

Enterococcus faecium harbouring *vanA*, *optrA* and *cfr(D)* on a linear plasmid in Canada: a CANWARD surveillance case

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Objectives: VRE resistant to potential therapeutic agents such as linezolid are an increasing concern in Canada and worldwide. Infections produced by these isolates have limited treatment options and are associated with poor clinical outcomes. We report an isolate of vancomycin-resistant *Enterococcus faecium* (VREfm); identified through the Canadian surveillance program, CANWARD, which exhibited linezolid resistance (LVREfm); and carried the *optrA* and *cfr(D)* genes on a linear plasmid.

Methods: Antimicrobial susceptibility testing was performed by broth microdilution and MICs were interpreted by 2025 CLSI breakpoints. WGS was performed to characterize resistance determinants and the associated plasmid.

Results: In 2021, a single LVREfm isolate was recovered from a blood culture of a 67-year-old male patient admitted to an ICU. The isolate was XDR but remained susceptible dose dependent to daptomycin (MIC = 2 mg/L). MLST identified the isolate as ST817, a sequence type not commonly found in our national collection. Vancomycin resistance was mediated by the *vanA* gene cluster, while linezolid resistance determinants included *optrA*, *cfr(D)* and the 23S rRNA gene mutation, G2576T. Vancomycin and linezolid resistance genes were co-located on a linear plasmid that exhibited over 94% sequence identity to previously described linear plasmids from India and the USA. Notably, the plasmid also encoded four toxin-antitoxin systems, potentially contributing to its stability and persistence.

Conclusions: This case highlights the appearance of linezolid resistance in VRE mediated by linear plasmids in Canada and underscores the importance of ongoing genomic surveillance to track the dissemination of MDR determinants in clinical settings.

Introduction

Enterococcus faecium is an ESKAPE pathogen associated with hospital-acquired infections and an XDR phenotype.¹ In Canada, vancomycin-resistant *E. faecium* (VREfm) predominantly carry the *vanA* gene.² Resistance to last-line antibiotics like linezolid further complicates treatment options and is a growing concern among VREfm. There are multiple mechanisms by which VREfm can become resistant to linezolid. Resistance may arise from G2576T or G2505A mutations in the 23S rRNA gene (*Escherichia coli* numbering) or from the presence of resistance genes *optrA*, *poxA* and *cfr* variants which are often located on

mobile genetic elements.^{3,4} The *optrA* and *poxA* genes encode ABC-F family proteins that confer resistance to phenicols and oxazolidinones, by protecting the bacterial ribosome from antibiotic binding.⁴ In contrast, *cfr(D)* encodes a 23S RNA methylase that provides resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A.⁴ Although reports of linezolid-resistant VREfm (LVREfm) remain scarce in Canada, with an average resistance rate 1.6% from 2018 to 2022,² cases have been documented internationally. Notably, *optrA*- and *vanA*-positive VREfm have been identified in multiple countries including Ireland (2020), India (2022) and the USA (2024).^{5–7} These genes can be carried on plasmids, and their ability to

move between bacteria raises concerns about horizontal gene transfer and widespread global distribution.

Until recently, most plasmids reported from VREfm were circular in structure, including Inc18-like, pRUM-like, pMG1-like and pHT-like plasmids.⁸ However, recent studies have reported the presence of linear plasmids carrying critical resistance genes such as *vanA* and *optrA*, suggesting a novel vector for resistance spread.^{6,7,9,10}

We report the first isolate of LVREfm harbouring a linear plasmid encoding *vanA*, *optrA* and *cfr(D)* identified in Canada and compare it with previously described linear plasmids harbouring the same resistance genes.

Materials and methods

Bacterial isolates

CANWARD is a Canadian antimicrobial resistance surveillance programme made up of tertiary-care medical centre sites in eight of the 10 Canadian provinces.¹¹ In CANWARD, medical centres submit bacterial pathogens from patients attending hospital clinical, emergency rooms, medical and surgical wards and ICUs to a central clinical microbiology laboratory (Health Science Centre, Winnipeg, Canada). In 2021, each site submitted consecutive bacterial isolates (one per patient, per infection site) from in-patients and outpatients; the submitted isolates consisted of 100 from respiratory infections, 25 from urinary tract infections, 25 from wound infections and 10 isolates per month from bloodstream infections.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by broth microdilution using both a custom-designed 96-well panel prepared in house and the GPALL1F Gram-positive Sensititre panels (Thermo Fisher Scientific, Waltham, MA, USA). Quality control strains ATCC 29213 and ATCC 29212 were tested concurrently in accordance with CLSI guidelines.¹² Antimicrobials agents tested included CLSI Tier 1 agents, ampicillin, penicillin and nitrofurantoin; Tier 2 agents, vancomycin, linezolid, daptomycin, high-level gentamicin, ciprofloxacin and levofloxacin; and Tier 3 agents, high-level streptomycin and tetracycline. Other antimicrobials tested included chloramphenicol, erythromycin, quinupristin/dalfopristin and rifampin. MICs were interpreted according to CLSI breakpoints for all agents tested.¹² Antimicrobial susceptibility testing of CANWARD isolates in 2021 identified a single isolate of LVREfm, N21-03014.

WGS and assembly

The LVREfm isolate N21-03014 was sequenced with Illumina MiSeq platforms and Oxford Nanopore Technologies (ONT). Genomic DNA was extracted using the Mag-Bind® Universal Pathogen DNA 96 kit (Omega Bio-Tek, Norcross, Georgia, USA). Short-read libraries were created with the NexteraXT library preparation kits (Illumina). Paired-end, 151 bp indexed reads were generated using the Illumina NextSeq™ platform. Long-read sequences were generated using the Rapid Barcoding Kit 96 (SQK-RBK110.96) on R9.4.1 flow cells with the MinION Mk1B (ONT, Oxford, Oxfordshire, UK). Read data were base called and demultiplexed with Guppy v6.5.7 using the Super High Accuracy model (ONT). ONT and Illumina reads were assembled with Flye v2.9.2 (<https://github.com/mikolmogorov/Flye>), hybrid Unicycler v0.5.0 (<https://github.com/rwwick/Unicycler>) and Hybracter v0.7.1 (<https://github.com/gbouras13/hybracter>). Assemblies were compared with ensure similar results were obtained from each assembler.

Bioinformatic analysis

Staramr v0.11.0 (<https://github.com/phac-nml/staramr>) was used to detect antimicrobial resistance genes using the ResFinder database

Table 1. Molecular and phenotypic traits of LVREfm isolate, N21-03014

Genotype		MIC (mg/L)																			Molecular typing	
Isolate no.	vanA	optrA	cfr(D)	23S rRNA position	257/6 T%	AMP	CHL	CIP	DAP	ERY	HL-GEN	HL-STR	LVS	LZD	NIT	PEN	SYN	RIF	TET	VAN	ST	CT
N21-03014	+	+	+	49.8		>8	>16	>2	2	>4	>500	>1000	>4	>8	>64	>8	>4	>4	>16	>32	817	8035

AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; DAP, daptomycin; ERY, erythromycin; HL-GEN, high-level gentamicin; HL-STR, high-level streptomycin; LVS, levofloxacin; NIT, nitrofurantoin; PEN, penicillin; SYN, quinupristin-dalfopristin; RIF, rifampin; TET, tetracycline; VAN, vancomycin. ST, sequence type; CT, complex type.

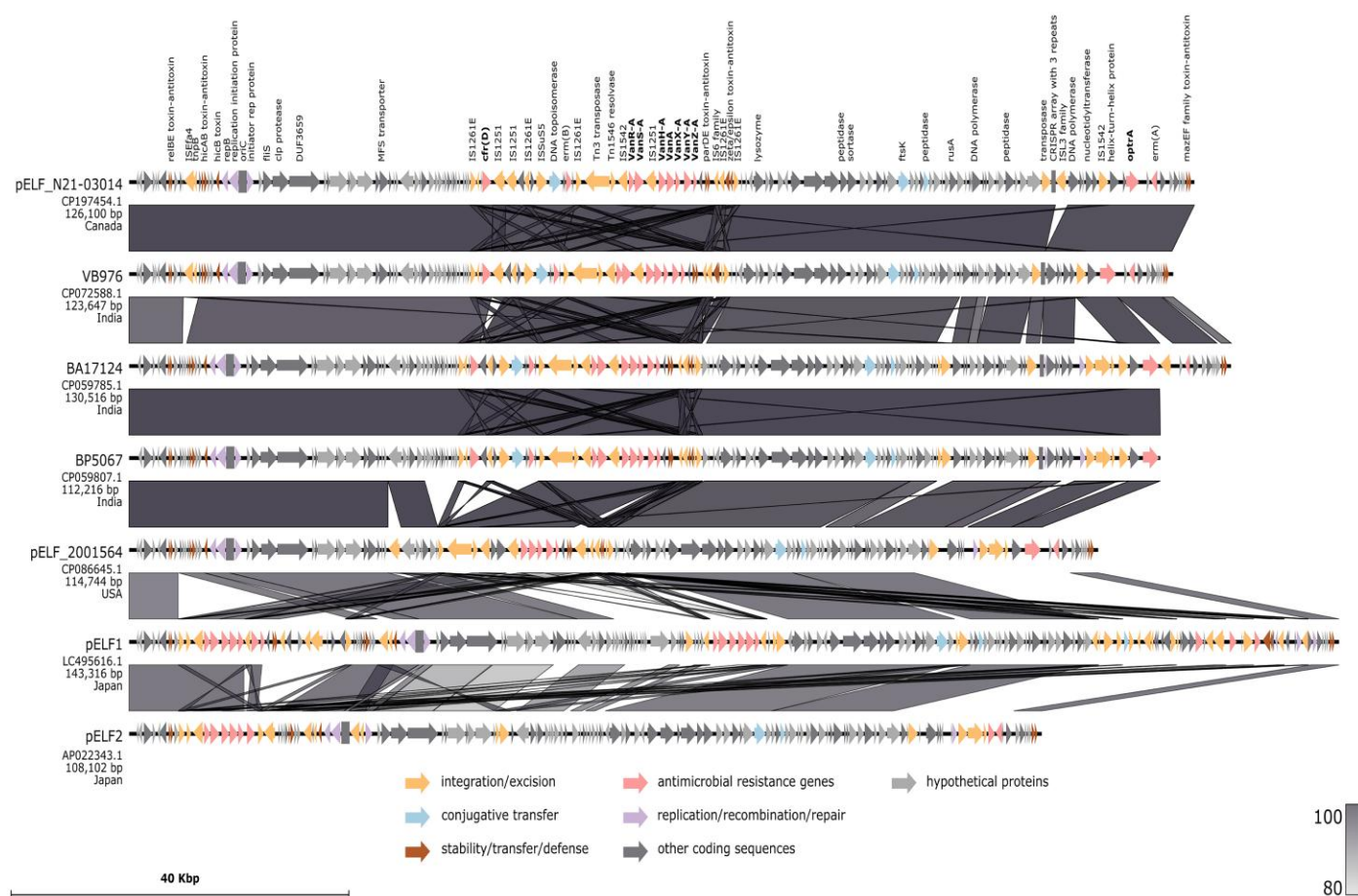


Figure 1. Comparison of this study's *vanA*- and *optrA*-positive linear plasmid, pELF_N21-03014 (CP197454.1), with six other highly similar *vanA*-/*optrA*-positive linear plasmids VB976 (CP072588.1), BA17124 (CP059785.1) and BP5067 (CP059807.1) from India and pELF_2001564 (CP086645.1) from the USA and pELF1 (LC495616.1) and pELF2 (AP022343.1) from Japan. Regions of similarity are connected by vertical blocks when there is a minimum of 500 bp and >80% BLASTN identity, and darker blocks indicate higher BLASTN identity. Coding sequences are coloured by function, and not all labels are displayed. Relevant antimicrobial resistance gene labels [*vanA*, *optrA* and *cfr(D)*] are bolded.

v2022-05-24¹³ and sequence type using the MLST database v2.23.0.¹⁴ LRE-Finder v1.0 (<https://cge.food.dtu.dk/services/LRE-finder/>) was used to detect 23S rRNA gene mutations at positions G2576T and G2505A.³ Antimicrobial resistance genes with 100% coverage and >90% identity were considered for analysis. SeqSphere+ software (Ridom, Muenster v9.0.10) was used to assess genetic relatedness using the *E. faecium* cgMLST scheme comprised of 1423 alleles.¹⁵

Plasmid analysis

Each assembler produced a putative linear plasmid contig with minor differences in size. The linear plasmid sequence (pELF_N21-03014) was determined by aligning the contigs to each other and to the pELF1 hairpin sequence (NZ_LC495616.1 coordinates 0-799) and pELF1 inverton sequence (NZ_LC495616.1 coordinates 142792-143316) via Web BLAST.¹⁶ Plasmid cluster was assigned via the default database and default Mash distances in MOB-suite v3.1.4 (<https://github.com/phac-nml/mob-suite>). The plasmid was annotated with Bakta v1.11.0 (<https://github.com/oschwengers/bakta>) using the full database v6.0. Plasmids were visualized and compared using Genofig v1.1.1 (<https://forge.inrae.fr/public-pgba/genofig/-/tree/main>). Toxin-antitoxin systems were screened using TADB v3.0 (<https://bioinfo-mml.sjtu.edu.cn/TADB3/>).

Nucleotide sequence accession numbers

Sequence read and genome assembly data was deposited in GenBank under BioProject PRJNA1279082.

Results

The CANWARD study collected a total of 2331 isolates in 2021; 13 (0.6%) of which were VREfm. From this subset, MIC data identified a single LVREfm (N21-03014) from a 67-year-old male in the ICU with a blood stream infection. Antimicrobial susceptibility testing identified the LVREfm isolate as XDR, showing resistance to 14 of the 15 antimicrobial agents tested and susceptible dose dependent to daptomycin (MIC = 2 mg/L) (Table 1). Molecular typing using MLST and cgMLST determined the isolate to be ST817 and CT8035, respectively.

Vancomycin resistance in the isolate was associated with the presence of the *vanA* gene, consistent with an MIC of >32 mg/L. The isolate was found to contain chromosomal mutations in the 23S rRNA gene, specifically at the G2576T position. The fraction of Ts at position 2576 was 49.8%, corresponding to three mutated

23S rRNA alleles, which is predictive of a linezolid-resistant phenotype. In addition, the isolate carried two linezolid-resistant genes, *optrA* and *cfr(D)*, which were co-located with *vanA* on a 126.1 kb linear plasmid (pELF_N21-03014, accession CP197454.1). Nine additional plasmids (1976–242 721 bp) were detected (BioProject PRJNA1279082). The linear topology of pELF_N21-03014 was inferred by identifying the hairpin and invertron ends that are characteristic of pELF-like plasmids.¹⁶ These features are present at opposing ends of the linear plasmid and were confirmed by their high sequence homology with 88% identity to the hairpin end and 98% identity to the invertron end. Comparative analysis of the complete linear plasmid demonstrated over 94% sequence identity to previously published linear plasmids also harbouring both *vanA* and *optrA* (Figure 1). WGS identified various genes related to transfer, integration/excision and replication (Figure 1). Similar to plasmids CP072588.1, CP059785.1, CP059807.1, and CP086645.1, MOB typing revealed pELF_N21-03014 had the same primary (AB369) and secondary (AK115) plasmid clusters, whereas replicon or relaxase types were not identified. Notably, this plasmid encoded at least four toxin–antitoxin systems, RelB/RelE, HicA/HicB, ParD/ParE and zeta/epsilon.^{17,18}

Discussion

The detection of a LVREfm isolate from a bloodstream infection in a Canadian patient represents an important finding in the ongoing battle against MDR pathogens. The XDR profile of LVREfm N21-03014 severely restricts treatment options for the patient, while the presence of vancomycin (*vanA*) and linezolid [*optrA* and *cfr(D)*] resistance genes on the same linear plasmid highlights the potential for horizontal gene transfer and raises concerns about its dissemination across enterococcal and other bacterial populations.

The plasmid from N21-03014 (pELF_N21-03014) was evaluated alongside four internationally sourced plasmids that also harboured the same vancomycin and linezolid resistance genes. The four linear plasmids compared were from India (accession no. CP072588.1, CP059785.1, CP059807.1)⁶ and the USA (accession no. CP086645.1).⁷ The high degree of sequence similarity of pELF_N21-03014 to previously published linear plasmids suggests that these plasmids have a conserved plasmid backbone and may act as efficient vectors for the dissemination of combined vancomycin and linezolid resistance among *Enterococcus* strains, indicating a potential international origin or transmission route.^{10,16} However, in the absence of conjugation experiments and detailed epidemiological data on the patient, definitive conclusions regarding transmission dynamics cannot be drawn.

From a clonality perspective, N21-03014 (ST817) is distinct from other *vanA*- and *optrA*-positive strains—such as ST1643, which predominates in India, and ST80, which is common in Italy, Ireland and the USA.^{6,7,9,19} Although ST817 is a single-locus variant of ST80 and a double-locus variant of ST1643, it represents a separate clonal lineage, indicating that dissemination of resistance genes is likely driven more by horizontal transfer of plasmids than clonal expansion of specific strains.²⁰ This highlights the complexity of resistance transmission pathways and the challenges it poses for epidemiological tracking and infection control.

Lastly, pELF_N21-03014 was found to have multiple toxin–antitoxin systems that appear to be conserved across similar plasmids and likely contribute to plasmid stability and maintenance within the host.^{17,18} Enhanced plasmid stability could facilitate long-term persistence in clinical settings. These characteristics raise concern about the potential for these plasmids to become endemic in hospital-associated *Enterococcus* populations, facilitating widespread resistance and dissemination.¹⁷

Conclusions

Our report marks the first description of a LVREfm harbouring *vanA*, *optrA* and *cfr(D)* on a linear plasmid in Canada. These findings emphasize the need for continuous genomic surveillance and incorporation of plasmid-focused analyses in AMR surveillance programs.

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Transparency declarations

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Melissa McCracken (Conceptualization, Writing—original draft), Nicole Lermينياux (Writing—review & editing), Heather Adam (Writing—review & editing), Melanie Baxter (Writing—review & editing), James Karlowsky (Writing—review & editing), George Golding (Writing—review & editing), and George G. Zhan (Writing—review & editing)

Disclaimer

The opinions expressed in this paper are those of the authors and do not necessarily represent those of Avir Pharma Inc., Iterum Therapeutics, Merck Canada Inc., Paladin Labs, Pfizer, Sandoz Canada, Shionogi Inc., Sunovion Pharmaceuticals Canada Inc., Venatorx Pharmaceuticals Inc., Verity Pharmaceuticals and Xediton Pharmaceuticals.

References

- 1 Rice LB. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J Infect Dis* 2008; **197**: 1079–81. <https://doi.org/10.1086/533452>
- 2 Canadian Nosocomial Infection Surveillance Program. Healthcare-associated infections and antimicrobial resistance in Canadian acute care hospitals, 2018–2022. *Can Commun Dis Rep* 2024; **50**: 179–96. <https://doi.org/10.14745/ccdr.v50i06a02>
- 3 Hasman H, Clausen PTL, Kaya H et al. LRE-Finder, a web tool for detection of the 23S rRNA mutations and the *optrA*, *cfr*, *cfr(B)* and *poxA* genes encoding linezolid resistance in enterococci from whole-genome sequences. *J Antimicrob Chemother* 2019; **74**: 1473–6. <https://doi.org/10.1093/jac/dkz092>
- 4 Schwarz S, Zhang W, Du XD et al. Mobile oxazolidinone resistance genes in Gram-positive and Gram-negative bacteria. *Clin Microbiol Rev* 2021; **34**: e0018820. <https://doi.org/10.1128/CMR.00188-20>
- 5 Egan SA, Corcoran S, McDermott H et al. Hospital outbreak of linezolid-resistant and vancomycin-resistant ST80 *Enterococcus faecium* harbouring an *optrA*-encoding conjugative plasmid investigated by whole-genome sequencing. *J Hosp Infect* 2020; **105**: 726–35. <https://doi.org/10.1016/j.jhin.2020.05.013>
- 6 Bakthavatchalam YD, Puraswani M, Livingston A et al. Novel linear plasmids carrying *vanA* cluster drives the spread of vancomycin resistance in *Enterococcus faecium* in India. *J Glob Antimicrob Resist* 2022; **29**: 168–72. <https://doi.org/10.1016/j.jgar.2022.03.013>
- 7 Kent AG, Spicer LM, Campbell D et al. Sentinel surveillance reveals phylogenetic diversity and detection of linear plasmids harboring *vanA* and *optrA* among enterococci collected in the United States. *Antimicrob Agents Chemothe* 2024; **68**: e0059124. <https://doi.org/10.1128/aac.00591-24>
- 8 Islam M, Sharon B, Abaragu A et al. Vancomycin resistance in *Enterococcus faecium* from the Dallas, Texas, area is conferred predominantly on pRUM-like plasmids. *mSphere* 2023; **8**: e0002423. <https://doi.org/10.1128/msphere.00024-23>
- 9 Cinthi M, Coccitto SN, Simoni S et al. The *optrA*, *cfr(D)* and *vanA* genes are co-located on linear plasmids in linezolid- and vancomycin-resistant enterococcal clinical isolates in Italy. *J Antimicrob Chemother* 2025; **80**: 1362–70. <https://doi.org/10.1093/jac/dkaf082>
- 10 Hashimoto Y, Suzuki M, Kobayashi S et al. Enterococcal linear plasmids adapt to *Enterococcus faecium* and spread within multidrug-resistant clades. *Antimicrob Agents Chemother* 2023; **67**: e01619-22. <https://doi.org/10.1128/aac.01619-22>
- 11 Zhanel GG, Adam HJ. Seventeen years of the CANWARD study (2007–2023). *J Antimicrob Chemother* 2025; **80**: ii2–4. <https://doi.org/10.1093/jac/dkaf216>
- 12 CLSI. *Performance Standards for Antimicrobial Susceptibility Testing—Thirty-Fifth Edition: M100*. 2025.
- 13 Bortolaia V, Kaas RS, Ruppe E et al. ResFinder 4.0 for predictions of phenotypes from genotypes. *J Antimicrob Chemother* 2020; **75**: 3491–500. <https://doi.org/10.1093/jac/dkaa345>
- 14 Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Res* 2018; **3**: 124. <https://doi.org/10.12688/wellcomeopenres.14826.1>
- 15 de Been M, Pinholt M, Top J et al. Core genome multilocus sequence typing scheme for high-resolution typing of *Enterococcus faecium*. *J Clin Microbiol* 2015; **53**: 3788–97. <https://doi.org/10.1128/JCM.01946-15>
- 16 Hashimoto Y, Taniguchi M, Uesaka K et al. Novel multidrug-resistant enterococcal mobile linear plasmid pELF1 encoding *vanA* and *vanM* gene clusters from a Japanese vancomycin-resistant enterococci isolate. *Front Microbiol* 2019; **10**: 2568. <https://doi.org/10.3389/fmicb.2019.02568>
- 17 Yang QE, Walsh TR. Toxin-antitoxin systems and their role in disseminating and maintaining antimicrobial resistance. *FEMS Microbiol Rev* 2017; **41**: 343–53. <https://doi.org/10.1093/femsre/fux006>
- 18 Hayes F. Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. *Science* 2003; **301**: 1496–9. <https://doi.org/10.1126/science.1088157>
- 19 Kavanagh NL, Kinnevey PM, Brennan GI et al. Co-carriage of diverse vancomycin-resistant *Enterococcus faecium* ST80-lineages by 70% of patients in an Irish hospital. *JAC Antimicrob Resist* 2025; **7**: dlaf065. <https://doi.org/10.1093/jacamr/dlaf065>
- 20 D'Costa V, McGrann KM, Hughes DW et al. Sampling the antibiotic resistome. *Science* 2006; **311**: 374–7. <https://doi.org/10.1126/science.1120800>