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vanA Gene Cluster in a Vancomycin-Resistant Clinical Isolate of *Bacillus circulans*

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We report on the cloning and sequencing of the *vanA* gene cluster present in the glycopeptide-resistant clinical isolate *Bacillus circulans* VR0709 (R. Fontana, M. Ligozzi, C. Pedrotti, E. M. Padovani, and G. Cornaglia, Eur. J. Clin. Microbiol. Infect. Dis. 16:473–474, 1997). The presence of a *vanA*-related gene in VR0709 was demonstrated in a PCR assay which permitted the specific amplification of an internal segment of *vanA*. Southern blotting suggested that the *vanA* gene was located in the chromosome in a 7.6-kb *Eco*RI fragment. DNA sequence analysis revealed the presence of all seven genes of the *vanA* cluster (*vanR*, *vanS*, *vanH*, *vanA*, *vanX*, *vanY*, and *vanZ*). The degree of identity between homologous proteins encoded by Tn*1546* and the chromosome of *B. circulans* VR0709 ranged from 87 to 95%. Neither PCR nor Southern blotting with specific primers and probes, respectively, showed the presence of open reading frames (ORFs) 1 and 2 which encode the transposase and the resolvase of Tn*1546*, respectively, the transposon found to carry the *vanA* gene cluster in enterococci. Determination of the sequences of the flanking regions of the *van* gene cluster of *B. circulans* revealed perfect inverted repeats of 10 bp which delineated a 9.2-kb region containing the *van* gene cluster and an ORF which encoded a putative protein (178 residues) which displayed a low level of identity (28%) to the resolvase of Tn*1546*. These results suggest that glycopeptide resistance in *B. circulans* VR0709 is associated with the acquisition of a *vanA* gene cluster which shows a high degree of homology with that of enterococci. In *B. circulans*, however, the cluster is not carried by Tn*1546* and is borne by the chromosome.

Most gram-positive bacteria are naturally susceptible to glycopeptide antibiotics (vancomycin and teicoplanin). Resistance to these compounds is an intrinsic property of some occasional human pathogens such as lactobacilli, leuconostoc, pediococci, *Erysipelothrix rhusiopathiae*, *Enterococcus gallinarum*, *Enterococcus casseliflavus*, and *Enterococcus flavescens* (22). Acquisition of glycopeptide resistance was first described in enterococci in 1988 (9, 20) and is mediated by three classes of related gene clusters that confer inducible resistance to high levels of vancomycin and teicoplanin (*vanA*), inducible resistance to various levels of vancomycin (*vanB*), or resistance to vancomycin and low levels of teicoplanin (*vanD*) (1, 4, 12, 14, 21).

VanA enterococci harbor a Tn*3*-like transposon (the prototype was designated Tn*1546*) which carries a cluster of seven genes (*vanR*, *vanS*, *vanH*, *vanA*, *vanX*, *vanY*, and *vanZ*) required for the phenotypic expression of resistance and which is generally located on large plasmids (1). The *vanB* cluster is carried by large conjugative elements and less frequently by plasmids (17). Transfer of genetic elements carrying *vanA* and *vanB* genes to susceptible enterococcal strains and, in the case of *vanA*, to strains of nonenterococcal species such as *Streptococcus sanguis*, *Streptococcus lactis*, *Streptococcus pyogenes*, *Bacillus thuringiensis*, *Staphylococcus aureus*, and listeriae of different species has been achieved under laboratory conditions (2, 3, 8, 10, 13). However, the natural spread of vancomycin resistance determinants among gram-positive bacteria other than enterococci has seldom been reported. To date, this phenomenon has been documented by the isolation from clinical specimens of one vancomycin-resistant strain of *Oerskovia turbata* and one strain of *Arcanobacterium* (*Corynebacterium*) *haemolyticum*, both of which harbor the *vanA* gene, and of one *Streptococcus bovis* strain that harbors the *vanB* gene (15, 16).

Recently, we described a glycopeptide-resistant strain of *Bacillus circulans* (a species naturally susceptible to these antibiotics) isolated from a catheter tip as part of routine activities at the Clinical Microbiology Laboratory of the Verona University Hospital (6). Since the natural dissemination of vancomycin resistance determinants among gram-positive bacteria is a matter of serious public health concern, we studied the mechanism of vancomycin resistance in our clinical isolate of *B. circulans*. We found that this strain harbored a *vanA*-like gene cluster with a high degree of homology with that of enterococci but was borne by the chromosome and was apparently carried by a genetic element other than Tn*1546*.

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MATERIALS AND METHODS

Bacterial strains. *B. circulans* VR0709 is a motile, catalase-positive, aerobic, spore-forming, gram-positive rod and was identified by the API CH and API 20E System biochemical profiles (bioMèrieux, Marcy l'Etoile, France). *B. circulans* ATCC 4513 was obtained from the American Type Culture Collection, Rockville, Md. *Enterococcus faecium* BM4147 carrying Tn*1546* in plasmid pIP816 was used as the reference strain. *Escherichia coli* DH5α was purchased from Stratagene (Cambridge, United Kingdom). Bacteria were routinely grown in brain heart infusion (BHI) broth and agar (Difco, Detroit, Mich.) at 37°C.

Susceptibility test. The MICs of vancomycin and teicoplanin were determined on Mueller-Hinton broth (MHB) or Mueller-Hinton agar (MHA) (Difco) by standard dilution techniques (11).

Inducibility of vancomycin resistance. Cultures of strain VR0709 were grown overnight in MHB at 37°C in the presence of subinhibitory concentrations of vancomycin (8 μg/ml) and were diluted to an A_{640} of 0.1 in fresh MHB with and without the same concentrations of vancomycin. The cultures were grown at 37°C with shaking, and A_{640} values were measured at regular intervals on a Pharmacia spectrophotometer.

Primers and digoxigenin-labeled probes. Primers A1 and A2 (*vanA*), B1 and B2 (*vanB*), C1 and C2 (*vanC1*), and D1 and D2 (*vanC2*, *vanC3*), selected by Dutka-Malen et al. (5), were used to specifically amplify the *van* genes. The primer sets used for amplification of ORFs 1 and 2 and the *van* genes of Tn*1546* were as described previously (7). When necessary, primers were also derived from the *B. circulans* DNA by sequencing. The primers were synthesized by Boehringer Mannheim, Mannheim, Germany. To be used as hybridization probes, the PCR products were labeled directly with digoxigenin (DIG)-dUTP by

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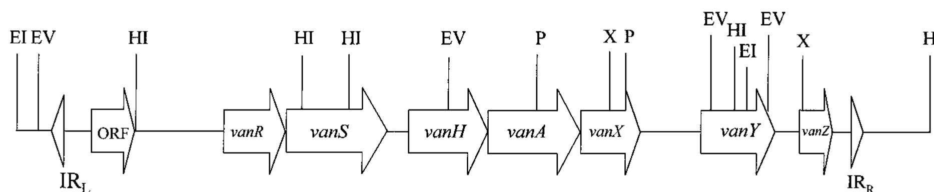

FIG. 1. Restriction map of the 9.2-kb *Eco*RI-*Hin*dIII fragment of *B. circulans* VR0709 containing the $vanA_{bc}$ cluster. EI, *Eco*RI; EV, *Eco*RV; HI, *Hin*dIII; P, *Pst*I; X, *Xba*I.

using a PCR DIG-labeling mix (Boehringer Mannheim). The reaction was run in a 100-μl volume under the PCR conditions described below.

Preparation of DNA. Plasmid DNA was prepared by a rapid alkaline lysis method (18). For preparation of total DNA, 3 ml of bacterial culture in BHI broth (37°C, 18 to 48 h) was centrifuged (14,000 × *g*, 10 min). After washing with 0.85% sterile NaCl, the bacteria were suspended in 400 μl of sucrose solution (6.7%), 25 μl of lysozyme (10 mg/ml) was added, and the mixture was incubated for 30 min at 37°C. Fifty microliters of 20% sodium dodecyl sulfate (SDS) was then added, and the mixture was incubated for 30 min and digested with 5 μl of proteinase K solution (20 mg/ml) at 37°C for 30 min. Genomic DNA was extracted twice with 1 volume of phenol-chloroform-isoamyl alcohol. After adding 0.1 volume of 3 M sodium acetate, DNA was precipitated in ethanol, air dried, and dissolved in 80 μl of sterile water.

PCR amplification. PCR was performed on a DNA thermal cycler (Mini-Cycler; GENENCO, Florence, Italy) in a final volume of 50 μl containing 250 ng of DNA as template, 0.5 μM (each) primer, 200 μM (each) deoxynucleoside triphosphate, and 2 U of *Taq* high-fidelity DNA polymerase (Boehringer Mannheim) in a 1× amplification buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$). The PCR mixtures were denatured (2 min at 94°C) and were then subjected to 35 cycles of amplification (1 min of annealing at 55°C, 1 min of elongation at 72°C, and 1 min of denaturation at 94°C) and a final elongation step of 72°C for 10 min. PCR products were resolved by electrophoresis in a 1% agarose gel stained with ethidium bromide.

DNA-DNA hybridization. Genomic and plasmid DNAs were digested with restriction enzymes as recommended by the manufacturer (Boehringer Mannheim) and were electrophoresed through a 0.8% agarose gel. The DNA restriction fragments were electroblotted onto a Hybond-N$^+$ nylon membrane (Amersham International, Amersham, United Kingdom) and were hybridized with the DIG-dUTP-labeled PCR products. Hybridizations were performed as follows: prehybridization and hybridization were carried out for 1 and 18 h, respectively, at 68°C in 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–0.02% SDS–1% (wt/vol) blocking reagent for nucleic acid hybridization (Boehringer Mannheim)–0.1% *N*-lauryl-sarcosine, followed by two washings in 0.2× SSC–0.1% SDS at 65°C for 15 min.

DNA cloning and sequencing. The sequence from *vanR* to *vanY* of the *B. circulans* chromosome was determined on cloned PCR fragments obtained by amplification of *B. circulans* DNA with primers derived from the sequence of Tn*1546* or based on the sequence obtained from fragments of *B. circulans* DNA. The region upstream from *vanR* was sequenced by cloning and sequencing first the 1.5-kb *Hin*dIII fragment containing *vanR* (Fig. 1) and then the fragment produced by inverse PCR carried out with a primer (3′ to 5′) based on the 5′ sequence of the 1.5-kb *Hin*dIII fragment and a primer (5′ to 3′) based on the 3′ end sequence of the 7.6-kb *Eco*RI fragment (Fig. 1). The sequence downstream from *vanZ* of *B. circulans* was determined by cloning and sequencing the 1.7-kb *Hin*dIII fragment containing *vanZ*. The amplification products were blunt ended and phosphorylated by standard procedures (18) and were then ligated into *Sma*I-digested, dephosphorylated SK plasmid. Fragments derived from enzymatic digestion were cloned into pUC18. M13 universal and reverse primers were used to sequence both strands of the inserts. Sequencing was performed with three independent PCR clones by the dideoxynucleotide termination method (19) with an ALF Sequencer and fluorescent dATP (Pharmacia, St. Albans, United Kingdom).

Nucleotide sequence accession numbers. The nucleotide sequences of the genes of the *B. circulans vanA* ($vanA_{bc}$) cluster have been assigned EMBL accession no. Y15704 ($vanA_{bc}$), Y15705 ($vanH_{bc}$), Y15706 ($vanR_{bc}$), Y15707 ($vanS_{bc}$), Y15708 ($vanX_{bc}$), Y17303 ($vanY_{bc}$), Y17304 ($vanZ_{bc}$), and Y17305 (ORF).

RESULTS AND DISCUSSION

***B. circulans* VR0709 properties.** *B. circulans* VR0709 is a motile, catalase-positive, aerobic, spore-forming, gram-positive rod identified as *B. circulans* by conventional biochemical methods. On BHI agar or MHA it formed ovoid colonies which swarmed after only a few days of incubation. Morphologically, the isolate showed the characteristic shape of aerobic bacilli. It formed rods (0.8 to 1 μm in width and 2 to 5 μm in length) that mostly contained a central or subterminal spore. Interestingly, the spore-forming ability was greatly reduced for bacteria grown in the presence of vancomycin at sub-MICs.

Strain VR0709 is the first natural clinical isolate of a vancomycin-resistant *Bacillus* described so far. It expressed resistance to glycopeptides in both liquid and solid media. In MHB, the MICs of vancomycin and teicoplanin were 64 and 32 μg/ml, respectively; in MHA the MICs were 256 and 32 μg/ml, respectively. The MICs of both antibiotics for strain ATCC 4513 were 0.5 μg/ml. Inducibility experiments showed that the rates of growth of *B. circulans* VR0709 were unaltered by exposure to vancomycin at sub-MICs, suggesting that expression of resistance to glycopeptides was constitutive in this organism (Fig. 2).

Identification and localization of vancomycin resistance determinant of *B. circulans* VR0709. The presence of the *van* genes was investigated in VR0709 by PCR carried out with primers from the *vanA*, *vanB*, and *vanC* genes of enterococci (5). A positive reaction was obtained with *vanA* primers only, which gave the expected 800-bp amplification product. No amplification of *B. circulans* ATCC 4513 DNA was obtained with the same sets of primers.

In enterococci the *vanA* gene cluster is found to reside on transposons that are similar or related to Tn*1546* and that are located either on plasmids or on the chromosome. *B. circulans*

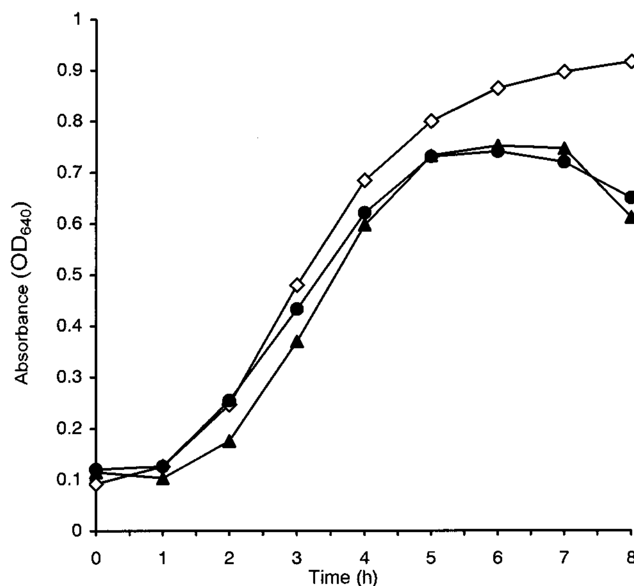

FIG. 2. Growth of *B. circulans* VR0709 in MHB in the absence of vancomycin (diamonds) and in the presence of 8 μg of vancomycin per ml after pregrowth in MHB with (black circles) and without (black triangles) the antibiotic. OD_{640}, optical density at 640 nm.

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TABLE 1. Comparison between the $vanA_{bc}$ and *vanA* gene clusters

B. circulans gene or sequence	Length (bp)	% Nucleotide identity	% Amino acid identity
IR_L-ORF[a]	195	33	
ORF[b]	534	54	28
ORF-*vanR*[c]	737	53	
vanR	696	91	89
vanS	1,140	93	92
vanS-vanH	213	89	
vanH	969	91	89
vanA	1,032	94	95
vanA-vanX	5	100	
vanX	609	95	94
vanX-vanY	434	73	
vanY	894	93	89
vanY-vanZ	154	76	
vanZ	486	91	87
vanZ-IR_R[d]	138	44	

[a] The nucleotide sequence was matched with the left terminal IR (IR_L)-ORF1 intervening sequence of Tn*1546*.

[b] The nucleotide and amino acid sequences were matched with ORF2 of Tn*1546*.

[c] The nucleotide identity was calculated by matching a 211-bp region upstream from $vanR_{bc}$ with ORF2-*vanR* intergenic sequence of Tn*1546*.

[d] The nucleotide sequence was matched with *vanZ*-IR_R intervening sequence of Tn*1546*.

VR0709 was found to harbor a small (7.28-kb) plasmid designated pVR0709; after *Eco*RI digestion the plasmid gave four fragments of 2.91, 2.15, 1.54, and 0.82 kb, respectively. None of these fragments hybridized with DIG-labeled amplicons of the enterococcal *vanA* gene, whereas the same probe reacted with a 7.6-kb *Eco*RI fragment of total DNA, suggesting a chromosomal location of the gene. In contrast, the *vanA* gene of Tn*1546* was confirmed to reside in a 4.1-kb *Eco*RI fragment of *E. faecium* BM4147 plasmid and total DNA.

Characterization of the *van* gene cluster of *B. circulans* VR0709. The presence of other genes of the *vanA* cluster in strain VR0709 was investigated by PCR performed with primers from ORFs 1 and 2 and from all the seven genes of the enterococcal cluster. If amplification products of the expected size were not obtained, the presence of a gene was investigated by probing the total DNA with DIG-labeled amplicons of the corresponding enterococcal gene. By using this strategy the presence of sequences homologous to all seven genes of the cluster but not to ORFs 1 and 2 was demonstrated.

To evaluate the homology of the *van* cluster of VR0709 with that of enterococci, the 9.2-kb *Eco*RI-*Hin*dIII fragment of *B. circulans* DNA (Fig. 1) was sequenced. It was found to contain sequences homologous to the *vanR*, *vanS*, *vanH*, *vanA*, *vanX*, *vanY*, and *vanZ* genes, in that order. The analysis of the nucleotide and the derived amino acid sequences showed a high degree of identity (93 to 96%) between homologous genes of Tn*1546* and *B. circulans vanA* gene clusters as well as the encoded proteins (87 to 95%) (Table 1). However, the degree of identity between the genes and the intergenic regions was less than would be expected if there had been the direct transfer of the gene cluster from enterococci, suggesting that these genes might have been acquired from a source different from that from which the enterococcal genes were acquired. No *Eco*RI sites were found in *B. circulans* $vanS_{bc}$, which explained the difference in the length of the *Eco*RI fragment which hybridized with the *vanA* probe. The genes of VR0709 were designated $vanR_{bc}$, $vanS_{bc}$, $vanH_{bc}$, $vanA_{bc}$, $vanX_{bc}$, $vanY_{bc}$, and $vanZ_{bc}$ and the entire cluster was designated $vanA_{bc}$.

The sequence of the van_{bc} cluster significantly diverged from that of Tn*1546* 6 bp upstream from the $vanR_{bc}$ start codon and immediately downstream from the $vanZ_{bc}$ stop codon (Fig. 3). A search for stop codons of each DNA strand in the nucleotide sequence 1.8 kb upstream from the translated initiation codon of $vanR_{bc}$ identified one open reading frame (ORF) transcribed in the same direction as the van_{bc} genes. The putative coding region in the ORF was assigned to positions 612 to 1146 of the 9.2-kb *Eco*RI-*Hin*dIII fragment. This was based on the presence of an in-frame ATG translation initiation codon preceded by a ribosome-binding site-like sequence. The corresponding 534-bp sequence could code for a protein of 178 amino acids and with a molecular mass of 21.564 Da. This protein showed a 28% amino acid sequence identity with the resolvase encoded by Tn*1546* ORF2 (Table 1). The ORF-$vanR_{bc}$ intergenic region was 731 bp long and did not show significant homology with the ORF2-*vanR* region of Tn*1546* up to the 6-bp sequence immediately upstream from the start codon of $vanR_{bc}$.

Sequence analysis of the region downstream from the *vanZ* stop codon did not identify any ORF. Perfect 10-bp inverted repeats (IRs; positions 416 to 425 and 8628 to 8637) that were not related to the IRs of Tn*1546* but that might delineate an 8,221-bp transposon-like element were found. No direct repeats suggestive of target sequence duplication were found.

These results suggest that Tn*1546*-like transposons were not components of the element responsible for the transfer of the *vanA* gene cluster in *B. circulans*. Heterogeneity of the *vanA* cluster was recently observed in clinical isolates of enterococci from the northeastern United States, but this was explained by insertion of IS*1251* in the *vanS-vanH* intergenic region of Tn*1546*-like transposons or by truncation of this element (7). The possibility that *B. circulans* VR0709 contained a truncated Tn*1546* lacking ORFs 1 and 2 was excluded by the very low level of identity in the sequence downstream from $vanZ_{bc}$ and the lack of the right terminal IR (IR_R) which ends Tn*1546*-like transposons. To the best of our knowledge this is the first report of the presence of the *vanA* cluster in a genetic element other than Tn*1546*.

In spite of the increasing number of nonenterococcal species found to be able to acquire the *van* cluster under laboratory or natural conditions, the clinical relevance of the resistance encoded by these genes still remains confined to enterococci (22). No serious infections or nosocomial outbreaks have been reported as being caused by nonenterococcal strains carrying *van* genes. In particular, it is surprising that clinical isolates of staphylococci carrying *van* genes have never been isolated, even though these microorganisms naturally exchange antibiotic resistance determinants with enterococci and the transfer of the *vanA* gene by conjugation from *E. faecalis* to *S. aureus* has been obtained under laboratory conditions (13). It is possible that the expression of the *van* genes in certain nonenterococcal species is not compatible with survival in natural environments (i.e., host biological fluids or tissues). The recent isolation of a clinical *S. aureus* isolate in which a reduced level of vancomycin susceptibility was associated with an increased level of synthesis of peptidoglycan precursors and PBPs 2 and 2′ (13) provides indirect support for this hypothesis and should predict that, in this species, clinically relevant resistance will be acquired by mechanisms other than those encoded by *van* gene clusters.

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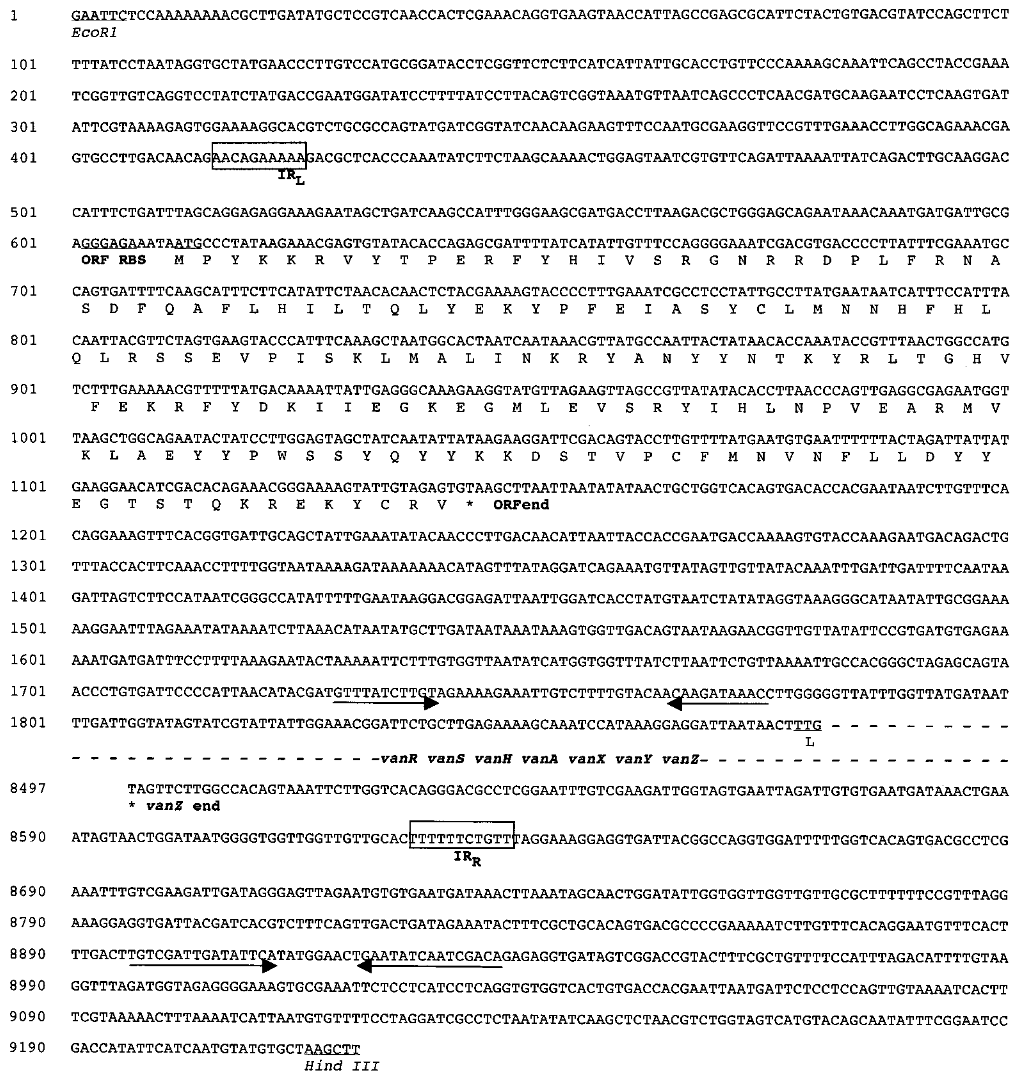

FIG. 3. Nucleotide sequence of the regions flanking the *vanA* gene cluster contained in the 9.2-kb *Eco*RI-*Hin*dIII fragment of *B. circulans* DNA. The left (IR_L) and right (IR_R) terminal IRs of the hypothetical transposon-like element containing the $vanA_{bc}$ cluster are boxed. The deduced amino acid sequence of the ORF is shown below the nucleotide sequence. Ribosome-binding sites (RBS) and translation initiation codons are underlined. The IRs in the ORF-$vanR_{bc}$ intergenic region and at the left extremity of the fragment are indicated by arrows.

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