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Running title: VanG-type vancomycin-resistant in *E. faecium*

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Synopsis

Objectives: to characterize phenotypically and genotypically the first *Enterococcus faecium* clinical isolate harbouring a *vanG* operon.

Methods: The antibiotic-resistance profile of *E. faecium* 16-346 was determined and its whole genome sequenced using the PacBio technology. Attempts to transfer vancomycin resistance by filter mating were performed and the inducibility of expression of the *vanG* operon was studied by reverse-transcription quantitative PCR **(**RT-qPCR) in the presence or absence of subinhibitory concentrations of vancomycin.

Results: *E. faecium* 16-346 was resistant to rifampicin (Minimum Inhibitory Concentration (MIC) > 4 mg/L) erythromycin (MIC > 4 mg/L), tetracycline (MIC > 16 mg/L) and vancomycin (MIC of 8 mg/L) but susceptible to teicoplanin (MIC of 0.5 mg/L). The strain harboured the *vanG* operon into its chromosome, integrated into a 45.5-kb putative mobile genetic element, similar to that of *E. faecalis* BM4518. We were unable to transfer vancomycin resistance from *E. faecium* 16-346 to *E. faecium* BM4107 and *E. faecalis* JH2-2. Lastly, transcription of the *vanG* gene was inducible by vancomycin.

Conclusions: This is to the best of our knowledge the first report of a VanG-type vancomycin-resistant strain of *E. faecium*. Despite the alarm pulled because of the therapeutic problems caused by vancomycin-resistant enterococci, our work shows that new resistant loci can still be found in *E. faecium.*

Introduction

Commensal bacteria of the human gut microbiota, enterococci have become major opportunistic pathogens.[1] In addition, vancomycin-resistant *Enterococcus faecium* (VREF) is currently a member of the list of bacteria recently edited by the World Health Organization (WHO), for which new antibiotics are urgently needed (http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/). This points to the worldwide emergence of this species, especially hospital-adapted strains belonging to clonal complex (CC) 17, that leads to major concerns in clinical settings.[2] Glycopeptide resistance is due to the presence of *van* operons that encode enzymes catalyzing the production of modified peptidoglycan precursors ending in D-Ala-D-Lac (VanA, B, D and M) or D-Ala-D-Ser (VanC, E, G, L and N).[2] These precursors have much less affinity for vancomycin than the D-Ala-D-Ala motif, leading to a resistant phenotype (MIC of vancomycin > 4 mg/L).[2] Moreover, these operons are tightly regulated by VanS/VanR-type two-component system, where VanS is a membrane-bound histidine kinase and VanR a transcriptional regulator.[3] Because some *van* operons are part of mobile genetic elements, the glycopeptide resistance has spread among enterococci.

The *vanG* operon, in which the VanG ligase allows synthesis of precursor ending in D-Ala-D-Ser, has only been detected in a few *Enterococcus faecalis* clinical isolates and confers moderate levels of resistance to vancomycin (MIC of 16 mg/L) but not to teicoplanin.[4,5] It is characterized by a "three component" regulatory system comprising an additional repressor ($VanU_G$) also involved in the control of the *vanG* operon transcription.[6]

We report, to the best of our knowledge, the first clinical isolate of *E. faecium* harbouring a *vanG* operon. The genome of this strain (*E. faecium* 16-346) was completely sequenced and we identified in the chromosome an acquired putative mobile genetic element containing the *vanG* locus.

Materials and methods

Bacterial strains and MIC determination

E. faecium 16-346 was obtained in 2016 from a fecal sample of a non-infected 40-year-old female patient hospitalized in the medical center of Sainte-Foy-l'Argentière, France.

E. faecium BM4107 and *E. faecalis* JH2-2 were used for filter mating experiments.

MICs of 20 antibiotics (chloramphenicol, daptomycin, gentamicin, linezolid, rifampicin, trimethoprim/sulfamethoxazole, quinupristin/dalfopristin, tetracycline, erythromycin, oxacillin, ampicillin, penicillin G, vancomycin, levofloxacin, tigecycline, moxifloxacin, clindamycin, streptomycin, ciprofloxacin and nitrofurantoin) were determined using a Sensititre™ automated system (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Whole genome sequencing and accession number

The whole genome sequence of the *E. faecium* 16-346 strain was determined with the PacBio technology (GATC Biotech, Konstanz, Germany). The sequence was annotated using the NCBI Prokaryotic Genome Annotation Pipeline.[7] The sequence was submitted to GenBank and assigned accession no. CP021849.

Filter mating

Transfer of vancomycin resistance from *E. faecium* 16-346 to *E. faecium* BM4107 and *E. faecalis* JH2-2 was attempted by filter mating. Transconjugants were selected on brain heart infusion (BHI) agar containing rifampicin (60 mg/L), fusidic acid (50 mg/L) and vancomycin (4 mg/L).

Reverse-transcription quantitative PCR (RT-qPCR) experiments

The expression of the *vanG* gene was assessed by RT-qPCR using RNAs extracted from bacterial cells (OD_{600} of 0.5) grown in the absence or in the presence of subinhibitory concentrations of vancomycin (i.e., 1 or 2 mg/L). PCR amplification was carried out with primers specific for *vanG* (*vanG*-F: (5'-TTCGTGCAGGCTCTTCCTTT-3'; *vanG*-R: 5'-CACAACCGACTTCAAAGCCG-3') or *adk* (housekeeping reference-gene) as described.[8]

Results and discussion

Characterization of *E. faecium* 16-346

E. faecium 16-346 was resistant to vancomycin (MIC of 8 mg/L). It was also resistant to rifampicin (MIC > 4 mg/L), erythromycin (MIC > 4 mg/L), and tetracycline (MIC > 16 mg/L) but remained susceptible to teicoplanin (MIC of 0.5 mg/L) and to the other antibiotics tested. Screening by specific PCR for the presence of the *vanA*, *vanB*, *vanD*, *vanM* and *vanN* genes, known to confer glycopeptide resistance in this species was negative.[2] Primers specific for the other *van* operons, not yet described in *E. faecium* were tested, and an amplification of the *vanG* gene was obtained. *vanG*-type operons have only been found in *E. faecalis* clinical isolates from Australia and Canada as well as in various *Clostridium* sp. [4,5,9,10] In the latter, *vanG* did not confer vancomycin resistance but they may however be considered as a possible reservoir. It has been shown that part of the microflora of the human gastrointestinal tract harboured the *vanG* gene.[11] Indeed, from 248 rectal swabs samples, Domingo and coworkers showed that 9.3% were positive for *vanG*, but none was associated with enterococci.[12] Since, to our knowledge, no VanG-type *E. faecium* have been identified so far, we further analyzed this strain by whole genome sequencing.

With a size of 2,736,595 bp, the genome harboured 2,824 putative genes (2,730 CDS) (Figure S1). This strain was plasmid free and belonged to sequence type ST121. Search for resistance

genes revealed the presence of the *vanG* operon, but also that of *erm*(Y) and *msr*(C), both involved in macrolide resistance, as well as that of *tet*(M) and *tet*(L) that confer tetracycline resistance (Table 1). These data were in agreement with the resistance phenotype of *E. faecium* 16-346.

Analysis of the *vanG* operon

The sequence of the *vanG* operon was very similar to those from *E. faecalis* BM4518, WCH9 and G1-0247 (Figure 1).[4,5,13] Indeed, the *van* genes (U_G, R_G, S_G, Y_G, W_G, G_G, XY_G, and T_G) were between 98.6% and 100% identical (Table 1). Thus, after VanN, VanG is the second ligase able to synthetize D-Ala-D-Ser-ending precursors in *E. faecium*.[14] This is consistent with low level vancomycin resistance (MICs of 8-16 mg/L), since the precursors ending with D-Ala-D-Ser present only a seven-fold less affinity for the antibiotic.[15] In $vanY_G$ of *E. faecalis* BM4518, a frameshift mutation is present.[13] In *E. faecium*, $vanY_G$ has no mutations as in the $vanY_G$ from *E. faecalis* G1-0247 strain.[5]

Twenty two bp imperfect inverted repeats (IR) framed the mobile genetic element (Figure 1). In *E. faecalis* BM4518, the element is 240 kb long, whereas, in *E. faecium*, its size was of 45.5 kb comprising 43 genes (Table S1). Of note, the sequence of the left IR was identical to that of *E. faecalis* G1-0247 (CGGTAGTACTTCTTTCCCACAA) and diverged from that of BM4518-by 2 bases (CGGTGGTACTGCTTTCCCACAA).[5,13] Surprisingly, the sequence of the left direct repeat (DR) (TGGA) was not the same as the TTGA sequence of the right DR (Figure 1). Based on the sequence of *E. faecium* Aus0004, TGGA appears as the motif present into the gene in which the element has been inserted[16]. It may be hypothesized that the target site of integration could be only the GA 2-bp sequence. To date, in *E. faecalis*, two types of *vanG* operon have been characterized, one from strain BM4518, WCH9 and G1-0247 (*vanG*), and another from N03-0233 (*vanG2*), lacking the $vanY_G$ gene and showing also a 2-bp (CA)

144 DR.[5] Interestingly, in all the VanG-type strains (clinical isolates or transconjugants) insertion
145 of the *vanG* cluster systematically occurred into the gene encoding a RNA methyltransferase,
146 whereas the *vanG2* is located into the *dctP* gene (encoding a subunits of the TRAP
147 dicarboxylate transporter).[13] This was not observed in *E. faecium* where the element was
148 integrated into a gene encoding a hypothetical protein (annotated EFAU004_00391) (Figure
149 1).
150
151 **Attempts to transfer and inductibility of expression of the *vanG* operon**
152 We were unable to transfer vancomycin resistance from *E. faecium* 16-346 to *E. faecium*
153 BM4107 and *E. faecalis* JH2-2. Similarly, McKessar and co-workers failed to transfer *vanG*
154 operons from *E. faecalis* to *E. faecium*.[4] In *E. faecalis* BM4518, *erm*(B) was co-transferred
155 with *vanG* and transconjugants could only be obtained on erythromycin-containing plates.[13]
156 In *E. faecium* 16-346, the mobile genetic element did not harbour *erm*(B) and macrolide
157 resistance was likely due to the presence of the *erm*(Y) and *msr*(C) genes located 231 kb and
158 522 kb downstream, respectively (Table 1). As in *E. faecalis* G1-0247 and N03-0233, the
159 functions necessary for the transfer should not efficient or the experimental conditions were
160 not adapted.
161 In order to test if *vanG* expression was inducible, RT-qPCR experiments were carried out
162 from cells grown with 0, 1 or 2 mg/L of vancomycin (corresponding to $^1/_8$ and ¼ of MIC,
163 respectively). As expected, *vanG* transcription was increased 3 and 5 fold ($p<0.05$) in the
164 presence of 1 and 2 mg/L of antibiotic, respectively. This strongly suggests that
165 transcriptional regulation may be similar to that of *E. faecalis* where the repressor $VanU_G$ and
166 the activator $VanR_G$ (both present in *E. faecium*) compete to the same P_{YG} promoter leading to
167 a rheostatic control of *vanG* operon expression.[6]
168

Conclusion

To our knowledge, this is the first report of a *vanG*-type locus acquired by a strain of *E. faecium*. This points out that the surveillance of VREF remains crucial to overcome the spread of this major opportunistic pathogen.

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Transparency declarations

None to declare.

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222

223

224 **Table 1**: Antimicrobial-resistance genes identified in the genome sequence of *E. faecium* 16-

225 246.

226

Gene	% Identity	Position in contig	Phenotype	Accession no.
vanT$_G$	100.00[a]	1442739..1444877	Vancomycin resistance	AY271782
vanXY$_G$	100.00[a]	1444870..1445634	Vancomycin resistance	AY271782
vanG	100.00[a]	1445631..1446680	Vancomycin resistance	AY271782
vanW$_G$	99.88[a]	1446682..1447527	Vancomycin resistance	AY271782
vanY$_G$	100.00[b]	1447603..1448442	Vancomycin resistance	DQ212986
vanS$_G$	99.82[a]	1448579..1449683	Vancomycin resistance	AY271782
vanR$_G$	100.00[a]	1449697..1450404	Vancomycin resistance	AY271782
vanU$_G$	100.00[a]	1450406..1450633	Vancomycin resistance	AY271782
tet(M)	97.45[c]	1707444..1709362	Tetracycline resistance	EU182585
tet(L)	99.85[d]	1709491..1710832	Tetracycline resistance	M29725
erm(Y)	81.51[e]	1715249..1715977	Macrolide resistance	AB014481
msr(C)	98.99[f]	2006190..2007668	Macrolide, Lincosamide Streptogramin resistance	AY004350

227 [a]Identity with the homologous gene from *E. faecalis* BM4518
228 [b]Identity with the homologous gene from *E. faecalis* G1-0247
229 [c]Identity with the homologous gene from *Streptococcus suis* T2S3
230 [d]Identity with the homologous gene from pLS1 plasmid of *Streptococcus agalactiae*
231 [e]Identity with the homologous gene from the pMS97 plasmid of *Staphylococcus aureus* RN4220
232 [f]Identity with the homologous gene from *E. faecium* TX2465

233

Legends

Figure 1: Schematic of the chromosome region harbouring the *vanG* operon of *E. faecium* 16-346, compared to the corresponding region of *E. faecalis* BM4518.

DR-L, direct repeat left; DR-R, direct repeat right; IR-L, inverted repeat left; IR-R, inverted repeat right.

Table S1: Genes identified in the mobile genetic element containing the *vanG* operon in *E. faecium* 16-346.

Figure S1: Circle representation of the genome of *E. faecium* 16-346 compared to four other *E. faecium* genomes.

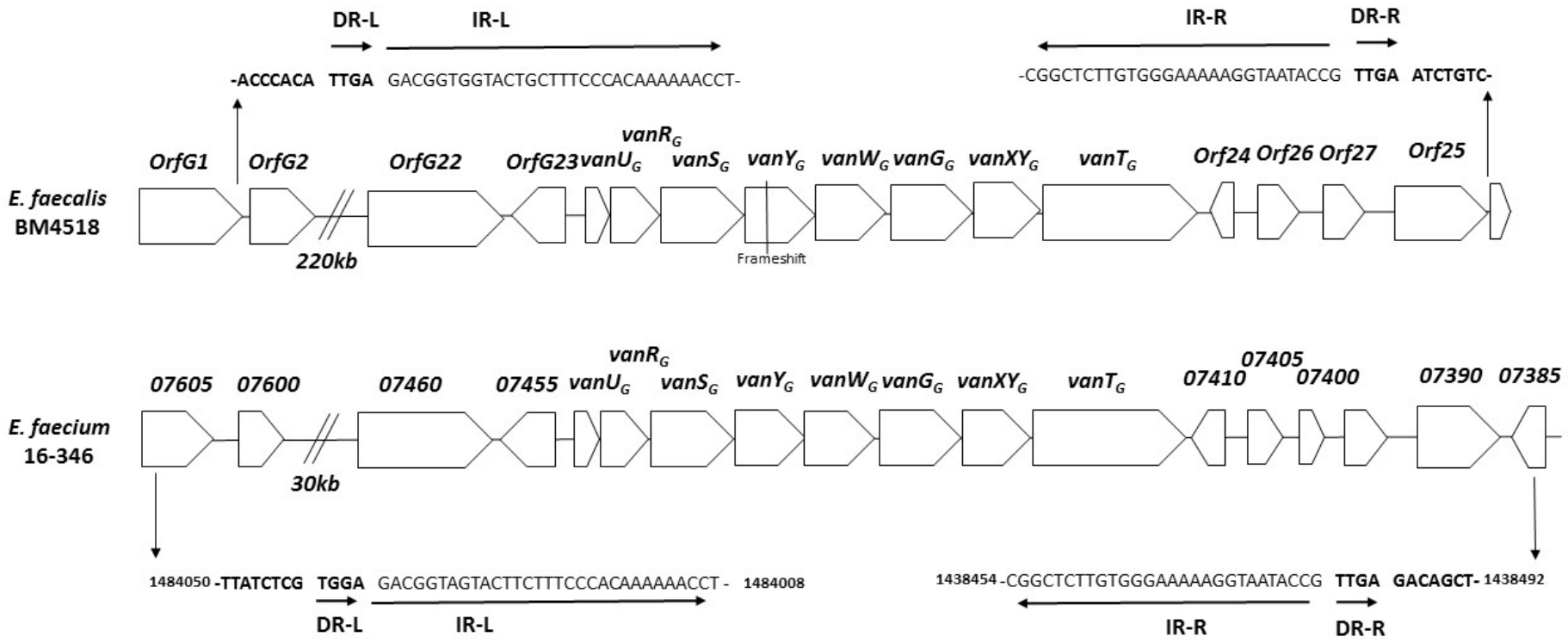
DR-L
IR-L
IR-R
DR-R
-ACCCACA TTGA GACGGTGGTACTGCTTTCCCACAAAAAACCT-
-CGGCTCTTGTGGGAAAAAGGTAATACCG TTGA ATCTGTC-
vanR_G
OrfG1 OrfG2 OrfG22 OrfG23 vanU_G vanS_G vanY_G vanW_G vanG_G vanXY_G vanT_G Orf24 Orf26 Orf27 Orf25
E. faecalis BM4518
220kb
Frameshift
vanR_G
07605 07600 07460 07455 vanU_G vanS_G vanY_G vanW_G vanG_G vanXY_G vanT_G 07410 07405 07400 07390 07385
E. faecium 16-346
30kb
1484050 -TTATCTCG TGGA GACGGTAGTACTTCTTTCCCACAAAAAACCT - 1484008
DR-L
IR-L
1438454 -CGGCTCTTGTGGGAAAAAGGTAATACCG TTGA GACAGCT- 1438492
IR-R
DR-R

Figure 1