

The first report of the *vanC_I* gene in *Enterococcus faecium* isolated from a human clinical specimen

Mingyue Sun, Yue Wang, Zhongju Chen, Xuhui Zhu, Lei Tian, Ziyong Sun⁺

Department of Laboratory Medicine, Tongji Hospital, Tongji Medical College,
Huazhong University of Science and Technology, Wuhan, Hubei, China

The *vanC_I* gene, which is chromosomally located, confers resistance to vancomycin and serves as a species marker for *Enterococcus gallinarum*. *Enterococcus faecium* TJ4031 was isolated from a blood culture and harbours the *vanC_I* gene. Polymerase chain reaction (PCR) assays were performed to detect *vanXYc* and *vanTc* genes. Only the *vanXYc* gene was found in the *E. faecium* TJ4031 isolate. The minimum inhibitory concentrations of vancomycin and teicoplanin were 2 µg/mL and 1 µg/mL, respectively. Real-time reverse transcription-PCR results revealed that the *vanC_I* and *vanXYc* genes were not expressed. Pulsed-field gel electrophoresis and southern hybridisation results showed that the *vanC_I* gene was encoded in the chromosome. *E. faecalis* isolated from animals has been reported to harbour *vanC_I* gene. However, this study is the first to report the presence of the *vanC_I* gene in *E. faecium* of human origin. Additionally, our research showed the *vanC_I* gene cannot serve as a species-specific gene of *E. gallinarum* and that it is able to be transferred between bacteria. Although the resistance marker is not expressed in the strain, our results showed that *E. faecium* could acquire the *vanC_I* gene from different species.

Key words: *Enterococcus gallinarum* - *Enterococcus faecium* - *vanC_I* gene

During the past two decades, glycopeptide-resistant enterococci, in particular *Enterococcus faecium*, have become increasingly widespread throughout the world and have been identified as multi-resistant opportunistic pathogens in hospitals and in the environment (e.g., in foods and animals) (Lebreton et al. 2011, Nomura et al. 2012). Since the first detection of vancomycin resistance in *E. faecium* in 1988, nine operons that confer resistance to glycopeptides have been distinguished based on the sequence of the structure for the resistance ligase (Leclercq et al. 1988, Uttley et al. 1988, Lebreton et al. 2011). These operons are classified according to the characteristics of the ligase gene, which can encode either a D-alanyl-D-lactate ligase or a D-alanyl-D-serine ligase. Genes that encode D-alanyl-D-lactate ligases include the *vanA*, *vanB*, *vanD* and *vanM* genes and those that encode D-alanyl-D-serine ligases include the *vanC_I*, *vanC_{II}*, *vanC_{III}*, *vanE*, *vanG*, *vanL* and *vanN* genes (Arthur et al. 1996, Courvalin 2006, Lebreton et al. 2011, Nomura et al. 2012). Resistance types can be acquired except for *vanC*-type resistance, which is thought to be intrinsic to *Enterococcus gallinarum* and *Enterococcus casseliflavus*. The *vanC_I* cluster is composed of five genes: *vanC_I*, *vanXYc*, *vanTc*, *vanRc* and *vanSc*. Three of these genes are involved in inducing resistance according to the following mechanism: *vanC_I* encodes a ligase that

synthesises the dipeptide D-Ala-D-Ser, which is added to the UDP-MurNAc-tripeptide; *vanXYc* encodes a D,D-dipeptidase-carboxypeptidase that hydrolyses D-Ala-D-Ala and removes D-Ala from UDP-MurNAc-pentapeptide; *vanT* encodes a membrane-bound serine racemase that provides D-Ser to the synthetic pathway (Arias et al. 2000). The *vanC_I* gene is thought to occur only in *E. gallinarum* and should therefore be useful for species identification (Ramotar et al. 2000). Furthermore, this gene is chromosomally located and has not been found in *E. faecium* until now. Since the *vanC_I* gene was first identified in vancomycin-susceptible *Enterococcus faecalis* strains isolated from pig manure samples in Germany, *vanC_I*-type *E. faecalis* of animal origin has been reported in Spain and Brazil. This finding emphasises that the chromosomal location of a gene in intrinsically resistant strains does not necessarily prevent gene transfer to another species, which is in contrast to traditional views (Schwaiger et al. 2012, de Garnica et al. 2013, de Moura et al. 2013). So far, there has been one report of the genetic location of the *vanC_I* gene isolated from cloacal swabs of broilers and this gene was detected on plasmid (de Moura et al. 2013). We also identified *vanC_I*-type *E. faecium* strain isolated from a blood culture.

In this study, *E. faecium* TJ4031 was susceptible to both vancomycin and teicoplanin, but harboured the *vanC_I* resistance gene. We also presented evidence showing that the *vanC_I* gene cluster was incomplete; the *vanC_I* and *vanXYc* genes were not expressed. Additionally, the resistance gene in this clinical isolate was located on the chromosome.

MATERIALS AND METHODS

Strains - *E. faecium* TJ4031 was isolated in 2012 from a blood culture from an outpatient in our hospital. This isolate was initially identified by Gram staining and bio-

doi: 10.1590/0074-0276140019

Financial support: China National Mega Project on Major Infectious Disease Prevention (2012ZX100004207-004)

+ Corresponding author: tj_szyong@163.com

Received 17 January 2014

Accepted 20 June 2014

chemical reaction as previously described (Facklam & Collins 1989). Furthermore, the Vitek2-Compact system (bioMérieux, France) was used and *Enterococcus*-specific polymerase chain reaction (PCR) was performed (Jackson et al. 2004). This strain was also distinguished from *E. gallinarum* by determining the *E. gallinarum* *sod* gene using the species-specific Egaspe primer set. The primers used are listed as follows: Egaspe F, GAACCAACAGGAGCCATCA; Egaspe R, ACCAAGCCCAGCCAGAAC (Poyart et al. 2000). TJ430, which was confirmed as *E. gallinarum* isolated from a clinical specimen and *E. faecalis* ATCC29212, was used as positive and negative controls, respectively, for the detection and expression analysis of the *vanC_I*, *vanXYc* and *vanTc* genes. TJ430 was also used as control strain to analyse the location of the *vanC_I* gene. *Salmonella* H9812 was used as a marker for pulsed-field gel electrophoresis (PFGE).

Antibiotic susceptibility testing - Minimum inhibitory concentrations (MICs) were determined according to a standard agar dilution procedure, as recommended by the Clinical and Laboratory Standards Institute (CLSI 2012). *E. faecalis* ATCC29212 was used as a control strain.

Molecular typing and genetic techniques - TJ4031 was typed by subjecting genomic DNA to PFGE using the CHEF II Mapper system (Bio-Rad, France) (Lebreton et al. 2011). Total DNA was digested with *Sma*I and then electrophoresed (6 V/cm, 17 h, pulse times of 1-17 s at 14°C) on an agarose gel. Total DNA from the H9812 marker was digested with *Xba*I. The plasmid size and content of the TJ4031 isolate were determined using the S1 nuclease method (Freitas et al. 2009a). The location of the *vanC_I* gene was determined using southern hybridisation. A *vanC_I* probe was constructed by labelling an internal amplification product of the *vanC_I* gene from TJ4031 with digoxigenin according to the manufacturer's protocol (Roche, Switzerland). The primers used to amplify the product are listed as follows: *vanC_I* F, 5'-ATGGCTGTATCCAAGGACTG-3'; *vanC_I* R, 5'-AGGCAATGGTGCTGGAC-3'.

The *E. faecium* TJ4031 isolate was subjected to multilocus sequence typing (MLST) analysis (Zheng et al. 2007, Freitas et al. 2009b). The alleles and sequence type (ST) were determined using the MLST database (efaecium.mlst.net). The new ST identified in this study has been deposited in the database.

PCR - *E. faecium* isolates were species identified with PCR using *Enterococcus*-specific primers combined with species-specific primers for the *E. gallinarum* *sod* gene (Poyart et al. 2000, Jackson et al. 2004). *E. faecium* TJ4031 was also evaluated to determine the presence of *vanA*, *vanB*, *vanC_I*, *vanC_{2/3}* and *vanXYc*, *vanTc* (Patel et al. 1998, Schwaiger et al. 2012).

Sequence analysis - The band from the *vanC_I*-positive strain was excised from the gel and then sequenced. Sequence similarity was compared using the NCBI Basic Local Alignment Search Tool database (ncbi.nlm.nih.gov).

RNA extraction and reverse transcription-PCR (RT-PCR) - Total RNA from strain TJ4031 grown in brain-heart-yeast extract broth at 37°C to an absorbance at

600 nm of 0.8 was isolated using an RNeasy Mini Kit (Qiagen, Germany). Transcripts were obtained using a RevertAid First Strand cDNA system kit (Fermentas) according to the manufacturer's instructions. Real-time RT-PCR was conducted in a final volume of 20 µL containing 10 µL SYBR Mix (NovoGene), 0.5 µM primers and 3 µL template cDNA. The reaction was performed using a Light Cycler 480 (Roche) under the following conditions: initial denaturation at 95°C for 7 min and 40 cycles of denaturation at 95°C for 10 s, annealing at 57°C for 15 s, elongation at 72°C for 30 s and cooling at 40°C for 30 s. The primers used in this procedure are as follows: *vanrtC_I* F, ATTGGTGCTTGATGCG; *vanrtC_I* R, CGGGAGTGCCAGTAAA; *vanXYc* F, GCAAAA-CAATGGGAACGACT; *vanXYc* R, ATCTCGAAAATGAGGGCAGA. Non-reverse-transcribed PCR controls were used to indicate the absence of genomic DNA contamination. ATCC29212 and TJ430 were used as negative and positive control strains, respectively.

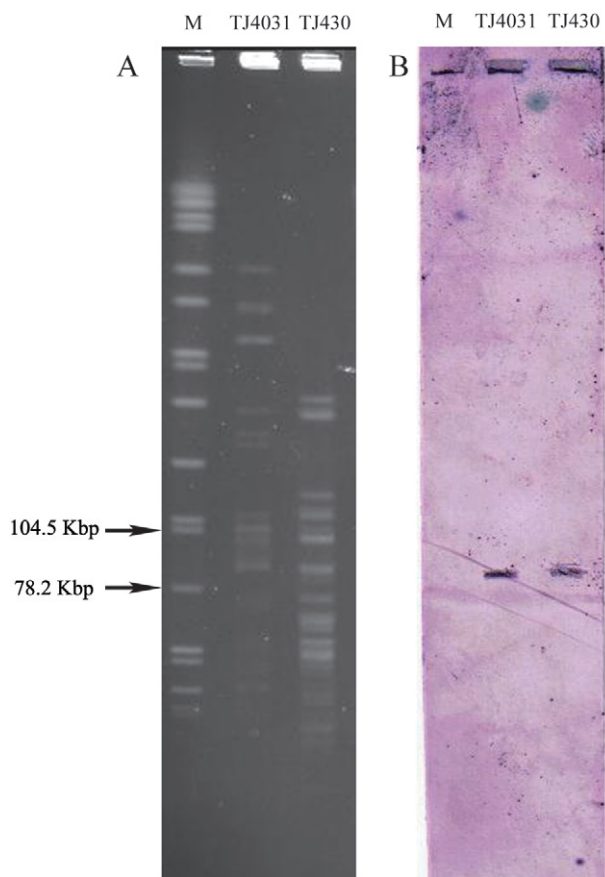
RESULTS

Characterisation of *E. faecium* TJ4031 - The clinical isolate TJ4031, which was isolated from a blood culture, was identified as *E. faecium* using conventional tests and the Vitek2-Compact system; species identity was confirmed by PCR. *E. gallinarum* was excluded as a possibility by a negative result in an *E. gallinarum* species-specific PCR. TJ4031 was susceptible to vancomycin (MIC = 2 µg/mL) and teicoplanin (MIC = 1 µg/mL). TJ4031 was also susceptible to chloramphenicol, linezolid, ciprofloxacin, levofloxacin and fosfomycin. By comparison, this strain was resistant to penicillin, rifampicin, erythromycin and nitrofurantoin. TJ4031 was positive for the *vanC_I* and *vanXYc* genes, but negative for the *vanTc* gene. The *vanC_I* gene sequence was deposited in GenBank (accession KF849246).

Identification of gene expression by real-time RT-PCR - RT-PCR assays failed to detect the corresponding *vanC_I* and *vanXYc* gene transcripts in the *vanC_I* gene positive *E. faecium* TJ4031 strain. The positive and negative controls worked as expected.

Molecular typing - The *E. faecium* TJ4031 isolate was categorised as a new ST according to MLST analysis and designated as ST837 (AtpA, 52; Ddl, 5; Gdh, 1; PurK, 1; Gyd, 1; PstS, 1; Adk, 3). This new ST showed the greatest similarity to the reported MLST type ST547 (AtpA, 15; Ddl, 5; Gdh, 1; PurK, 1; Gyd, 1; PstS, 1; Adk, 3) based on the information from the database (MLST database URE) (efaecium.mlst.net/). ST837 was a single-locus variant of ST547. Neither ST547 nor ST837 belong to the well-characterised clonal complex (CC) 17, which is found in a hospital-adapted and epidemic *E. faecium* strain cluster.

Genetic location of *vanC_I* gene in *E. faecium* TJ4031 - Figure shows the restriction endonuclease pattern of the *vanC_I* genotype in the *E. faecium* TJ4031 strain after PFGE with *Sma*I was performed. The location of the *vanC_I* gene was determined using southern blot hybridisation with a *vanC_I* probe. PFGE hybridisation analysis results showed that the *vanC_I* probe hybridised



A: pulsed-field gel electrophoresis of *SmaI* digested genomic DNA from TJ4031 and TJ430 strains. The first lane is H9812 digested with *XbaI* as marker with relevant sizes indicated to the gel; B: hybridisation of southern transfer of DNA in left panel using an internal fragment of *vanC1* as a probe. The result showed that the probe hybridised to the chromosome and the size was between 78.2-104.5 Kbp.

to the chromosomal DNA band and that the size of the macrorestriction fragment was between 78.2-104.5 Kbp (Figure). However, S1-PFGE analysis results showed that a plasmid was absent in the TJ4031 isolate (data not shown). The results indicated that the *vanC₁* gene was located in the chromosome.

DISCUSSION

The *vanC* intrinsic resistance genotype is associated with several enterococcal species, including *E. gallinarum* (*vanC₁*), *E. casseliflavus* (*vanC₂*) and *E. flavescens* (*vanC₃*). These *vanC* operons are chromosome associated and testing *E. faecium* or other *Enterococcus* species for the presence of *vanC₁* is considered unnecessary because this gene is thought to be species-specific for *E. gallinarum* (Leclercq et al. 1992, Schwaiger et al. 2012). However, the *vanC₁* gene has also been detected in *E. faecalis* strains isolated from pig manure samples (Germany), sheep bulk tank milk samples (Spain) and cloacal swabs of broilers (Brazil).

In our study, expected biochemical reactions were observed in the *vanC₁* genotype-positive *E. faecium* TJ4031. *E. faecium* was also analysed using the Vitek2-

Compact system. Moreover, the *Enterococcus* and *E. gallinarum*-specific PCR results of this study are consistent with those of other bacteriological studies (Patel et al. 1998, Arias et al. 2000, Zheng et al. 2007, de Moura et al. 2013). Our study failed to detect the corresponding *vanC₁* and *vanXYc* genes in a *vanC₁* genotype-positive strain with real-time RT-PCR assays and similar results have been previously reported (Schwaiger et al. 2012, de Moura et al. 2013). This result could be attributed to a non-functional *vanC₁* gene cluster that has been transferred from a bacterial community to our strain or to a failed recombination event that inserted a non-functional gene and removed beneficial DNA (Lawrence et al. 2001, de Moura et al. 2013). In a previous study, the *vanC₁* gene was found on a plasmid (de Moura et al. 2013). However, our study showed that the *vanC₁* genotype of the *E. faecium* TJ4031 isolate contained no plasmid; this procedure was repeated in triplicate to verify our initial findings. The possible explanation is that megaplasmids, which cannot be detected with current techniques, or bacteria without plasmids may be present. Furthermore, the *vanC₁* gene was successfully hybridised to the chromosome band using southern blot, showing that this gene was located in this chromosome.

E. faecium TJ4031 may have acquired the *vanC₁* gene via a horizontal gene transfer from a natural carrier (*E. gallinarum*) or from a carrier of animal origin (*E. faecalis*) (Schwaiger et al. 2012, de Garnica et al. 2013, de Moura et al. 2013) because strains from human-adapted CCs that cause enterococcal infection may be recovered from farm and companion animals and strains from CCs commonly found among animals have also been isolated from humans. Furthermore, other studies have revealed several cases of animal-human transmission of vancomycin-resistant enterococci, resulting in frequent infections of healthy humans that closely interact with animals (Damborg et al. 2009, Freitas et al. 2009b, 2011, Willems & van Schaik 2009, Larsen et al. 2010).

This study was the first to identify the *vanC₁* gene in a vancomycin-susceptible *E. faecium* strain isolated from the blood culture of a patient in China. Our result is important because the *vanC₁* gene is often used to identify *E. gallinarum*; without this gene, species may be erroneously identified. This result also emphasises that the chromosomal location of a gene in an intrinsically resistant strain does not necessarily prevent transfer to other species, thereby contributing to species diversity. Furthermore, the *vanC₁*-type vancomycin resistance gene was encoded on the chromosome. Our data indicate that *vanC₁*-type *E. faecium* strains could be detected in humans. Even if the strain was phenotypically susceptible to vancomycin, the fact that *E. faecium* are able to naturally acquire *vanC₁* from the bacterial community is a cause for concern because the possibility that complete gene clusters and functional genes will be transferred and expressed cannot be ruled out. This study underlines that *E. faecium* are very potent resistance gene collectors and possibly donors (Schwaiger et al. 2012). We should always monitor enterococci in not only human clinical isolates, but also in the commensals from diverse habitats.

REFERENCES

- Arias CA, Courvalin P, Revnolds PE 2000. *vanC* cluster of vancomycin-resistant *Enterococcus gallinarum* BM4174. *Antimicrob Agents Chemother* 44: 1660-1666.
- Arthur M, Revnolds P, Courvalin P 1996. Glycopeptide resistance in enterococci. *Trends Microbiol* 4: 401-407.
- CLSI - Clinical and Laboratory Standards Institute 2012. *Performance standards for antimicrobial susceptibility testing*, 22nd informational supplement M100-S13, CLSI, Villanova, 188 pp.
- Courvalin P 2006. Vancomycin resistance in Gram-positive cocci. *Clin Infect Dis* 42 (Suppl. 1): S25-S34.
- Damborg P, Top J, Hendrickx AP, Dawson S, Willems RJ, Guardabassi L 2009. Dogs are a reservoir of ampicillin-resistant *Enterococcus faecium* lineages associated with human infections. *Appl Environ Microbiol* 75: 2360-2365.
- de Garnica ML, Valdezate S, Gonzalo C, Saez-Nieto JA 2013. Presence of the *vanC* gene in a vancomycin-resistant *Enterococcus faecalis* strain isolated from ewe bulk tank milk. *J Med Microbiol* 62: 494-495.
- de Moura TM, Cassenego APV, Campos FS, Ribeiro AML, Franco AC, d'Azevedo PA, Frazzon J, Frazzon APG 2013. Detection of *vanC_I* gene transcription in vancomycin-susceptible *Enterococcus faecalis*. *Mem Inst Oswaldo Cruz* 108: 453-456.
- Facklam RR, Collins MD 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. *J Clin Microbiol* 27: 731-734.
- Freitas AR, Coque TM, Novais C, Hammerum AM, Lester CH, Zervos MJ, Donabedian S, Jensen LB, Francia MV, Baquero F, Peixe L 2011. Human and swine hosts share vancomycin-resistant *Enterococcus faecium* CC17 and CC5 and *Enterococcus faecalis* CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids. *J Clin Microbiol* 49: 925-931.
- Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L 2009a. Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of *Enterococcus faecalis* from Portugal. *J Antimicrob Chemother* 63: 1104-1111.
- Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L 2009b. Dispersion of multidrug-resistant *Enterococcus faecium* isolates belonging to major clonal complexes in different Portuguese settings. *Appl Environ Microbiol* 75: 4904-4908.
- Jackson CR, Fedorka-Cray PJ, Barrett JB 2004. Use of a genus and species-specific multiplex PCR for identification of enterococci. *J Clin Microbiol* 42: 3558-3565.
- Larsen J, Schönheyder HC, Lester CH, Olsen SS, Porsbo LJ, Garcia-Migura L, Jensen LB, Bisgaard M, Hammerum AM 2010. Porcine-origin gentamicin-resistant *Enterococcus faecalis* in humans, Denmark. *Emerg Infect Dis* 16: 682-684.
- Lawrence JG, Hendrix RW, Casjens S 2001. Where are the pseudogenes in bacterial genomes? *Trends Microbiol* 9: 535-540.
- Lebreton F, Depardieu F, Bourdon N, Fines-Guyon M, Berqer P, Camiade S, Leclercq R, Courvalin P, Cattoir V 2011. D-Ala-d-Ser *vanN*-type transferable vancomycin resistance in *Enterococcus faecium*. *Antimicrob Agents Chemother* 55: 4606-4612.
- Leclercq R, Derlot E, Duval J, Courvalin P 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. *N Engl J Med* 319: 157-161.
- Leclercq R, Dutka-Malen S, Duval J, Courvalin P 1992. Vancomycin resistance gene *vanC* is specific to *Enterococcus gallinarum*. *Antimicrob Agents Chemother* 36: 2005-2008.
- Nomura T, Tanimoto K, Shibayama K, Arakawa Y, Fujimoto S, Ike Y, Tomita H 2012. Identification of *vanN*-type vancomycin resistance in an *Enterococcus faecium* isolate from chicken meat in Japan. *Antimicrob Agents Chemother* 56: 6389-6392.
- Patel R, Piper KE, Rouse MS, Steckelberg JM, Uhl JR, Kohner P, Hopkins MK, Cockerill FR 3rd, Kline BC 1998. Determination of 16S rRNA sequences of enterococci and application to species identification of non-motile *Enterococcus gallinarum* isolates. *J Clin Microbiol* 36: 3399-3407.
- Poyart C, Quesnes G, Trieu-Cuot P 2000. Sequencing the gene encoding manganese-dependent superoxide dismutase for rapid species identification of enterococci. *J Clin Microbiol* 38: 415-418.
- Ramotar K, Woods W, Larocque L, Toye B 2000. Comparison of phenotypic methods to identify enterococci intrinsically resistant to vancomycin (*vanC* VRE). *Diagn Microbiol Infect Dis* 36: 119-124.
- Schwaiger K, Bauer J, Hörmansdorfer S, Mölle G, Preikschat P, Kämpf P, Bauer-Unkauf I, Bischoff M, Hölzel C 2012. Presence of the resistance genes *vanC_I* and *pbp5* in phenotypically vancomycin and ampicillin susceptible *Enterococcus faecalis*. *Microb Drug Resist* 18: 434-439.
- Uttley AH, Collins CH, Naidoo J, George RC 1988. Vancomycin-resistant enterococci. *Lancet* 1: 57-58.
- Willems RJ, van Schaik W 2009. Transition of *Enterococcus faecium* from commensal organism to nosocomial pathogen. *Future Microbiol* 4: 1125-1135.
- Zheng B, Tomita H, Xiao YH, Wang S, Li Y, Ike Y 2007. Molecular characterization of vancomycin-resistant *Enterococcus faecium* isolates from mainland China. *J Clin Microbiol* 45: 2813-2818.