Resistance to glycopeptides in enterococci has become an increasingly worrisome problem in clinical practice. Resistance is observed most commonly in Enterococcus faecium, which is often resistant to other classes of antibiotics such as β-lactams and aminoglycosides, and to a lesser extent in Enterococcus faecalis (19). In these species, resistance to glycopeptides is mediated by mobile elements which encode the production of enzymes under the control of a two-component regulatory system. These enzymes result in the synthesis of late peptidoglycan precursors ending in D-Ala-D-Lac whereas VanB-type strains remain susceptible to teicoplanin because both antibiotics are inducers, strains are characterized by a high level of resistance to vancomycin. Enterococci that require the presence of vancomycin in the culture medium and transcriptional activation of the resistance genes. Two proteins are essential for the production of peptidoglycan precursors ending in D-Ala-D-Lac, namely, a dehydrogenase (VanH or VanHB) which synthesizes D-Lac from pyruvate and a ligase (VanA or VanB) which ligates D-Lac to D-Ala. Two proteins (a D-depeptidase [VanX or VanXa] and a D-carboxypeptidase [VanY or VanYb]) are responsible for the catabolism of D-Ala-D-Ala termini, by cleaving the D-Ala-D-Ala dipeptide or by removing the ultimate D-Ala from the pentapeptide precursor. VanA-type strains are characterized by a high level of resistance to vancomycin and teicoplanin because both antibiotics are inducers, whereas VanB-type strains remain susceptible to teicoplanin because this glycopeptide is not an inducer of the vanB operon.

More recently, enterococci that require the presence of vancomycin in the culture medium for effective growth have been isolated from patients treated for long periods with this antibiotic. E. faecalis strains with this phenotype have been isolated from urine or blood samples (14, 15, 28), and E. faecium strains have been isolated from feces or blood samples (7, 16, 31). Similar mutants have also been isolated from animals treated with vancomycin (5) or obtained in vitro by growing bacteria in the presence of the antibiotic (6, 26). The mechanism responsible for vancomycin dependence has been studied in two in vitro mutants. In each case, a unique mutation in the ddl gene encoding the host D-Ala-D-Ala ligase was found. The mutations were responsible for inactivation of the enzyme following synthesis of a truncated protein (26) or replacement of an amino acid essential for substrate binding (6). The bacteria grew only in the presence of vancomycin, since this antibiotic was required for induction of the resistance proteins and, therefore,
for synthesis of peptidoglycan from D-Ala-D-Lac- instead of D-Ala-D-Ala-containing precursors.

In the present study, we report the mechanism of vancomycin dependence in clinical strains of *E. faecalis* with the vanB genotype isolated from three patients treated with vancomycin for prolonged periods. We also studied in vitro mutants of one of these strains which either reverted to a nondependent highly glycopeptide-resistant phenotype or became dependent on both vancomycin and teicoplanin for growth.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The isolates used in this work are described in Table 1 and Fig. 1. *E. faecalis* BM4386 is an isolate from the bloodstream of a patient at Hahnemann University (Philadelphia, Pa.) previously treated with vancomycin for a *Staphylococcus aureus* infection. BM4387 and TJ310 (15) are urinary tract isolates from patients at Thomas Jefferson University Hospital (Philadelphia, Pa.) who had received vancomycin. TJ282, a non-vancomycin-dependent strain (15), and TJ310 were isolated from one patient, but TJ282 was isolated several days earlier than TJ310 was.

Three types of mutants with distinct phenotypes were obtained by growing...
comycin and teicoplanin were determined after 48 h of incubation by the method revertant mutants (BM4388, BM4389, and BM4390) and the nondependent grown in the same medium supplemented with 10 μg of vancomycin per ml, and the teicoplanin-dependent in vitro mutant (BM4391) was (BHI; Difco Laboratories, Detroit, Mich.) supplemented with 10 μg of teicoplanin per ml. The revertant mutants (BM4388, BM4389, and BM4390) and the nondependent clinical strain TJ332 were grown in unsupplemented BHI or in BHI containing 4 to 10 μg of vancomycin per ml for induction of the resistance genes.

Phenotypic and genotypic characterization of the strains. The MICs of vancomycin and teicoplanin were determined after 48 h of incubation by the method of Sneath et al. (27) with 103 CFU per spot on BHI or on BHI supplemented with 50 mM D-Ala-D-Ala (to determine the MICs of teicoplanin for the vancomycin-dependent strains) or with 4 or 10 μg of vancomycin per ml (to induce the expression of resistance genes). Susceptibility to antibiotics other than glycopeptides was determined by disk agar diffusion on BHI or BHI supplemented with 10 μg of vancomycin per ml (for vancomycin-dependent strains) or 10 μg of teicoplanin per ml (for teicoplanin-dependent BM4391). Enterococci were identified at the species level by a PCR assay (8) with DNA from E. faecalis (11) and the vanB (12) and vanA (8) genes and confirmed by Southern hybridization of EcoRI-digested total DNA (21) with a vanB probe (11) labeled with [-32P]dCTP (Radiochemical Center, Amersham, United Kingdom) by nick translation using E. faecalis V583 (vanA) as a positive control. The glycopeptide resistance genotype was determined by a PCR assay with the primers for vanB (12) and vanA (8) genes.

The vancomycin-dependent clinical strains were grown in brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) supplemented with 10 μg of vancomycin per ml, and the teicoplanin-dependent in vitro mutant (BM4391) was grown in the same medium supplemented with 10 μg of teicoplanin per ml. The revertant mutants (BM4388, BM4389, and BM4390) and the nondependent clinical strain TJ332 were grown in unsupplemented BHI or in BHI containing 4 to 10 μg of vancomycin per ml for induction of the resistance genes.

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Zero-integrated field gel electrophoresis. Genomic DNA embedded in agarose gel (18) was digested overnight at 37°C with 100 U of NotI endonuclease. The restriction fragments were separated with a software-assisted zero-integrated field gel electrophoresis apparatus according to the manufacturer’s recommendations (Auto Base; Q-Life System Inc.). This apparatus was used with ROM card no. 3 that optimizes DNA fragment separation in the 8- to 500-kb range.

Amplification, cloning, and sequencing of the ddi, vanS, vanR, and vanG genes. Purified total DNA was used as a template for amplification by PCR, using Pfu polymerase and the oligodeoxynucleotide primers for the ddi gene of E. faecalis (11) and the vanS, vanR, and vanG genes (6). Amplification was carried out in a 100-μl volume containing 50 pmol of each oligonucleotide primer, 50 nmol of each of the 2 deoxynucleoside 5’ triphosphate, reaction buffer, 100 ng of DNA, and 1.25 U of DNA Pfu polymerase. A total of 30 cycles of PCR were done: 1 cycle consisted of 1 min at 94°C for denaturation, 1 min at 54°C for annealing, and 2 min 30 s at 72°C for polymerization. The PCR products were purified from agarose gels (Seephagsa kit; Pharmacia, Uppsala, Sweden), cloned into pCR-Blunt (Invitrogen, Leek, The Netherlands), and sequenced by the dideoxy-chain termination method (24), using T7 DNA polymerase (Sequenase kit; U.S. Biochemical Corp., Cleveland, Ohio) and [α-32P]dATP (Amersham Radiochemical Center).

Extraction and analysis of peptidoglycan precursors. Extraction and analysis of the peptidoglycan precursors were performed by the procedures of Reynolds and coworkers (22). In brief, enterococci were grown in BHI medium supplemented with yeast extract and glycopeptides (if induction of resistance was required). Ramoplanin was added to inhibit peptidoglycan synthesis, and incubation was continued for one half of the mean generation time to cause peptidoglycan synthesis. The restriction fragments were separated with a software-assisted zero-integrated field gel electrophoresis apparatus according to the manufacturer’s recommendations (Auto Base; Q-Life System Inc.). This apparatus was used with ROM card no. 3 that optimizes DNA fragment separation in the 8- to 500-kb range.

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RESULTS AND DISCUSSION

Clinical isolates. (i) Characterization of the strains. We have studied three isolates of vancomycin-dependent (VmA) E. faecalis of the vanB genotype. A vancomycin-resistant (Vmr) VanB-type E. faecalis TJ332 isolated from the same patient as TJ310 but isolated a few days earlier was included in the investigation. The three VmA E. faecalis strains isolated from different patients at various hospitals and at different times were considered different strains since they had distinct restriction fragment patterns following pulsed-field gel electrophoresis (Fig. 2) and differed in their susceptibility to antibiotics other than glycopeptides. TJ310 was resistant to gentamicin; BM4386 was resistant to gentamicin, streptomycin, and erythromycin; and BM4387 was also resistant to chloramphenicol. All were highly resistant to vancomycin and remained susceptible to teicoplanin when their growth was triggered by D-Ala-D-Ala (Table 1). However, they displayed variable levels of resistance to teicoplanin in the presence of 10 μg of vancomycin per ml to induce expression of the resistance genes.

In contrast to TJ310, TJ328 was not dependent on vancomycin for growth but was resistant to low levels of this antibiotic (Table 1). Like TJ310, TJ328 had the vanB gene and was resistant to gentamicin. However, it was also resistant to streptomycin and erythromycin and differed from TJ310 by two bands in its restriction fragment pattern (Fig. 2). A previously published pattern of TJ328 and TJ330 using Smal digests also demonstrated two band differences between these isolates (15). Although TJ328 is probably clonally related to TJ310, it may not be its direct progenitor in vivo.

(ii) Sequence of the ddi gene. It was recently shown that vancomycin dependence of mutants obtained in vitro was associated with mutations in the host ddi gene leading to synthesis of an inactive D-Ala-D-Ala ligase (6, 26). We therefore sequenced the ddi genes of the three VmA clinical isolates and VmA TJ328. The ddi sequence of the latter was identical to that of E. faecalis V583 (11). In contrast, a mutation in the ddi gene of the VmP strains was found (Fig. 3). The ddi genes of BM4386 and BM4387 had a single point mutation at codon 295 (GAT to GTT) and 316 (ACT to ATT), leading to Asp-to-Val and Thr-to-Ile substitutions, respectively. The ddi gene of TJ310 had suffered a deletion of 6 bp (GAT GCA AAA to
GAA) at codons 251 to 253, leading to the substitution of Asp-Ala-Lys by Glu.

(iii) Analysis of peptidoglycan precursors. To ascertain whether the mutations had impaired D-Ala:D-Ala ligase activity, we determined the nature and the relative amounts of the peptidoglycan precursors synthesized by the hosts (Fig. 4, left panel). This was assessed by inhibiting peptidoglycan synthesis with the antibiotic ramoplanin for a short period when bacteria were in the exponential phase of growth and investigating the accumulation of the precursors that could no longer be used. In the absence of induction by vancomycin, only TJ282 was able to grow and synthesized quasiexclusively UDPMurNac pentapeptide. Induction by $4 \text{mg}$ of vancomycin per ml resulted in the activity of both glycopeptide-susceptible and -resistant pathways of peptidoglycan synthesis, as indicated by the presence of pentapeptide (34%), pentadepsipeptide (50%), and tetrapeptide (16%) in the precursor pool. As expected, VmD strains synthesized exclusively pentadepsipeptide (when these strains were grown in the presence of $10 \mu g$ of vancomycin per ml, confirming their exclusive reliance on the resistance pathway of peptidoglycan synthesis for growth).

(iv) DD-Dipeptidase assays. The expression levels of the resistance proteins were determined by measuring the VanX$_B$ DD-dipeptidase activity (2). This enzyme can serve as a reporter since the vanY$_B$, vanW$_B$, vanH$_B$, vanB, and vanX$_B$ genes are transcribed from a single promoter (10). As illustrated in Fig. 5 (left panel), the activity was negligible in uninduced TJ282. Although VmD strains produced exclusively resistant precursors, VanX$_B$ was synthesized at relatively low levels in BM4387 and TJ310, even though the resistance operon was switched on. VanX$_B$ and VanY$_B$ activities are not required in these strains because D-Ala-D-Ala and hence pentapeptide are not synthe-

FIG. 3. Schematic representation of the D-Ala:D-Ala ligase of E. faecalis. The positions of the amino acids implicated in the binding of D-Ala1, D-Ala2, and ATP are indicated by black, dotted, and hatched bars, respectively. Substitutions relative to the deduced DDI sequence of TJ282 (and the corresponding mutations in parentheses) are indicated in bold italic characters.

<table>
<thead>
<tr>
<th>STRAIN (phenotype)</th>
<th>251-253</th>
<th>295</th>
<th>316</th>
</tr>
</thead>
<tbody>
<tr>
<td>TJ282 (Vm$^D$)</td>
<td>DAK (GAT GCA AAA)</td>
<td>D (GAT)</td>
<td>T (ACT)</td>
</tr>
<tr>
<td>BM4386 (Vm$^D$)</td>
<td>DAK (GAT GCA AAA)</td>
<td>V (GTT)</td>
<td>T (ACT)</td>
</tr>
<tr>
<td>BM4387 (Vm$^D$)</td>
<td>DAK (GAT GCA AAA)</td>
<td>D (GAT)</td>
<td>I (ATT)</td>
</tr>
<tr>
<td>TJ310 (Vm$^D$)</td>
<td>E (GAA)</td>
<td>D (GAT)</td>
<td>T (ACT)</td>
</tr>
<tr>
<td>BM4388 (Vm$^R$ Te$^D$)</td>
<td>EYK (GAA TAC AAA)</td>
<td>D (GAT)</td>
<td>T (ACT)</td>
</tr>
<tr>
<td>BM4389 (Vm$^R$ Te$^D$)</td>
<td>E (GAA)</td>
<td>D (GAT)</td>
<td>T (ACT)</td>
</tr>
<tr>
<td>BM4390 (Vm$^R$ Te$^D$)</td>
<td>E (GAA)</td>
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<td>BM4391 (Vm$^D$ Te$^D$)</td>
<td>E (GAA)</td>
<td>D (GAT)</td>
<td>T (ACT)</td>
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FIG. 4. Relative proportions of pentapeptide (cross-hatched bars), tetrapeptide (black bars), tripeptide (white bars), and pentadepsipeptide (hatched bars) synthesized by clinical isolates (left panel) and in vitro mutants obtained from TJ310 (right panel). Precursors were determined from noninduced bacteria (left bar of the pair of bars for each strain) or from bacteria induced (right bar of each pair of bars) and by growth in the presence of $10 \mu g$ of vancomycin per ml (BM4386, BM4387, TJ310, BM4389, and BM4390), 4 $\mu g$ of vancomycin per ml (TJ282 and BM4388), or 10 $\mu g$ of teicoplanin per ml (BM4391). NA, not applicable.
with a dimensional structure of a VmD mutant, it was reported that a nonsense mutation in the presence of vancomycin in the culture medium. In a previous study of vancomycin but remained susceptible to teicoplanin, even after induction by vancomycin. The latter observation could be due to a relatively low level of expression of the resistance proteins, resulting in a pentadepsipeptide/pentapeptide ratio insufficient to increase the teicoplanin MIC (2).

Taken together, these data confirm that vancomycin dependence results from impairment of the host D-Ala:D-Ala ligase activity. Peptidoglycan synthesis in these cells is reliant on production of the resistance proteins, which is dependent on the presence of vancomycin in the culture medium. In a previous study of a VmD mutant, it was reported that a nonsense mutation gave rise to a truncated protein, resulting in loss of D-Ala:D-Ala ligase activity (26). In the current investigation, the three clinical VmD isolates had a base pair substitution or deletion in the ligase gene. Such mutations can result in loss of substrate binding or catalytic activity if the substituted amino acid is at a critical functional position. This is illustrated by an in vitro VmD mutant of E. faecalis in which a Ser 281-to-Ile substitution suppressed a hydrogen bond essential for the interaction with D-Ala-2 (13, 25). The three-dimensional structure of Escherichia coli Ddl cocrystallized with a S,R-methylphosphinate inhibitor and ATP has been determined (13), and this enzyme has been analyzed by mutagenesis experiments (25). The Asp 295 residue mutated in BM4386 corresponds to an invariant position in the ligases (9). It is believed to form a hydrogen bond via a water molecule with D-Ala-2 and to participate therefore in substrate recognition and/or catalysis, since substitution of the corresponding amino acid (Asp 257) in E. coli ligase totally abolished enzyme activity (25). The mutation at position 316 in BM4387 does not affect an amino acid previously reported to be directly implicated in enzyme activity. However, this Thr residue appears to be highly conserved in the D-Ala:D-Ala, D-Ala:D-Lac, and D-Ala:D-Ser ligases from various bacterial species (9), suggesting that it could also play an important role in either enzyme activity or conformation. Thr 316 is close to four other conserved residues (Glu 311, Asn 313, Ser 319, and Met 320), and Ser 281, which in E. coli corresponds to the penultimate amino acid, has a key role in the interaction with D-Ala-2 (25). By analogy with the observations made on the corresponding Lys 215 mutant in the E. coli ligase (13, 25), loss of the highly conserved Lys at position 253 of TJ310 ligase may have impaired the enzyme activity by suppressing a residue involved in the transfer of the phosphate group of ATP. The concomitant lack of amino acids at positions 251 and 252 in this strain affects nonconserved residues (9), and therefore, the potential impact of this deletion cannot be established.

In vitro mutants. We obtained revertants of VmD strain TJ310 by growing TJ310 in the absence of vancomycin (Table 1 and Fig. 1) as described previously (7, 16). The mutants were classified according to their susceptibility (VmD TeR strain BM4388) or resistance (VmD TeR BM4389 and BM4390) to teicoplanin. Vancomycin and teicoplanin-dependent mutant (VmD Ddl) BM4391 was selected by growing TJ310 in the presence of 10 μg of vancomycin per ml and 1 μg of teicoplanin per ml. All the mutants displayed the same resistance phenotype to antibiotics other than glycopeptides as did the parental strain TJ310.

(i) Vancomycin-resistant, teicoplanin-susceptible revertants. Loss of vancomycin dependence by VmD strain BM4388 prompted us to examine the sequence of the ddl gene. Compared with the ddl sequence of VmD parental strain TJ310, the sequence of the mutant had an insertion of 6 bp (TAC AAA) corresponding to positions 252 to 253 (Fig. 3). Interestingly, this mutation resulted in a triplet of amino acids at positions 251 to 253, of which two are different from those in wild-type E. faecalis ligase (Glu-Tyr instead of Asp-Ala), but Lys 253, thought to be essential for activity, was present in both. Most surprisingly, the same mutation was found in the four independent revertants sequenced, suggesting that only a few combinations of amino acids can restore ligase activity. As expected, in the absence of vancomycin, strain BM4388 synthesized peptidoglycan precursors qualitatively similar to those produced by TJ282 (Fig. 4, right panel). Following preincubation with 4 μg of vancomycin per ml, the proportions of pentapeptide and tetrapeptide, respectively, were 34 and 16% in VmD strain BM4388 and 83% in VmD strain BM4388, and pentadepsipeptide represented 83% of the total precursors in the latter strain. This increase in the percentage of resistant pre-

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FIG. 5. VanX DD-dipeptidase activity in clinical isolates (left panel) and in vitro mutants of TJ310 (right panel). The enzymatic activity was measured in extracts prepared from noninduced strains (black bars) or from strains induced (hatched bars) by growth in the presence of 10 μg of vancomycin per ml, except for BM4391 which was induced with 10 μg of teicoplanin per ml. Data are the means ± standard deviations from three independent experiments. NA, not applicable; ND, not detectable.
cursors generated by VanHB and VanB correlated with higher VanXB activity (Fig. 5, right panel) and accounts for high-level vancomycin resistance of this strain. However, despite increased production of the resistance proteins, higher than in VmD BM4386, strain BM4388 was resistant to low levels of teicoplanin only under inducing conditions. This is due to the fact that almost complete elimination of the pentapeptide is required to achieve resistance to this antibiotic (2). No mutations were found in the \textit{vanR} \textit{B} and \textit{vanS} \textit{B} genes encoding the two-component regulatory system in this mutant (Fig. 6).

(ii) Vancomycin and teicoplanin-resistant revertants. Vm' Te' BM4389 and BM4390 revertants had the same mutation in the \textit{ddl} gene as their VmD parent TJ310 did (Fig. 3). They synthesized only pentadepsipeptide precursors, even in the absence of induction (Fig. 4, right panel), indicating that peptidoglycan synthesis used the resistance pathway exclusively. Moreover, VanXB enzymatic activity in the revertants was as high as that of induced BM4388, even under noninducing conditions (Fig. 5, right panel). The lack of pentapeptide precursors explains, as in VmD BM4386, the high level of teicoplanin resistance. Taken together, these data demonstrate that the resistance genes must be expressed constitutively. We therefore determined the sequences of the \textit{vanS} \textit{B} and \textit{vanR} \textit{B} genes which encode the two-component regulatory system in this mutant (Fig. 6).

(iii) Vancomycin- and teicoplanin-dependent mutants. VmD TeD mutant BM4391 had the same mutation in its \textit{ddl} gene as the VmD progenitor TJ310 did (Fig. 3). This strain also syn-

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{Schematic representation of the VanS\textit{B} sensor. The putative membrane-associated sensor domain (white) containing transmembrane segments (black) and the cytosolic kinase domain (cross-hatched) are depicted. H, N, G1, F, and G2 refer to conserved amino acid motifs present in histidine kinases. Amino acids in the sequence underlined for TJ310 correspond to the G2 domain. The substitutions relative to VanS\textit{B} of TJ310 (and the corresponding mutations in parentheses) are indicated in bold italic characters.}
\end{figure}

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{STRAIN (phenotype)} & 232 & 247 & 401-411 \\
\hline
TJ310 (Vm\textsuperscript{D}) & S (TCT) & E (GAG) & \text{---} \\
BM4388 (Vm\textsuperscript{R} Te\textsuperscript{D}) & S (TCT) & E (GAG) & \text{---} \\
BM4389 (Vm\textsuperscript{R} Te\textsuperscript{D}) & S (TCT) & K (AAG) & \text{---} \\
BM4390 (Vm\textsuperscript{R} Te\textsuperscript{D}) & F (TTT) & E (GAG) & \text{---} \\
BM4391 (Vm\textsuperscript{D} Te\textsuperscript{D}) & S (TCT) & E (GAG) & \text{---} \\
\hline
\end{tabular}
\end{table}

\begin{align*}
\text{SRKSGRSGGLGL} \\
(\text{AGG AGA AAA AGT GGG CCA AGC GGT TTG GGG CTT})
\end{align*}
the sized only pentadepsipeptide precursors and had a low level of VanX_B activity (right panels of Fig. 4 and 5). The only difference observed in comparison with parental TJ310 was an alteration of induction specificity, since BM4391 could grow in the presence of either vancomycin or teicoplanin. No mutation was found in the vanR_R gene of BM4391, but a mutation was identified in its vanS_B gene. This consisted of a 18-bp deletion (AGC AGA AAA AGC GGG CGA) affecting amino acids 402 to 407 and leading to loss of part of the conserved G2 domain. Since certain transmitters (AgrORF2, NarQ, and NarX) possess a single nucleotide binding domain, loss of the G2 block should not necessarily impair ATP binding. However, it has been proposed that the G2 block could also play a role in the modulation of enzyme conformation (20). If ATP were unable to bind at this site in BM4391, it might impair the stabilizing role of ATP on the molecule conformation without affecting enzyme activity (20). It is conceivable that this change could result in recognition of teicoplanin, since a single amino acid substitution in the sensor domain is sufficient to cause a similar change in induction specificity (6).

Emergence of VmD strains in clinical settings currently appears to be more of a curiosity than problem of clinical concern (28, 30). The inability of these bacteria to survive in the absence of vancomycin makes them, a priori, easy to eliminate, simply by discontinuing antibiotic therapy. However, VmD enterococci are rarely isolated in routine laboratory practice because of their particular nutritional requirements. Growth is obtained only on medium supplemented by d-Ala-d-Ala, vancomycin, or other inducers of vancomycin resistance gene expression (e.g., bacitracin and moenomycin). Moreover, VmD mutants have also been isolated from patients not receiving vancomycin. Therefore, they constitute a reservoir of vancomycin resistance genes which could be transferred to other bacteria. More importantly, VmD strains can easily revert, at least in vitro, to a nondependent, highly resistant phenotype, and may therefore require particular attention. Screening for VmD isolates in infected patients is therefore advisable.

ACKNOWLEDGMENTS

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