A), and *ant(9)-Ia* genes were located on the ca. 35- to 40-kb pIP848 conjugative plasmid present at five copies per cell and that *erm*(C) was carried by one of the other plasmids in VRSA-7 (data not shown).

Sequences of the *ddl* genes of VRSA-6 and VRSA-7. Vancomycin dependence results from mutations that impair the host Ddl (39). The sequences of the *ddl* gene of strain VRSA-7 and the *ddl* gene of strain VRSA-6, which was used as a control, were determined; and the deduced amino acid sequences were compared (Fig. 3). The *ddl* sequence of VRSA-6 was identical to that of vancomycin-susceptible *S. aureus* COL (GenBank accession no. NC_002951), whereas the Ddl of VRSA-7 had a single point mutation at position 308 that led to an asparagine-to-lysine ($N_{308}K$) substitution. The asparagine at position 308 corresponds to one of the highly conserved residues of the active site of Ddls, and its substitution is therefore likely to affect the activity of the enzyme (Fig. 3).

Functionality of Ddl of VRSA-7. To the best of our knowledge, mutation of the N_{308} residue in the Ddl has not been described previously. It has been demonstrated that the introduction of a *ddl* gene coding for a functional ligase into VanD-type glycopeptide-resistant *E. faecium* BM4339 (vancomycin MIC = 64 μg/ml), which has an impaired Ddl, restores its susceptibility to glycopeptides (8). To determine if the $N_{308}K$ mutation was responsible for the loss of activity of the Ddl, the *ddl* genes from strains VRSA-7 and VRSA-6 were cloned under the control of the P_2 promoter in pAT79 (4); and the recombinant plasmids obtained, pAT516 (pAT79ΩP_2ddl_{VRSA-7}) and pAT515 (pAT79ΩP_2ddl_{VRSA-6}), respectively, were electrotransformed into BM4339. Transformant BM4339/pAT516 (P_2ddl_{VRSA-7}) had the same level of vancomycin resistance as BM4339 (vancomycin MIC = 64 μg/ml), whereas BM4339/pAT515 (P_2ddl_{VRSA-6}) was less resistant (MIC = 16 μg/ml).

The ca. 41-kDa C-terminal His_6-tagged VRSA-6 Ddl and VRSA-7 Ddl overproduced from *E. coli* BL21(DE3)pLys harboring plasmid pAT518 [pET28a(+)Ωddl_{VRSA-6}] or pAT519 [pET28a(+)Ωddl_{VRSA-7}], respectively, were obtained as soluble proteins and were >99% pure, as determined by SDS-PAGE analysis (data not shown). The M_r of Ddl VRSA-7, determined by size-exclusion chromatography, was estimated to be 82,000, suggesting that the dimeric structure was preserved. Kinetic analysis indicated that the VRSA-6 Ddl had K_ms of 1.2 mM and 17 mM for D-Ala subsite 1 (K_{m1}) and D-Ala subsite2 (K_{m2}), respectively, and k_{cat}s of 1,905 min^{-1} (Table 2). The k_{cat} of the $N_{308}K$ mutant Ddl of VRSA-7 was found to be 250-fold lower than that of the VRSA-6 Ddl. The $N_{308}K$ mutation also affected the binding affinity of D-Ala at subsite 2, where the K_{m2} was 4.5-fold higher than that of the VRSA-6 Ddl. Overall, the VRSA-7 Ddl was approximately 1,000-fold less efficient than the VRSA-6 Ddl, as indicated by the relative k_{cat}/K_{m2} catalytic efficiency values (Table 2). The K_m values for ATP were similar, indicating that the $N_{308}K$ mutation did not affect the ATP affinity binding site. The crystal structure of the Ddl of *S. aureus* COL (StaDDl) in complex with ADP has been reported previously (23). However, the crystal structure of substrate analog-bound StaDDl is not available, and the role of the N_{308} residue is not clearly identified. However, modeling

FIG. 3. Alignment of the amino acid sequences of VRSA-6 Ddl, VRSA-7 Ddl, and *E. coli* DdlB. The amino acid substitution $N_{308}K$ is indicated by an arrow. Numbers refer to the positions of the amino acids in DdlB. Conserved residues are highlighted in dark gray. The active-site residues conserved in all Ddls are underlined.

of the homology with other ligases suggests that the side chain nitrogen of N_{308}, the side chain of R_{291}, and the main chain amide of G_{312} form an oxyanion hole that probably interacts with the phosphate of the D-alanyl-phosphate intermediate. The $N_{308}K$ mutation thus presumably affects the binding of the transition-state intermediate, leading to the loss of activity.

Expression of *vanA* gene cluster. The level of expression of the *vanA* operon in strains VRSA-7 and VRSA-6 was determined by comparative analysis of the nature and relative amounts of cytoplasmic peptidoglycan precursors (Fig. 4). In the absence of induction by vancomycin, pentapeptides were the main late precursors synthesized by VRSA-6 (86%), whereas induction by vancomycin resulted in the presence of pentapeptides (22%), pentadepsipeptides (64%), and tetrapeptides (14%). Strain VRSA-7 synthesized mainly pentadepsipeptide precursors (ca. 80%) without or with induction. The synthesis of very low levels of pentapeptide precursors (15%) in VRSA-7 could be due to the residual activity of the host ligase or to that of the VanA D-Ala:D-Lac ligase, which can also synthesize D-Ala–D-Ala (7). The loose regulation of the *vanA* operon by the VanR-VanS system, which allows low-level expression of the *vanA* operon in the absence of induction (3), combined with a gene dosage effect due to the location of the *vanA* gene cluster on a multicopy plasmid, could account for the significant expression of the *vanA* operon in the absence of an inducer in VRSA-7. These data, combined with the facts that VRSA-7 grows more efficiently in the presence of glycopeptides and that the sequences of *vanR* and *vanS* in this strain had no mutations, indicate that the expression of the *vanA* operon is inducible.

Methicillin resistance in VRSA-7. To date, all *S. aureus* isolates that have acquired the *vanA* operon also possess the *mecA* gene. Methicillin resistance is due to the synthesis of an

TABLE 2. Kinetic parameters of Ddl VRSA-6 and Ddl VRSA-7 ligases for D-Ala–D-Ala synthesis

Ddl source	K_{m1}[a] (mM)	K_{m2}[b] (mM)	k_{cat} (min^{-1})	k_{cat}/K_{m2} (min^{-1}/mM)	Relative k_{cat}/K_{m2}	ATP K_m[c] (mM)
VRSA-6	1.2	17	1,925	113	1	0.70
VRSA-7	ND[d]	76.5[e]	7.6[f]	0.01	0.9×10^{-3}	0.86

[a] K_{m1}, K_m at subsite 1.
[b] K_{m2}, K_m at subsite 2.
[c] Determined with 10 mM D-Ala for the VRSA-6 Ddl and 80 mM D-Ala for the VRSA-7 Ddl.
[d] ND, not detectable.
[e] The highest substrate concentration tested.
[f] Activity was measured with 50 μg (1.25 μM) of enzyme.

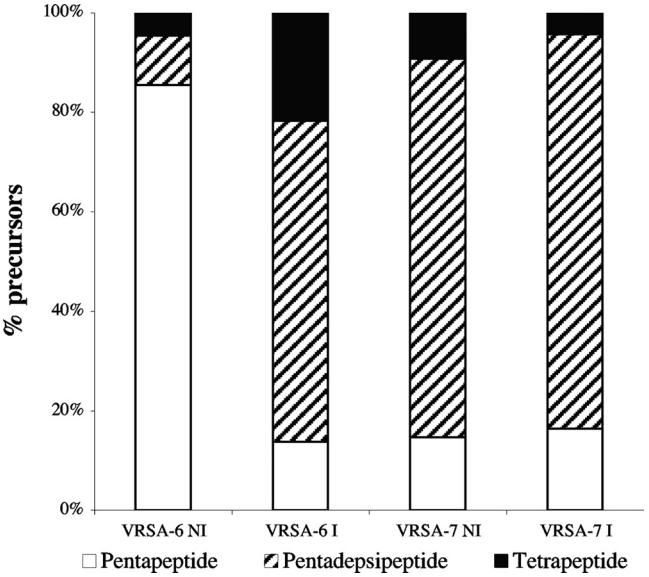

FIG. 4. Cytoplasmic peptidoglycan precursors in VRSA-6 and VRSA-7. NI, not induced; I, induced by growth in the presence of 8 μg/ml of vancomycin.

additional PBP, PBP 2′, encoded by the *mecA* gene, which exhibits a low affinity for β-lactams (31). PBP 2′ is the only transpeptidase that remains active in the presence of β-lactams in the medium (11). It has been demonstrated that pentadepsipeptide peptidoglycan precursors ending in D-Ala–D-Lac are not substrates for PBP 2′ (35), resulting in a strong synergism between glycopeptides and ß-lactams against VRSA isolates (17, 29). Strain VRSA-7 was susceptible to oxacillin (MIC = 0.125 μg/ml), even in the absence of glycopeptides in the culture medium. Determination of the sequence of a 2,291-bp PCR fragment containing the *mecA* gene and its promoter revealed no mutations. The PBP 2′ protein, which has an expected size of 76 kDa, was detected by Western blotting with an anti-PBP 2′ monoclonal antibody (data not shown). It therefore appears that inactivation of the VRSA-7 Ddl and acquisition of the *vanA* gene cluster, which are responsible for a lack of peptidoglycan precursors ending in D-Ala–D-Ala and the synthesis of precursors mainly ending in D-Ala–D-Lac, respectively, result in the loss of resistance to ß-lactams, despite the presence of a functional *mecA* gene.

In summary, the $N_{308}K$ mutation in the Ddl of strain VRSA-7 has impaired the activity of the enzyme, and thus, the strain must rely on the vancomycin-inducible activity of the acquired VanA D-Ala:D-Lac ligase for growth. The basal level of expression of the *vanA* operon due to a combination of loose regulation by the two-component regulatory system and a gene dosage effect accounts for the slow growth of VRSA-7 in the absence of vancomycin, whereas following induction, full expression of the operon allows the normal growth of the strain. The synthesis of pentadepsipeptide peptidoglycan precursors by VRSA-7, even in the absence of vancomycin in the medium, that cannot be processed by PBP 2′ is responsible for susceptibility to ß-lactams. Thus, it is conceivable that patients infected with this type of strain could be treated with ß-lactams, despite the presence of a functional *mecA* gene, a hypothesis that remains to be critically tested in an animal model. The prevalence of clinical isolates of VRSA partially dependent on vancomycin for growth may be underestimated, since these strains can be missed in routine laboratory practice because of their particular nutritional requirement.

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