Genomic and expression analysis of the vanG-like gene cluster of Clostridium difficile

Johann Peltier,1 Pascal Courtin,2 Imane El Meouche,1 Manuella Catel-Ferreira,3 Marie-Pierre Chapot-Chartier,2 Ludovic Lemée1 and Jean-Louis Pons4

Correspondence
Jean-Louis Pons
jean-louis.pons@parisdescartes.fr

1Laboratoire G.R.A.M., EA 2656 IFR 23, UFR de Médecine Pharmacie, 76183 Rouen Cedex, France
2INRA UMR1319 Micalis, Domaine de Vilvert, F-78352 Jouy-en-Josas, France
3University of Rouen, Laboratoire Polymères Biopolymères Surfaces, UMR 6270 & FR 3038 CNRS, IFRMP23, 76821 Mont Saint Aignan, France
4Laboratoire Ecosystème Intestinal, Probiotiques, Antibiotiques (EA 4065, IFR IMTCE), Université Paris Descartes, 75270 Paris, France

Primary antibiotic treatment of Clostridium difficile intestinal diseases requires metronidazole or vancomycin therapy. A cluster of genes homologous to enterococcal glycopeptides resistance vanG genes was found in the genome of C. difficile 630, although this strain remains sensitive to vancomycin. This vanG-like gene cluster was found to consist of five ORFs: the regulatory region consisting of vanR and vanS and the effector region consisting of vanG, vanXY and vanT. We found that 57 out of 83 C. difficile strains, representative of the main lineages of the species, harbour this vanG-like cluster. The cluster is expressed as an operon and, when present, is found at the same genomic location in all strains. The vanG, vanXY and vanT homologues in C. difficile 630 are co-transcribed and expressed to a low level throughout the growth phases in the absence of vancomycin. Conversely, the expression of these genes is strongly induced in the presence of subinhibitory concentrations of vancomycin, indicating that the vanG-like operon is functional at the transcriptional level in C. difficile. Hydrophilic interaction liquid chromatography (HILIC-HPLC) and MS analysis of cytoplasmic peptidoglycan precursors of C. difficile 630 grown without vancomycin revealed the exclusive presence of a UDP-MurNAc-pentapeptide with an alanine at the C terminus. UDP-MurNAc-pentapeptide [D-Ala] was also the only peptidoglycan precursor detected in C. difficile grown in the presence of vancomycin, corroborating the lack of vancomycin resistance. Peptidoglycan structures of a vanG-like mutant strain and of a strain lacking the vanG-like cluster did not differ from the C. difficile 630 strain, indicating that the vanG-like cluster also has no impact on cell-wall composition.

INTRODUCTION

Clostridium difficile, a Gram-positive spore-forming bacterium, is the major cause of intestinal diseases associated with antibiotic therapy (Viswanathan et al., 2010). Clinical manifestations in humans range from asymptomatic colonization or mild diarrhoea to pseudomembranous colitis and death (Kelly & LaMont, 1998). The main virulence factors have been identified as toxin A and toxin B. Recent outbreaks have led to increasing morbidity and mortality and have been associated with a new highly virulent strain (BI/NAP1/027) of C. difficile (Warny et al., 2005). Metronidazole and vancomycin are the two main antibiotics used for treatment of C. difficile infections (CDIs). Metronidazole is used as the first-line treatment mainly due to its lack of potential for selection of vancomycin-resistant Enterococcus. Vancomycin is the second drug of choice, used as a therapy for serious CDI cases, for metronidazole-intolerant patients, and for treatment of recurrences which are a frequent feature of CDIs (Gerding et al., 2008; Zar et al., 2007). While these antibiotics are the primary treatment, fidaxomicin, monoclonal antibodies and intestinal microbiota transplantation are promising new treatment options, especially regarding prevention of recurrent disease (Tschudin-Sutter et al., 2012).

Abbreviations: CDI, C. difficile infection; HILIC, hydrophilic interaction liquid chromatography; MLST, multilocus sequence typing; RAM, retrotransposition-activated marker.

Two supplementary tables and two figures are available with the online version of this paper.
Resistance to glycopeptide antibiotics among Gram-positive bacteria may be either acquired or naturally expressed (Arthur et al., 1996b). Glycopeptide resistance was first described for enterococci (Leclercq et al., 1988), for which nine resistance genotypes (vanA, vanB, vanC, vanD, vanE, vanG, vanI, vanM and vanN) have been described (Boyd et al., 2008; Courvalin, 2006; Lebreton et al., 2011; Xu et al., 2010). The vanF genotype has been also described in the biopesticide Paenibacillus popilliae (Patel et al., 2000). Glycopeptide resistance is due to the synthesis of modified peptidoglycan precursors with low affinity for these antibiotics (Arthur et al., 1996a). Two types of modified precursors have been identified, those with pentapeptides terminating in D-Ilanine–D-lactate (D-Ala–D-Lac) (VanA, VanB, VanD, VanF and VanM phenotypes), which exhibit a 1000-fold-lower binding affinity for vancomycin, and those terminating in D-alanine–D-serine (D-Ala–D-Ser) (VanC, VanE, VanG, VanL and VanN phenotypes), whose affinity for vancomycin is reduced 7-fold (Arthur et al., 1996a).

D-Ala–D-Ser peptidoglycan precursors are produced by D-Ala–D-Ser ligases, which belong to the D-Ala–D-Xligase superfamily and are phylogenetically close to the D-Ala–D-Lac ligase in Enterococcus faecalis (Meziane-Cherif et al., 2012). D-Ala–D-Ser peptidoglycan precursors have also been characterized in Clostridium innocuum and related to intrinsic resistance to vancomycin (David et al., 2004).

Two distinct vanG operons, vanG1 and vanG2, have been described in E. faecalis (Boyd et al., 2006; Depardieu et al., 2003; McKessar et al., 2000). The VanG phenotype corresponds to low-level resistance to vancomycin and susceptibility to teicoplanin. This resistance results from the acquisition of an operonic cluster of genes consisting in vanG of three regulatory genes (vanUC1, vanRC1, vanSC1) and five effector genes (vanYG, vanWG, vanG, vanXYG, vanTC1) transcribed from two distinct promoters (Depardieu et al., 2003). The vanG2 operon harbours the same genetic organization but lacks the vanYG gene.

In C. difficile, the genome sequence of strain 630 revealed the presence of a cluster of genes with high similarity to the vanG cluster of enterococci, although this strain remains susceptible to vancomycin (Sebaihia et al., 2006). In the present work, we report the genetic organization, genetic location and intraspecific phylogeny of the vanG-like cluster of C. difficile. We show that, although the genes of this cluster are expressed at the transcriptional level and their transcription is induced by vancomycin, cytoplasmic peptidoglycan precursors contain only classical pentapeptide precursors ending in D-Ala–D-Ala, corroborating the lack of vancomycin resistance.

**METHODS**

**Bacterial strains and culture conditions.** C. difficile 630 (Sebaihia et al., 2006) and C. difficile 630Aerm (Hussain et al., 2005), which is a spontaneously cured derivative of strain 630 and allows selection of Clostridium mutants, were used in all experiments. An additional set of 83 C. difficile isolates selected as representative of the main clusters that we previously defined by multilocus sequence typing (MLST) (Lemee et al., 2004; Lemée & Pons, 2010), and recovered from various hosts and geographical sources, were used to study the phylogenetic origin of the vanG-like operon. All C. difficile strains were cultured on blood agar (Oxoid), BHI agar (Difco) or BHI broth (Difco) at 37 °C in an anaerobic environment [80 % N2, 10 % CO2, and 10 % (v/v) H2].

**General DNA techniques.** Chromosomal DNA extraction from C. difficile colonies was performed using the InstaGene Matrix kit (Bio-Rad). PCRs were done with a reaction volume of 25 μl by using GoTaq Green Master (Promega) or Advantage II Polymerase Mix (BD Biosciences). The primers used for this work (Eurofins MWG Operon) are listed in Table 1. PCR products and plasmids were purified using a NucleoSpin Extract II kit and a Nucleospin plasmid kit (Machery-Nagel), respectively.

**MIC determinations.** E-Test measurements of MICs of vancomycin were performed on Brucella agar supplemented with 5 % blood from bacterial suspensions at 3 McFarland turbidity (Bonnet et al., 2013). Agar plates were incubated anaerobically (37 °C) for 48 h, and MICs were determined following the manufacturer’s instructions (AB Biodisk). E-Test measurements of MICs of vancomycin and teicoplanin against C. difficile 630 were also performed on BHI agar using the same experimental protocol, as subsequent experiments in this study were performed using BHI broth.

**RNA isolation and quantitative real-time PCR.** For each growth phase, samples of total RNA of C. difficile were isolated from cells grown without or with subinhibitory concentrations of vancomycin (1/4 MIC, 0.375 μg ml−1 on BHI) or teicoplanin (1/4 MIC, 0.25 μg ml−1 on BHI) with the RNAsesy Mini kit (Qiagen). Samples were treated with two different DNases, Dnase I (Sigma) and Turbo DNA-free kit (Ambion) as per the respective manufacturer’s instructions. The total RNA purity and puerity were spectrophotometrically measured (NanoVue, GE) and 2 μg total RNA was reverse transcribed using the Omniscript enzyme (Qiagen) and random 15 mer primers (Eurofins MWG Operon). A total of 6 ng cDNA was used for subsequent PCR amplification with the IQ SYBR green Supermix (Bio-Rad) and the appropriate primers (0.5 μM each, final concentration). Specific primers used for PCR amplification were designed with Beacon Designer software (PREMIER Biosoft International) to produce ampiclons of equivalent length (about 100 bp) (Table 1). Quantification of 16S rRNA gene levels was used as an internal control. Amplification, detection (with automatic calculation of the threshold value) and real-time analysis were performed in duplicate and with RNA samples from three biological replicates for each condition by using the CFX96 real-time PCR detection system (Bio-Rad). The value used for the comparison of gene expression levels was the number of PCR cycles required to reach the threshold cycle (Ct). Expression of vanG, vanXY and vanY-like genes was calculated as fold changes using the formula: Fold changes=2−ΔΔCt, with ΔΔCt=(Ct gene X − Ct 16S rRNA) vancomycin treated − (Ct gene X − Ct 16S rRNA) non-treated.

**Construction of C. difficile vanG mutant by using the ClosTron system.** The ClosTron system was used as described by Heap et al. (2007), in conjunction with the commercially available TargetT gon knock-out system kit (Sigma-Aldrich). Briefly, the algorithm available on the TargetT Design Site was used to identify intron insertion sites within vanG. The primers (Table 1) designed by the algorithm to re-target the group II intron on pMTL007 to vanG were used with universal primer EBS and intron template DNA to generate a 353 bp DNA fragment by overlap PCR according to the manufacturer’s instructions. The resultant PCR product was cloned into the HindIII and BsgI restriction sites of pMTL007 and the construct was transfected into Escherichia coli TOP10. The fidelity of the cloned inserts was verified by DNA sequencing using pMTL007-specific primers pMTLSeq-F and pMTLSeq-R (Table 1). Plasmid
Table 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>vanT1-R</td>
<td>CGACCCACTATTGCTGCTCT</td>
<td>Internal to vanT1-like</td>
</tr>
<tr>
<td>vanXYT-R</td>
<td>ATACTAGTAAACAAAGATCGGCC</td>
<td>Internal to vanT1-like</td>
</tr>
<tr>
<td>RT16S-F</td>
<td>GAGGAAAGGIGGAIGACGT</td>
<td>Real Time-quantitative PCR (RT-qPCR) gene ADN</td>
</tr>
<tr>
<td>RT16S-R</td>
<td>AGICCCGGAAGCATTACAC</td>
<td>RT-qPCR gene ADN 16S</td>
</tr>
<tr>
<td>RTvanG-F</td>
<td>TGTGGTCGAGGGTTTCTGAT</td>
<td>RT-qPCR gene vanG</td>
</tr>
<tr>
<td>RTvanG-R</td>
<td>TGACGACCCCTACCTCAAAACCA</td>
<td>RT-qPCR gene vanG</td>
</tr>
<tr>
<td>RTvanXY-F</td>
<td>CTCGCGGTGACAAATGGAAA</td>
<td>RT-qPCR gene vanXY</td>
</tr>
<tr>
<td>RTvanXY-R</td>
<td>AGCAAGGGCTGCTGATGTT</td>
<td>RT-qPCR gene vanXY</td>
</tr>
<tr>
<td>RTvanT-F</td>
<td>CTATGGCTATGCGGATGTT</td>
<td>RT-qPCR gene vanT</td>
</tr>
<tr>
<td>RTvanT-R</td>
<td>CGACCCACTATTGCTGCTTAC</td>
<td>RT-qPCR gene vanT</td>
</tr>
<tr>
<td>RTddl-F</td>
<td>CACTCTCCTGAGTGGGACCTC</td>
<td>RT-qPCR gene ddl</td>
</tr>
<tr>
<td>RTddl-R</td>
<td>AACCATGAGACAGTGGATGAGG</td>
<td>RT-qPCR gene ddl</td>
</tr>
<tr>
<td>van out-F</td>
<td>GAGGATATTATAGGAGCTG</td>
<td>Upstream of vanG cluster</td>
</tr>
<tr>
<td>van out-R</td>
<td>CAACAGTGGTGAAGGCTG</td>
<td>Downstream of vanG cluster</td>
</tr>
<tr>
<td>van in-R</td>
<td>CCTATGTATGAAACCACAGAC</td>
<td>Left end of vanG cluster</td>
</tr>
<tr>
<td>IBS-vanG</td>
<td>AAAAAGCTTATAATTTACCTT</td>
<td>Intron retargeting</td>
</tr>
<tr>
<td>EBS1d-vanG</td>
<td>CAGATTGTACAACAGATGTTG</td>
<td>Intron retargeting</td>
</tr>
<tr>
<td>EBS2-vanG</td>
<td>TGAACTGGCGATTTCCAAATTTCCGG</td>
<td>Intron retargeting</td>
</tr>
<tr>
<td>EBS universal</td>
<td>CAGATTGTACAAACAGATGTTG</td>
<td>Intron retargeting</td>
</tr>
<tr>
<td>RAM-F</td>
<td>ACGGTTGCTGACCTAATTTACGGAATAGCTG</td>
<td>erm-AM</td>
</tr>
<tr>
<td>RAM-R</td>
<td>ACGGTTGCTGACCTAATTTACGGAATAGCTG</td>
<td>erm-AM</td>
</tr>
<tr>
<td>vanG-mutF</td>
<td>GATGGAAACAGTGGAGG</td>
<td>Mutagenesis confirmation</td>
</tr>
<tr>
<td>vanG-mutR</td>
<td>GGTACTTTTATACCTTCTTGAATGAC</td>
<td>Mutagenesis confirmation</td>
</tr>
<tr>
<td>pMTLS eq-F</td>
<td>GGGATCTCTCAGAGTGG</td>
<td>Intron sequencing</td>
</tr>
<tr>
<td>pMTLS eq-R</td>
<td>CAGATTGTACCAACAGATGTT</td>
<td>Intron sequencing</td>
</tr>
</tbody>
</table>

pMTL007::Cdi-vanG-489s retargeted the group II intron to insert into the vanG gene in the sense orientation immediately after the 489th nucleotide in the coding sequence. This derivative pMTL007 plasmid was transformed into the conjugative donor Escherichia coli HB101 (RP4) and then transferred via conjugation into the C. difficile strain ATCC 43300 (erm B). Successful C. difficile transconjugants were selected by subculturing on BHI agar containing cefoxitin (25 mg l⁻¹) and thiamphenicol (15 mg l⁻¹). Then, the integration of the group II intron RNA into vanG was selected by plating onto BHI agar with erythromycin (5 mg l⁻¹). Erythromycin-resistant (and thiamphenicol-resistant) C. difficile colonies are produced, following plasmid loss and insertion of the group II intron into the chromosome, which is accompanied by splicing out of the group I intron from the ermB retrotransposon-activated marker (RAM). Moreover, genomic DNA of erythromycin-resistant transconjugants was isolated, and subjected to PCR using primers flanking vanG, vanG-mutF and vanG-mutR (Table 1), to verify that the group II intron had inserted into the correct target gene. In addition to erythromycin resistance being demonstrated phenotypically, confirmatory PCR using the RAM-F/RAM-R primer pair (Table 1) was also performed.

Extraction and analysis of the peptidoglycan nucleotide precursor pools. Peptidoglycan precursors were extracted and analysed according to established procedures (Billot-Klein et al., 1994) with some modifications. Cultures of C. difficile were grown in the absence or presence of vancomycin (0.375 μg ml⁻¹) to an optical density at 600 nm of 0.5, bacitracin was added (768 μg ml⁻¹) when vancomycin is absent and 100 μg ml⁻¹ when vancomycin is added and incubation continued for 2 h to amplify the amount of cell-wall precursors (Severin et al., 2004). The cells were rapidly cooled in an ice bath and harvested by centrifugation. Low-molecular-mass cytoplasmic components were extracted with 1.1 M formic acid for 30 min at 4 °C before centrifugation. The supernatant was concentrated by lyophilization. The cytoplasmic components were then separated by hydrophilic interaction liquid chromatography (HILIC-HPLC) using a Nucleodur HILIC (3 μm, 125 x 4 mm; Macherey-Nagel). Precursors were eluted at 1 ml min⁻¹ flow rate for 5 min with 70 % acetonitrile in 50 mM ammonium formate pH 5 buffer, then with a 10 min linear gradient to 64 % acetonitrile in buffer and then a 5 min linear gradient to 40 % acetonitrile in buffer. Column temperature was 50 °C. The eluted compounds were detected by absorption at 262 nm. Fractions containing the main peaks were analysed by MALDI-TOF MS in the negative mode with a Voyager DE STR mass spectrometer (Applied Biosystems) and samples were mixed with same the volume of 6-aza-2-thioguanine as matrix prepared by dissolving 1 mg 6-aza-2-thioguanine in 1 ml ethanol/20 mM aqueous ammonium citrate (1:1, v/v). Separation and analysis were performed from at least five different precursor extracts for each condition. UDPMurNAc-t-Ala-o-Glu-DAP-o-Ala-o-Ser ([M-H]⁻ 1208.3) was synthesized by UKBAKWAN and was used as a standard in HILIC-HPLC analysis and MS.
Digestion of the entire cytoplasmic extract with R39 D,D-carboxypeptidase (kind gift from P. Hols, Université catholique de Louvain, Belgium) was performed as previously described by Billot-Klein et al. (1994).

Peptidoglycan structure analysis. C. difficile PG structure was analysed by reversed-phase HPLC as described previously (Peltier et al., 2011).

RESULTS AND DISCUSSION

Genetic organization of the vanG-like cluster of C. difficile

Complete sequencing of the C. difficile 630 genome revealed the presence of a cluster of genes with high similarity to the vanG-type vancomycin resistance locus from E. faecalis (Sebaihia et al., 2006). This vanG-like locus is 6153 nt long and encompasses five ORFs (Fig. 1a), with between 60 and 83% amino acid sequence identity with gene products of the vanG cluster of E. faecalis BM4518 or E. faecalis N03-0233 (Table 2). The 5' end of the locus contains two genes homologous to the vanRG and vanSG two-component regulatory system. The vanSG-like gene is followed 51 nt downstream by a stem–loop structure \( \Delta G = -24.4 \text{ kcal mol}^{-1} \). This 25 inverted repeat may form a secondary structure which could function as a Rho-independent terminator, suggesting that, like the vanG1 and vanG2 operons of E. faecalis (Depardieu et al., 2003), these two genes are transcribed separately from the remainder of the locus. Following the regulatory genes, the resistance region consists of vanG-, vanXYG- and vanTG-like genes, encoding putative D-Ala–D-Ser ligase, bifunctional D,D-dipeptidase and serine racemase, respectively. The vanG-like gene cluster is located between two loci (CD1623 and CD1629) transcribed in the same direction and encoding, respectively, a putative nitric oxide reductase flavoprotein and a hypothetical protein. The structural organization of the vanG-like cluster of genes shows that this locus is bordered by two stem–loops (Fig. 1a, b) that may function as transcriptional Rho-independent terminators for the CD1623 locus and for vanT. The first stem–loop structure (IR1) \( \Delta G = -25.2 \text{ kcal mol}^{-1} \) is located 416 nt upstream of CD1623.

![Diagram](http://mic.sgmjournals.org)

**Fig. 1.** Genetic organization of the vanG-like cluster of C. difficile 630. (a) Schematic organization of the vanG-like cluster and flanking regions. Large arrows represent the ORFs and indicate the direction of transcription. The genes of the vanG-like cluster are represented by open arrows and flanking genes by hatched arrows. The primers used for analysis of vanG-like effector genes cluster transcription are indicated by thin black arrows. The putative Rho-independent transcriptional terminators (Te) are indicated. (b) Nucleotide sequences of the left and right ends of the vanG-like cluster. The different IR sequences are represented by arrows. Identical nucleotides between the IR1 and IR2 sequences are underlined. (c) Transcriptional analysis of the vanG-, vanXYG- and vanTG-like effector genes. Electrophoresis of the Reverse Transcription-PCR (RT-PCR) products obtained from the vancomycin-induced strain using primers vanXYT-R and RTvanG-F. Incubations were carried out in the presence (lane 1) or absence (lane 2) of reverse transcriptase. A control with added genomic DNA shows the expected size of the band with the primer set used (lane 3).
the vanR<sub>G</sub>-like initiation codon and the second (IR<sub>2</sub>) \((\Delta G^\circ = -22.6 \text{ kcal mol}^{-1})\) is located 118 nt downstream of the vanT<sub>G</sub>-like termination codon. Interestingly, the nucleotide sequence of inverted repeats IR<sub>2</sub> is very close to the first 19 nt of inverted repeats IR<sub>1</sub> (16 of 19 nt) (Fig. 1b).

To analyse the transcription of the vanG-like gene cluster in <i>C. difficile</i> 630, we extracted and purified total RNA from cultures grown in the presence of vancomycin, and performed reverse transcription with primer vanT1-R (Fig. 1a and Table 1) internal to vanT<sub>G</sub>-like. The cDNA obtained was amplified with primers vanXYT-R and RTvanG-F internal to vanT<sub>G</sub>-like and vanG-like, respectively (Fig. 1a and Table 1). A PCR product with the expected size of ca. 1.5 kb was obtained (Fig. 1c), indicating that the vanG-like, vanXY<sub>G</sub>-like and vanT<sub>G</sub>-like genes are co-transcribed in a unique polycistronic mRNA, similarly to the vanG operons of <i>E. faecalis</i> (Depardieu et al., 2003).

**Delineation and insertion site of the vanG-like cluster**

<i>In silico</i> research among currently available <i>C. difficile</i> genomes revealed the lack of a vanG-like operon in <i>C. difficile</i> QCD-23m63, which is very distant to strain 630 phylogenetically. As the complete genome sequence of this strain is not yet available, PCR with primers van out-F and van out-R (Table 1) flanking the missing region and sequencing were performed in strain E112, which is phylogenetically close to strain QCD-23m63. Sequence analysis confirmed the lack of a vanG-like gene cluster in this genomic location. Moreover, PCR performed with primers internal to the vanG-like operon suggested strongly that this cluster was not present elsewhere in the genome of this strain. Comparative analysis of this sequence with regions flanking the vanG-like operon in <i>C. difficile</i> 630 allowed us to delineate the vanG-like putative genetic element (Fig. 2), which encodes only the five genes encompassing the vanG-like operon. Consequently, this element does not appear to encode genes involved in either excision, integration or conjugation, as usually found in transposable elements. Interestingly, the left end of the gene cluster is located in the intergenic space upstream of the vanR<sub>G</sub>-like gene within the transcriptional terminator IR<sub>1</sub> and the right end is located in the intergenic space downstream of the vanT<sub>G</sub>-like gene within the transcriptional terminator IR<sub>2</sub> (Fig. 2a). As the vanG-like gene cluster is flanked by the very close inverted repeats IR<sub>1</sub> and IR<sub>2</sub>, it is tempting to assume that these inverted repeats could be assimilated to insertion elements (Mahillon et al., 1999) lacking transposases, bordering the 6732 bp vanG-like genetic element (Fig. 2b). Precisely, the left end is located at a site 8 bp downstream from the CD1623 gene stop codon and the right end is located at a site 165 bp upstream from the CD1629 translation start codon. No target site duplication flanking the vanG-like genetic element and usually produced in the transposition mechanism seems to occur upon insertion. The lack of insertion sequences that produce a target site duplication has been previously reported, especially for Tn5520 of <i>Bacteroides</i>.
*fragilis* (Vedantam et al., 1999) and a mobile element of *Sulfolobus islandicus* (Berkner & Lipps, 2007).

A 30 bp DNA sequence, which is absent in strain 630 harbouring the *vanG*-like operon, was found in strain E112 in the region corresponding to the insertion of the genetic element (Fig. 2a). In addition, a 21 bp perfect inverted repeat (IR3) was identified in strain E112 in a region overlapping this unrelated 30 bp sequence, as reported by Ammam et al. (2012) (Fig. 2a). This stem–loop structure (ΔG = –26.6 kcal mol\(^{-1}\)) could constitute the transcriptional terminator of the CD1623 locus. This transcriptional terminator should be abolished during the transposition of the *vanG*-like genetic element but its loss should be balanced by the insertion of the inverted repeats IR2 with the genetic element. This observation suggests that the transcriptional terminator IR2 constitutes the target DNA for the insertion of the genetic element (Fig. 2b). The Tn7 transposon of *Escherichia coli* also targets transposition to a transcriptional terminator and consequently inserts at a high frequency into a single specific site. Transposition of Tn7 abolishes termination of the *glmS* transcription at this site but the transcripts terminate at a new site located within the transposon. This site-specific insertion is not deleterious to the host and provides a ‘safe site’ for transposon insertion (Gay et al., 1986; Peters & Craig, 2001).

**Distribution of the *vanG*-like gene cluster in *C. difficile* lineages**

Eighty-three isolates representative of the main lineages described for *C. difficile* were selected from the MLST database of *C. difficile* (http://www.pasteur.fr/recherche/...
genopole/PF8/mlst) (Lemee et al., 2004; Lemée & Pons, 2010), to analyse the occurrence of the vanG-like operon within the different lineages of the species. DNA from each strain was amplified with both the forward primer van out-F and the reverse primer van out-R flanking the putative genetic element and the reverse primer van in-R located in the left end of the element in a single duplex reaction. The expected size of the DNA fragment produced by the van out-F/van out-R primer combination was 355 bp in strains lacking the vanG-like gene cluster and the expected size of the van out-F/van in-R product was 167 bp in strains harbouring this cluster. PCR products of one or the other of the two expected sizes were obtained in all tested strains. Results indicate that 57 of the 83 strains studied harbour the vanG-like operon, indicating a high prevalence of the genetic element in various lineages of the species (Fig. 3). PCR performed with primers internal to the vanG-like operon confirmed that this cluster was not present elsewhere in the genome of the 26 remaining strains. This result is consistent with the study of Ammam et al. (2012), in which this cluster was detected in 35 out of 41 clinical isolates. Interestingly, the 26 strains lacking the vanG-like cluster correspond to the most divergent MLST lineages within the species, such as isolates A+B+CDT+; PR078/126; tox.V; ST49/50 or A-B+CDT-; PR017; tox.VIII; ST45 [PR, PCR ribotype; tox, toxinotype according to Rupnik et al. (1998); ST, sequence type]. In contrast, hypervirulent strains corresponding to isolates A+B+CDT+; PR027; tox.III; ST3 harbour the vanG-like cluster (Fig. 3). The phylogenetic origin of this cluster remains difficult to specify. The data obtained from the MLST analysis suggest an acquisition rather than a loss of the genetic element in various lineages of C. difficile, such as isolates A+B+CDT+; PR078/126; tox.V; ST49/50 or A-B+CDT-; PR017; tox.VIII; ST45 [PR, PCR ribotype; tox, toxinotype according to Rupnik et al. (1998); ST, sequence type]. In contrast, hypervirulent strains corresponding to isolates A+B+CDT+; PR027; tox.III; ST3 harbour the vanG-like cluster (Fig. 3). The phylogenetic origin of this cluster remains difficult to specify. The data obtained from the MLST analysis suggest an acquisition rather than a loss of the genetic element in various lineages of C. difficile, such as isolates A+B+CDT+; PR078/126; tox.V; ST49/50 or A-B+CDT-; PR017; tox.VIII; ST45 [PR, PCR ribotype; tox, toxinotype according to Rupnik et al. (1998); ST, sequence type]. In contrast, hypervirulent strains corresponding to isolates A+B+CDT+; PR027; tox.III; ST3 harbour the vanG-like cluster (Fig. 3). The phylogenetic origin of this cluster remains difficult to specify. The data obtained from the MLST analysis suggest an acquisition rather than a loss of the genetic element in various lineages of C. difficile.

### Vancomycin susceptibility of strains harbouring the vanG-like operon

A previous study revealed that strain 630, which harbours the vanG-like gene cluster, and strain CF2, which lacks these genes, are both susceptible to vancomycin (Sebaiha et al., 2006). We investigated if MICs of vancomycin could vary between strains harbouring the vanG-like operon and strains lacking this element. From the 83 isolates used to study the distribution of the vanG-like cluster (Fig. 3), 26 strains representative of the main MLST lineages were selected, among which 13 harboured the vanG-like locus and 13 lacked this locus (Table S1, available in Microbiology Online). No significant difference in vancomycin MICs could be detected between these strains, with MICs ranging from 0.5 to 2 µg ml⁻¹, which classify all strains as susceptible to vancomycin (Table S1).

### Transcriptional analysis of the vanG-like gene cluster

To determine if the lack of vancomycin resistance in strains of C. difficile harbouring the vanG-like gene cluster is due to a defective transcription of effector genes, we analysed by quantitative real-time PCR the transcription of vanG-, vanXY- and vanT-like genes during growth of C. difficile 630 with or without vancomycin. A transcript was detectable from cells grown in the absence or presence of vancomycin for the three genes, indicating a significant transcriptional expression in both conditions. Moreover, the results showed a strong induction of the vanG-like gene transcription in the presence of vancomycin, especially in mid-exponential growth phase (OD600 of 0.5; +190-fold). This induction was also pronounced in early exponential (OD600 of 0.3; +65-fold) and late exponential growth phase (OD600 of 1; +77-fold) and reached the minimum amount in stationary phase (OD600 of 1.4; +11-fold) (Fig. 4).

Transcription of the vanXYG and vanTG genes was also induced in cells exposed to vancomycin but with a maximum induction in mid-exponential growth phase of 114-fold for vanXYG and only 12-fold for vanTG (Table S1). The difference observed in the induction level of the vanG, vanXYG and vanTG genes is probably directly due to their position on the operonic structure, as the closest genes to the promoter sequence can be more efficiently transcribed than genes located in the 3’ end. As the expression of vanTG (which encodes the serine racemase) is only weakly induced by vancomycin, it was tempting to speculate that the absence of vancomycin resistance could be explained by insufficient production of D-serine. To address this hypothesis, we measured the MIC of vancomycin in BHI agar supplemented with the highest serine concentration not affecting bacterial growth. As it is not known whether D-serine can be transported into C. difficile, we tested supplementation of the growth medium with D-serine or L-serine (10 and 20 mM, respectively). The addition of either D-serine or L-serine did not affect susceptibility to vancomycin, suggesting that the lack of vancomycin resistance is not due to limited input of D-serine for the synthesis of D-Ala–D-Ser ending precursors. The transcription level of the ddl gene, encoding the intrinsic D-Ala–D-Ala ligase in C. difficile, was also investigated and as expected was not modified in the presence of vancomycin.
(data not shown). The vanG-like transcription was also analysed in mid-exponential growth phase in the presence of teicoplanin (1/4 MIC) but no induction was observed (data not shown). These data are consistent with the VanG phenotype, which confers low-level resistance to vancomycin but no resistance to teicoplanin in *E. faecalis* (Depardieu et al., 2003).

Taken together, these results indicate that the vanG-like operon of *C. difficile* is functional at the transcriptional level and is induced by vancomycin but not teicoplanin. Induction of effector genes of the vanG-like operon by vancomycin also suggests that the regulatory proteins VanRG-like and VanSG-like are functional.

**Fig. 3.** Distribution of the vanG-like operon within 83 isolates representative of the main MLST phylogenetic lineages described for *C. difficile*. The denomination of strains is as follows: ‘strain number’/’toxinotype’ (according to Rupnik et al., 1998)/’PCR-ribotype’/’presence (+) or absence (−) of the vanG-like operon’. Moreover, the strains harbouring the vanG-like gene operon are in bold. The diamonds indicate the strains for which PCR product was sequenced. PR, PCR-ribotype; tox, toxinotype; ST, sequence type; ND, not determined.
Analysis of peptidoglycan precursors

In a previous study, we showed that vancomycin had no impact on peptidoglycan structure in *C. difficile* (Peltier et al., 2011). To investigate further the possible expression of the vanG-like operon, we analysed in the present work the cell-wall precursors in *C. difficile* 630 grown in the presence (1/4 MIC) or absence of vancomycin, by blocking the cell-wall assembly with the addition of bacitracin (Severin et al., 2004). In the absence of vancomycin, bacitracin at 770 μg ml⁻¹ was added to completely stop the bacterial growth, but the addition of the same bacitracin concentration in culture grown in the presence of vancomycin led to a rapid lysis of the cells, due to a synergistic effect of the two antibiotics. Consequently, the concentration of bacitracin used in the presence of vancomycin was adjusted to 100 μg ml⁻¹ to completely stop the bacterial growth without inducing cellular lysis.

The cytoplasmic pools of peptidoglycan precursors were separated by HILIC-HPLC and peaks were analysed by MALDI-TOF MS in the negative mode. In cells grown without vancomycin, only one molecular species corresponding to a peptidoglycan precursor was detected, with a *m/z* value of 1192.3 ([M-H⁻]) corresponding to UDP-MurNAc-pentapeptide. Of note, identical results were obtained using ramoplanin instead of bacitracin to block the cell-wall assembly. Surprisingly, HILIC-HPLC analysis of cytoplasmic peptidoglycan precursors extracted from cells grown in the presence of vancomycin also revealed only UDP-MurNAc-pentapeptide[D-Ala], although transcriptional induction of the vanG-like operon was confirmed in the same samples. No UDP-MurNAc-pentapeptide[D-Ser] could be detected by HILIC-HPLC and MALDI-TOF analysis (Table S2).

Effect of the inactivation or absence of the vanG-like cluster on the peptidoglycan structure

Although the vanG-like gene products look like they could substitute D-Ala–D-Ser, they might have another effect on cell-wall composition. To address this question, we used the CloStron system to create an independent insertional mutant of *C. difficile* 630Δerm in which the vanG-like gene was inactivated. The general gene inactivation process is represented schematically in Fig. S1(a). Insertion of the group II intron into the target genes was verified by PCR using specific internal primers (Fig. S1b). Peptidoglycan structure of the corresponding mutant strain was determined and compared with that of the *C. difficile* 630Δerm parental strain. The muropeptide profile of the mutant strain was exactly the same as that of the parental strain (Fig. S2), indicating that the presence of the vanG-like gene has no impact on the cell-wall composition.

We recently showed that the peptidoglycan of *C. difficile* is mainly connected by 3–3 cross-links generated by L,D-transpeptidases, instead of the more classical 4–3 cross-links generated by D,D-transpeptidation (Peltier et al., 2011). L,D-Transpeptidases use acyl donors containing a tetrapeptide stem, generated by the activity of a D,D-carboxypeptidase (Lavollay et al., 2009; Mainardi et al., 2008; Sacco et al., 2010). However, no UDP-MurNAc-tetrapeptide was detected among cytoplasmic peptidoglycan precursors of *C. difficile* (Table S2). These data suggest that the tetrapeptide substrate of L,D-transpeptidase in *C. difficile* is produced by a D,D-carboxypeptidase active either on lipid-linked precursors (lipid I or II) or after translocation of the precursors to the outside surface of the membrane.

Taken together, these data indicate that transcriptional induction of the vanG-like operon by vancomycin does not result in the production of modified D-Ala–D-Ser precursors in our conditions, a result that corroborates the absence of vancomycin resistance, as precursors terminating in D-Ala–D-Ala have high affinity for glycopeptides. The serine racemase does not seem to be implicated in the lack of D-Ala–D-Ser precursors, as bypass by the addition of D-serine or L-serine did not affect vancomycin susceptibility. Further structural and functional studies of the *C. difficile* VanG D-Ala-D-Ser ligase, as recently performed in *E. faecalis* (Meziane-Cherif et al., 2012), may help to explain the lack of accumulation of D-Ala–D-Ser precursors in *C. difficile* and its sensitivity to vancomycin.

**Fig. 4.** Quantitative Real Time-PCR (RT-PCR) analyses of gene transcription of the vanG-like operon of *C. difficile* 630 with or without vancomycin treatment. Cultures were grown with or without vancomycin (1/4 MIC) and samples were taken at different times of cell growth (recorded as OD 600 nm). Relative induction values are given as fold changes (growth with vancomycin relative to growth without vancomycin), calculated by the 2^ΔΔCt method. Error bars indicate SD.
We also analysed the cell-wall composition of a strain lacking the vanG-like cluster, strain E25, which is one of the most phylogenetically close to the 630 strain among those that do not harbour the vanG-like cluster (Fig. 3). The muropeptide profile of the E25 strain was very close to that of the 630 strain (Fig. S2), confirming that the vanG-like cluster does not affect the peptidoglycan structure of C. difficile.

In summary, the present work characterizes the wide distribution of the vanG-like gene cluster of C. difficile in the main phylogenetic lineages of the species, describes its genomic insertion site in strain 630, and shows that, despite vancomycin-induced transcription of this operon, C. difficile synthesizes D-Ala–D-Ala peptidoglycan precursors and thus retains vancomycin susceptibility.

ACKNOWLEDGEMENTS

We thank Neil Fairweather (Imperial College, London, UK) for critical reading of the manuscript. This work was supported by the University of Rouen, Rouen University Hospital and INRA, Jouy-en-Josas, France.

REFERENCES


Edited by: A. O'Neill