Polyclonal emergence of *vanA* vancomycin-resistant *Enterococcus faecium* in Australia

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Objectives: To investigate the genetic context associated with the emergence of *vanA* VRE in Australia.

Methods: The whole genomes of 18 randomly selected *vanA*-positive *Enterococcus faecium*patient isolates, collected between 2011 and 2013 from hospitals in four Australian capitals, were sequenced and analysed.

Results: *In silico* typing and transposon/plasmid assembly revealed that the sequenced isolates represented (in most cases) different hospital-adapted STs and were associated with a variety of different Tn*1546* variants and plasmid backbone structures.

Conclusions: The recent emergence of *vanA* VRE in Australia was polyclonal and not associated with the dissemination of a single 'dominant' ST or *vanA*-encoding plasmid. Interestingly, the factors contributing to this epidemiological change are not known and future studies may need to consider investigation of potential community sources.

Introduction

Enterococcus faecium became prominent as a nosocomial pathogen in the 1990s with the emergence of hospital-adapted clones (a polyclonal subpopulation grouped together and previously designated as clonal complex 17) associated with antimicrobial resistance.[1]Although first identified in the USA, these *E. faecium* clones quickly spread around the world, resulting in the widespread use of vancomycin as first-line therapy for the treatment of hospital-acquired enterococcal infections. Not surprisingly, VRE emerged shortly thereafter in North America and Europe. Since then VRE have been detected worldwide, including Australia.

The epidemiology of vancomycin resistance differs between regions, with the use of antibiotics in agriculture (such as avoparcin in Europe) thought to be a contributing factor. Irrespective of these differences, vancomycin resistance is acquired (in most cases) through the acquisition of either the *vanA* or the *vanB* operon, carried by the transposons Tn*1546* and Tn*1549* (and structural variants

thereof), respectively. While the spread of these transposons amongst enterococcal populations is largely facilitated by MDR plasmids, integration (of the transposon) into the chromosome can occur, particularly in the context of *vanB*; however, this is not required for operon stability or function.

Although the first VRE reported in Australia was a *vanA*-positive *E. faecium* in 1995, vancomycin resistance in enterococci has largely been mediated by a chromosomally encoded *vanB* determinant,[2,3] which was associated with ~36.5% of all *E. faecium* and 98% of all VRE bacteraemia isolates in 2011.[4] Unexpectedly, in 2012 *vanA* was reported in 32% of vancomycin-resistant *E. faecium* isolated (predominantly from rectal surveillance specimens) in Western Australia.[5] The proportion of vancomycin-resistant *E. faecium* bacteraemia isolates in Australia harbouring *vanA* genes increased from <1% in 2011[4] to 9.5% in 2014.[6] Some Australian centres have even witnessed a dramatic shift, with *vanA* replacing *vanB* VRE since 2012.[7] In order to investigate the genetic context associated with this phenomenon, a random selection of *vanA*-positive *E. faecium* isolates from hospitals in four Australian capital cities were collected and subjected to WGS. All isolates were obtained from patients between 2011 and 2013 and corresponded with the 'emergence' of *vanA* within each institution.

Methods

DNA manipulations

DNA was extracted from each isolate using the ISOLATE Genomic DNA Kit (Bioline, London, UK). DNA fragments were PCR-amplified using BioTaq (Bioline) and capillary sequencing was performed by Macrogen Inc. (Seoul, Korea).

Isolates and vanA screening

Eighteen *vanA E. faecium* isolates collected between 2011 and 2013 were obtained from hospitals in Brisbane ($n = 5$), Melbourne ($n = 5$), Perth ($n = 5$) and Sydney ($n = 3$) and confirmed as *vanA* positive by conventional PCR using primers *vanA*-F (5-atgaatagaataaaaggcaatactg-3) and *vanA*-R (5-

tcacccctaacgctaatac-3). All isolates demonstrated similar phenotypes with resistance to ampicillin, vancomycin and teicoplanin (MICs >8, 2 and 4 mg/L, respectively).

To examine the genomic context of *vanA*-positive isolates relative to *vanB*, the dataset was supplemented by a single isolate from each city (collected during the same time period). During this period three STs (ST203, ST341 and ST796) accounted for 90% of all *vanB*-positive isolates.[8,9] Of these, only a single ST (ST203) was disseminated across Australia, accounting for 54% of all *vanB* VRE.

WGS analysis

All isolates underwent WGS following library preparation using either the Ion Plus Fragment Library kit (Life Technologies, Carlsbad, USA) or the NEBNext Fast DNA Fragmenon & Library Prep Set (New England Biolabs, Ipswich, USA). Sequencing was performed on the Ion Torrent PGM (Life Technologies) according to the manufacturer's instructions. *De novo* assemblies were constructed using SPAdes software v3.5,[10] with *in silico* MLST performed against the generated contigs. For isolates where no match was obtained, for one or more alleles, a mapped approach was taken using the closed genome of the *E. faecium* ST203 isolate AUS0085 (GenBank accession number CP006620). Identified ST groups were assigned to clonal complex 17 using eBURST 3 (efaecium.mlst.net), which defines groupings if ≥5/7 allele loci are shared between isolates. A maximum-likelihood phylogenetic tree was generated from the core single-nucleotide variants using RAxML v8.2.8[11] with tree visualization and manipulation performed within Figtree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

Transposon and plasmid assembly

Transposon assembly for all isolates was accomplished via mapping reads to the Tn*1546*structure of the *E. faecium* isolate BM4147 (GenBank accession number M97297), in conjunction with manual curation using CLC Genomics Workbench v5.5 (CLCbio, Aarhus, Denmark). Based on BLASTn matches of *de novo* assembled contigs, a similar approach was taken for the assembly and closure of pJEG40 and pJEG43, using the previously characterized plasmids pS177 (GenBank accession number

NC_014959) and pRE25 (GenBank accession number NC_008445), respectively. Gaps in plasmid read mappings were closed (and assemblages confirmed) via PCR amplification and capillary sequencing. *In silico* plasmid replicon typing for all isolates was performed using BLASTn and the Gram-positive database available via the PlasmidFinder 1.3 website (https://cge.cbs.dtu.dk/services/PlasmidFinder/).

The complete sequences of pJEG40 and pJEG43 have been deposited in GenBank and assigned accession numbers KX810025 and KX810026, respectively.

Results and discussion

Eighteen isolates from four capital cities representing nine different hospitals were sequenced. These isolates represented both clinical [bloodstream and urine infection ($n = 5$)] and screening ($n = 13$) isolates. *De novo* assembly resulted in an average of 215 contigs with a mean *N*50 of >30 kb per isolate. From the 43 300 SNPs detected relative to the reference (Aus0085), 39 167 mutations were present in all isolates, leaving 4133 to construct the phylogeny. *In silico* MLST identified 12 different STs, with multiple STs observed in each capital city except in the case of Sydney, which only had ST80 isolates. Identical MLST isolates (ST80, ST203 and ST18) differed on a core genome level confirming non-identical isolates between Australian cities and institutions (Figure 1). Overall, 90% of isolates belonged to CC17, suggesting *vanA* has largely emerged from within the hospital-adapted clones (data not shown). Furthermore, the data suggest that *vanA* acquisition has occurred within the circulating clones, with all *vanB* ST203 isolates sharing the same ancestry.

Additionally, analysis of WGS reads revealed that nine Tn*1546* structural variants were associated with the sequenced isolates (Figure 2a). These variants differed by the type and position of IS elements located within the transposon and in most cases the transposase genes *orf1-orf2* were partially or completely deleted, preventing independent transposition (i.e. observed Tn*1546* variants were likely anchored to their host plasmid backbone). The plasmid backbone structures associated with the most common Tn*1546* variants, ii and iv, were completely assembled for two isolates and

designated pJEG040 (Efm008) and pJEG043 (Efm0038), respectively (Figure 2b). Subsequent read mapping using these plasmids as a reference revealed identical transposon variants were mostly associated with the same plasmid backbone (Figure 2a). Note that, with the exception of Tn*1546* variant vi, which is also associated with a pJEG40-like backbone structure, sequence reads from the other isolates did not map to (or support assembly of Tn*1546* variants with) pJEG040- or pJEG043-like plasmids. In this respect, *in silico* plasmid replicon typing indicated the presence of multiple plasmid backbone structures (in these isolates) that could contain the Tn*1546*variant (Figure 2a).

It is also interesting to note that while pJEG040 and pJEG043 lack obvious conjugation/mobilization genes (Figure 2b), they were detected in multiple STs from across Australia. This indicates these plasmids are able to spread between strains, most likely with the help of co-resident transmissible plasmids; analysis of unassembled sequence contigs revealed the presence of conjugation-related genes. In this regard, pJEG40 is very similar to the pRUM-like plasmid pS177 (data not shown), which has mediated transfer of high-level vancomycin resistance to MRSA clinical isolates, most likely via co-integration with a conjugative plasmid present in the VRE donor.[12]

The presence of multiple Tn*1546* variants in association with different STs and plasmid backbone structures suggests the emergence of *vanA* VRE in Australia during the 2011–13 period was not driven by the dissemination of a single 'dominant' ST or *vanA*-encoding plasmid. Furthermore, based on the sequences of pJEG040 and pJEG043, there are no obvious co-selectable plasmid-encoded phenotypes that might confer a selective advantage.

In context of the hospital setting, it seems unlikely that *vanA* VRE could have emerged in multiple different clones across Australia simultaneously, especially as the human gut does not have a *vanA* reservoir; contrarily, studies have shown that the *vanB* determinant is commonly carried by anaerobic gut commensals.[13,14] Additionally, given the diversity within Australian hospital practice regarding *vanB* VRE treatment (e.g. use of teicoplanin), it is also unlikely that hospital antimicrobial VRE therapy is responsible for this genotypic shift. Therefore, possible explanations for the Australia-wide polyclonal emergence of *vanA* VRE include multiple previous introductions (of *vanA* VRE)

coupled with an unknown selection pressure. Alternatively, the *vanA* operon has been recently introduced via a community source (e.g. the food chain) and acquired by already circulating *E. faecium* clones, which are then enriched for via antimicrobial use in hospitals.

Ultimately, the factors contributing to this epidemiological change are not known and future studies may need to consider investigation of potential community sources. Regardless, despite the initial diversity observed, it is predicted (based on our *vanB* experience) that certain clones are likely to become dominant over time.

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Transparency declarations

None to declare.

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Figure 1. Unrooted maximum-likelihood phylogenetic tree. Branch tips indicate isolates' state of origin (New South Wales, orange; Queensland, green; Victoria, turquoise; Western Australia, red). The smallest number of single nucleotide variants (SNVs) occurs between Efm0038 and Efm0064 (18 SNVs). Grey circles indicate ST groupings. Asterisks denote ST203 *vanB* VRE (single isolate included from each city; collected during the same time period). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

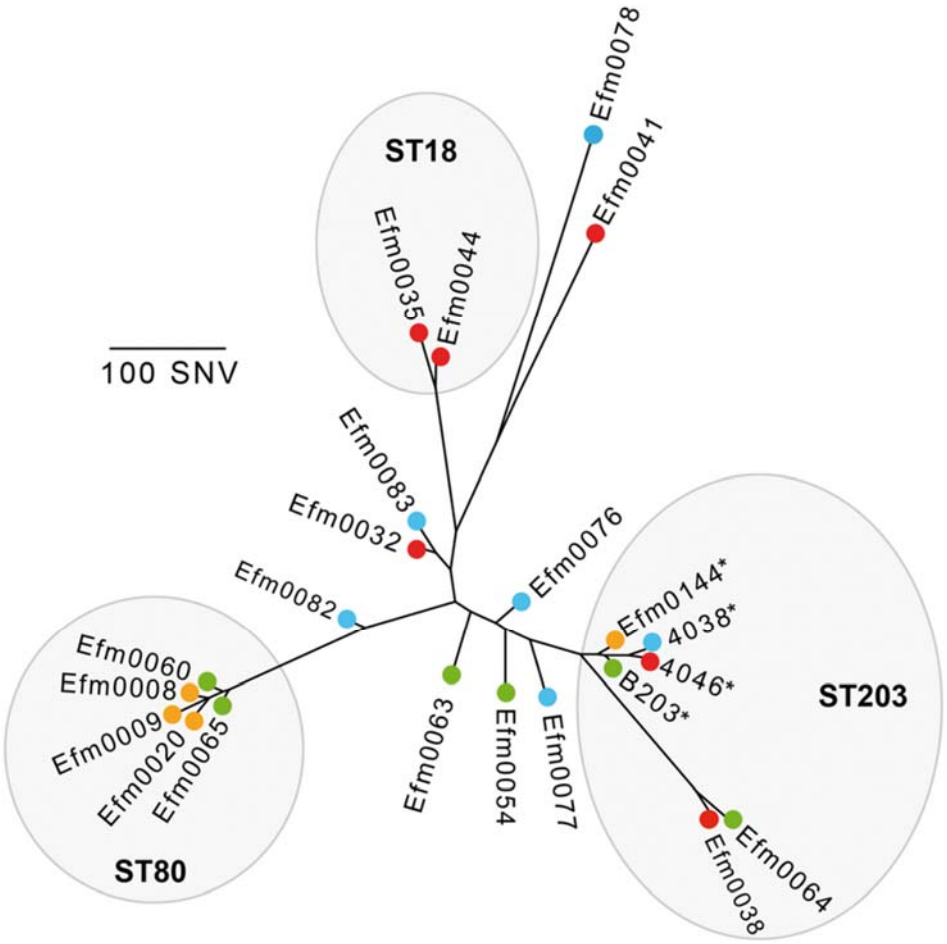
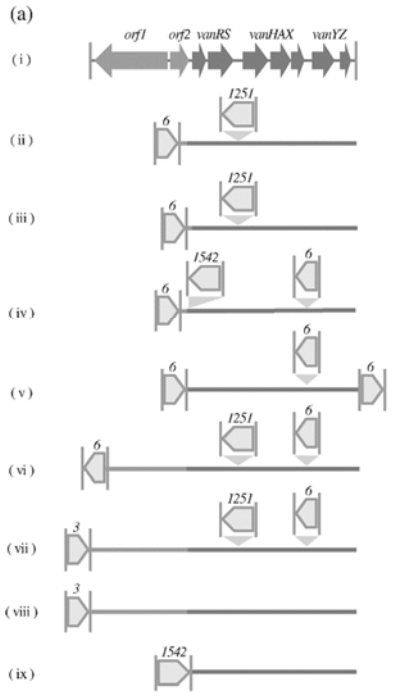


Figure 2. Structural features of Tn*1546* variants (a) and plasmids pJEG040 and pJEG043 (b).
The *orf1-orf2* and *van* operon regions of the Tn*1546* variants are represented by orange and blue lines, respectively (i–ix). Selected genes (arrows) of the plasmid maps are annotated: plasmid replication, red; transposon-related, orange; non-glycopeptide resistance, green; glycopeptide resistance, blue; all others [including antitoxin/toxin (AT/T) systems], white. Other features represented are: plasmid backbones, grey lines; ISs, orange pentagons (IS number indicated below or above; direction is with respect to the transposase gene); inverted repeats, vertical orange lines. This figure appears in colour in the online version of *JAC*and in black and white in the print version of *JAC*.



Strain	State[a]	ST	Year	Tn*1546* Structure	Plasmid Backbone[b]	Replicon Types[c]
Efm0041	WA	450	2012	i	ND	22,US1,US12,US15
Efm0060	QLD	80	2013	ii	pJEG040*	2,11,17,18,US15
Efm0065	QLD	80	2013	ii	pJEG040	2,11,17,US15
Efm0008	NSW	80	2011	ii	pJEG040	1,2,17,US15
Efm0009	NSW	80	2012	ii	pJEG040*	2,17,US7
Efm0020	NSW	80	2013	ii	pJEG040	1,2,11,17,US15
Efm0083	VIC	796	2012	ii	pJEG040	2,11,17,US15
Efm0044	WA	18	2012	iii	ND	17,US12,US15
Efm0035	WA	18	2012	iv	ND	US15
Efm0038	WA	203	2012	iv	pJEG043	1,2,7,11,14,US15
Efm0064	QLD	203	2013	iv	pJEG043	1,2,7,11,US15
Efm0063	QLD	721	2013	iv	pJEG043	1,2,7,11,US15
Efm0032	WA	555	2012	v	ND	2,11,18,US15
Efm0054	QLD	772	2012	vi	pJEG040*	17,US15
Efm0077	VIC	896	2013	vi	ND	US15
Efm0076	VIC	78	2012	vii	ND	2,11,17,US15
Efm0078	VIC	137	2013	viii	ND	2,US15
Efm0082	VIC	192	2013	ix	ND	17,US15

[a]NSW, New South Wales; QLD, Queensland; VIC, Victoria; WA, Western Australia. [b]ND, not determined. *contains an insertion or deletion within the pJEG40 backbone structure. [c]Plasmid replicon types present in each isolate; pJEG40 and pJEG43 types are coloured red.