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1 **Genetic characterization of a VanG-type vancomycin-resistant *Enterococcus faecium***
2 **clinical isolate**

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

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23

24 **Synopsis**

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

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

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

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

43

44 **Introduction**

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

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

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

69

70 **Materials and methods**

71 **Bacterial strains and MIC determination**

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

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

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

81

82 **Whole genome sequencing and accession number**

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

87

88 **Filter mating**

89 Transfer of vancomycin resistance from *E. faecium* 16-346 to *E. faecium* BM4107 and *E.*
90 *faecalis* JH2-2 was attempted by filter mating. Transconjugants were selected on brain heart
91 infusion (BHI) agar containing rifampicin (60 mg/L), fusidic acid (50 mg/L) and vancomycin
92 (4 mg/L).

93

94 **Reverse-transcription quantitative PCR (RT-qPCR) experiments**

95 The expression of the *vanG* gene was assessed by RT-qPCR using RNAs extracted from
96 bacterial cells (OD₆₀₀ of 0.5) grown in the absence or in the presence of subinhibitory
97 concentrations of vancomycin (i.e., 1 or 2 mg/L). PCR amplification was carried out with
98 primers specific for *vanG* (*vanG*-F: (5'-TTCGTGCAGGCTCTTCCTTT-3'; *vanG*-R: 5'-
99 CACAACCGACTTCAAAGCCG-3') or *adk* (housekeeping reference-gene) as described.⁸

100

101 **Results and discussion**

102 **Characterization of *E. faecium* 16-346**

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

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

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

123

124 **Analysis of the *vanG* operon**

125 The sequence of the *vanG* operon was very similar to those from *E. faecalis* BM4518, WCH9
126 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*)
127 were between 98.6% and 100% identical (Table 1). Thus, after VanN, VanG is the second
128 ligase able to synthesize D-Ala-D-Ser-ending precursors in *E. faecium*.¹⁴ This is consistent
129 with low level vancomycin resistance (MICs of 8-16 mg/L), since the precursors ending with
130 D-Ala-D-Ser present only a seven-fold less affinity for the antibiotic.¹⁵ In *vanY_G* of *E. faecalis*
131 BM4518, a frameshift mutation is present.¹³ In *E. faecium*, *vanY_G* has no mutations as in the
132 *vanY_G* from *E. faecalis* G1-0247 strain.⁵

133 Twenty two bp imperfect inverted repeats (IR) framed the mobile genetic element (Figure 1).
134 In *E. faecalis* BM4518, the element is 240 kb long, whereas, in *E. faecium*, its size was of
135 45.5 kb comprising 43 genes (Table S1). Of note, the sequence of the left IR was identical to
136 that of *E. faecalis* G1-0247 (CGGTAGTACTTCTTTCCCACAA) and diverged from that of
137 BM4518-by 2 bases (CGGTGGTACTGCTTTCCCACAA).^{5,13} Surprisingly, the sequence of
138 the left direct repeat (DR) (TGGA) was not the same as the TTGA sequence of the right DR
139 (Figure 1). Based on the sequence of *E. faecium* Aus0004, TGGA appears as the motif present
140 into the gene in which the element has been inserted¹⁶. It may be hypothesized that the target
141 site of integration could be only the GA 2-bp sequence. To date, in *E. faecalis*, two types of
142 *vanG* operon have been characterized, one from strain BM4518, WCH9 and G1-0247 (*vanG*),
143 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 $\frac{1}{8}$ and $\frac{1}{4}$ 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

169 **Conclusion**

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

173

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178 **Transparency declarations**

179 None to declare.

180

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182

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220 *Enterococcus faecium* genome. *J Bacteriol* 2012; **194**: 2334-41.

221

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.
<i>vanT_G</i>	100.00 ^a	1442739..1444877	Vancomycin resistance	AY271782
<i>vanXY_G</i>	100.00 ^a	1444870..1445634	Vancomycin resistance	AY271782
<i>vanG</i>	100.00 ^a	1445631..1446680	Vancomycin resistance	AY271782
<i>vanW_G</i>	99.88 ^a	1446682..1447527	Vancomycin resistance	AY271782
<i>vanY_G</i>	100.00 ^b	1447603..1448442	Vancomycin resistance	DQ212986
<i>vanS_G</i>	99.82 ^a	1448579..1449683	Vancomycin resistance	AY271782
<i>vanR_G</i>	100.00 ^a	1449697..1450404	Vancomycin resistance	AY271782
<i>vanU_G</i>	100.00 ^a	1450406..1450633	Vancomycin resistance	AY271782
<i>tet(M)</i>	97.45 ^c	1707444..1709362	Tetracycline resistance	EU182585
<i>tet(L)</i>	99.85 ^d	1709491..1710832	Tetracycline resistance	M29725
<i>erm(Y)</i>	81.51 ^e	1715249..1715977	Macrolide resistance	AB014481
<i>msr(C)</i>	98.99 ^f	2006190..2007668	Macrolide, Lincosamide Streptogramin resistance	AY004350

227 ^aIdentity with the homologous gene from *E. faecalis* BM4518228 ^bIdentity with the homologous gene from *E. faecalis* G1-0247229 ^cIdentity with the homologous gene from *Streptococcus suis* T2S3230 ^dIdentity with the homologous gene from pLS1 plasmid of *Streptococcus agalactiae*231 ^eIdentity with the homologous gene from the pMS97 plasmid of *Staphylococcus aureus* RN4220232 ^fIdentity with the homologous gene from *E. faecium* TX2465

233

234 **Legends**

235

236

237 **Figure 1:** Schematic of the chromosome region harbouring the *vanG* operon of *E. faecium* 16-
238 346, compared to the corresponding region of *E. faecalis* BM4518.

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

241

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

244

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

247

248

249

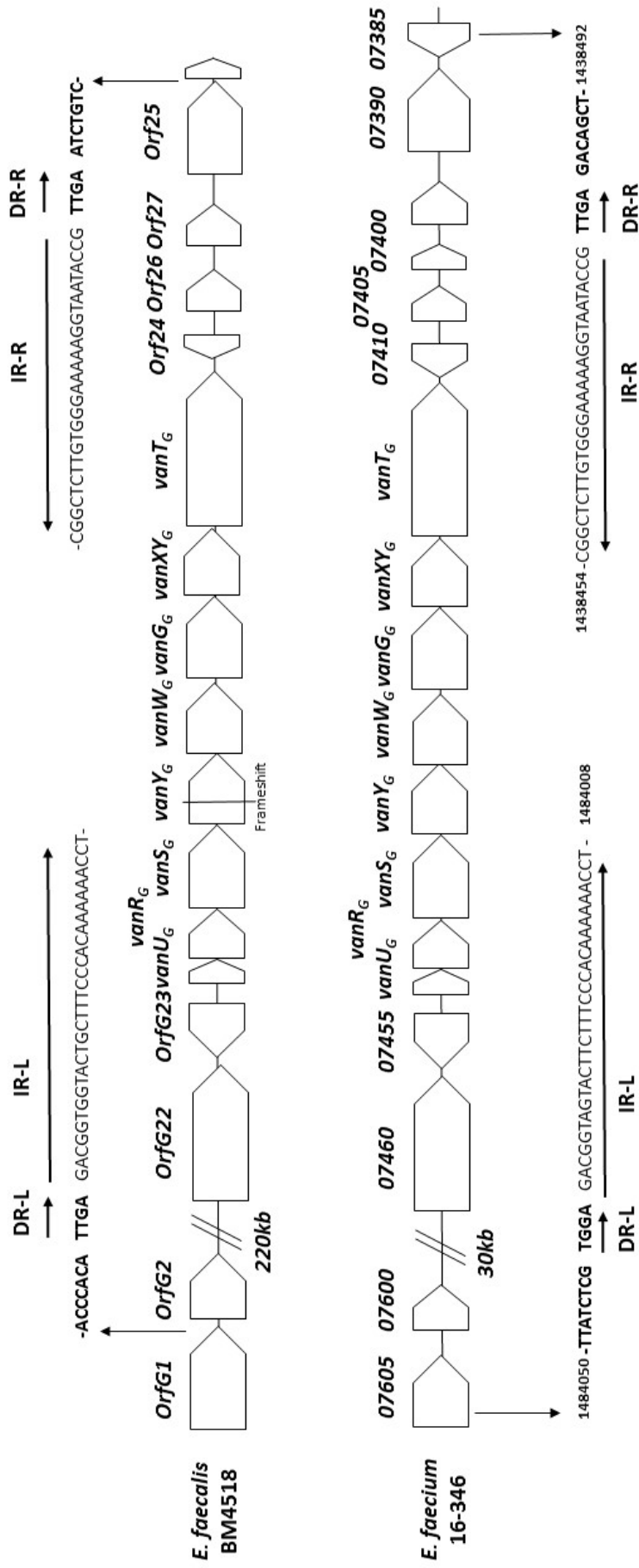


Figure 1