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The first *vanE*-type vancomycin resistant *Enterococcus faecalis* isolates in Norway – phenotypic and molecular characteristics

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ABSTRACT

Objectives: We aimed to characterize the *vanE* cluster and its genetic support in the first Norwegian *vanE*-type isolates and assess genetic relatedness to other *vanE* isolates.
Methods: Two *vanE*-type vancomycin resistant *Enterococcus faecalis* (*vanE*-VRE*fs*) isolates (E1 and E2) recovered from the same patient 30 months apart were examined for antimicrobial susceptibility, genome sequence, vancomycin resistance induction, *vanE* transferability, genome mutation rate, and phylogenetic relationship to *E. faecalis* closed genomes and two *vanE*-VRE*fs* from North America.
Results: The ST34 E1 and E2 strains expressed low-level vancomycin resistance and susceptibility to teicoplanin. Their *vanE* gene clusters were part of a non-transferable Tn*6202*. The histidine kinase part of $vanS_E$ was expressed although a premature stop codon (E1) and insertion of a transposase (E2) truncated their $vanS_E$ gene. The vancomycin resistance phenotype in E1 was inducible while constitutive in E2. E1 showed a 125-fold higher mutation rate than E2. Variant calling showed 60 variants but nearly identical chromosomal gene content and synteny between the isolates. Their genomes also showed high similarity to another ST34 *vanE*-VRE*fs* from Canada.
Conclusion: In-depth genomic analyses of the first two *vanE*-VRE*fs* found in Europe identified a single chromosomal insertion site of two variants of *vanE*-conferring Tn*6202*. Single nucleotide polymorphism (SNP) and core genome multilocus sequence type (cgMLST) analyses show the genomes are different. This can be explained by the high mutation rate of E1 and acquisition of different mobile genetic elements; thus, we believe the two isolates from the same patient are genetically related. Genome similarities also suggest relatedness between the Canadian and Norwegian *vanE*-VRE*fs*.

1. Introduction

Enterococcus faecalis (*Efs*) is an opportunistic pathogen causing invasive infections in which vancomycin is a valid treatment option in cases of penicillin allergy and resistance to other antibiotics [1]. In vancomycin-resistant enterococci (VRE), the *van* gene clusters encode mechanisms that replace the D-Ala-D-Ala terminal side-chain residues of peptidoglycan precursors which reduce the binding affinity to vancomycin. Among the ten known vancomycin-resistant gene clusters, *vanC, E, G, L,* and *N* encode the sidechain change to D-Ala-D-Ser, while *vanA, B, D, M,* and *P* mediate a change to D-Ala-D-Lac [1,2].

The *vanA* and *vanB* are the dominant acquired *van*-types worldwide, mostly found in *Enterococcus faecium*, while *vanE* has been described only in a few reports from North America and Australia and only as chromosomally located in *Efs* [2–5]. The *vanE* gene cluster consists of five genes: *vanE* (D-Ala–D-Ser ligase), $vanXY_E$

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(D,D-dipeptidase/D,D-carboxypeptidase), *vanT*$_E$ (serine racemase), and the regulatory operon with *vanR*$_E$ and *vanS*$_E$ [5]. VanR and VanS work as a two-component signal transduction system regulating the expression of *van* genes in response to the extracellular glycopeptide antibiotics. Vancomycin induces autophosphorylation in the membrane-bound sensor protein VanS, which consequently phosphorylates the transcription activator VanR, resulting in inducible vancomycin resistance [6]. Constitutive expression of vancomycin resistance has been reported in a VanE strain with a truncated *vanS*$_E$ gene [4]. Only one previously reported *vanE*-VRE*fs* strain (N00–410) has been analysed by whole-genome sequencing (WGS) [4,5,7–9].

We describe a detailed pheno- and genotypic analyses of the first two *vanE*-VRE*fs* isolates in Norway and compare the isolates with WGS data from previously reported *vanE* isolates.

2. Material and methods

2.1. Case description and data collection

An elderly hospitalised male with several comorbidities, including an urostomy after cystoprostatectomy, underwent surgery for a lumbar prolapse in 2016. During the hospital stay, a *vanE*-VRE*fs* (isolate E1) was isolated from an asymptomatic polymicrobial bacteriuria, including *vanE*-VRE*fs* >100.00 CFU/mL, associated with a permanent urinary catheter. He was discharged without antibiotic therapy and readmitted twice after ten and fifteen months, with negative VRE rectal and urine screening samples. During the latter hospitalization, he was treated with cefotaxime because of a Chronic Obstructive Pulmonary Disease exacerbation. Thirty months after isolate E1, a second *vanE*-VRE*fs* (isolate E2) was recovered from a chronic lower back wound sample obtained by the patient's general practitioner. No antibiotics were prescribed as treatment for the wound infection. In addition to E1 and E2, we genome sequenced the first *vanE* type strain BM4405 (1999/the US) [7] and got access to the genome sequence (PRJNA951724) of N00–410 (Canada/2002) [5].

2.2. Species identification, antimicrobial susceptibility testing, and van genotype determinations

Initial species identification was performed by MALDI-TOF (Bruker Daltonik GmbH, Bremen, Germany). The Sensititre EUENCF plate (Thermo Scientific, Waltham, MA), as well as ComASP^{TM} Vancomycin (Liofilchem, Roseto Degli Abruzzi, Italy), were used to determine the minimum inhibitory concentrations (MICs) of vancomycin, teicoplanin, ampicillin, gentamicin, and linezolid. Agar dilution and gradient MIC Test Strips (Liofilchem) were used to determine rifampicin and fusidic acid MICs, respectively. Minimum inhibitory concentrations were interpreted according to EUCAST clinical breakpoints v.12.0 and level of glycopeptide resistance defined according to VRE literature [6]. Polymerase chain reaction was performed for initial *van*-typing using primers described previously [7].

2.3. DNA extraction, library preparation, and genome sequencing

For short-read sequencing, genomic DNA extraction and quantification were performed as described [10]. Samples were sequenced at the Genomic Support Center Tromsø^{TM} using NextSeq550 system of Illumina platform as previously explained [10]. For long-read sequencing, the genomic DNA was extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Genomic DNA quantification was done as described for Illumina sequencing. Long-read sequencing was performed in the Norwegian Sequencing Centre (NSC) using the SMRT cell 8M (Sequel II) of PacBio platform, which resulted in reads sized from 10 to 20 kb.

2.4. PacBio reads assembly and analyses (Q20 reads)

PacBio circular consensus sequence reads of accuracy >Q20 were assembled using Unicycler v0.4.7 [11] and the assemblies were annotated by Prokka [12]. Antimicrobial resistance (AMR) genes and plasmids were identified using bacterial AMR reference gene database (PRJNA313047) and PlasmidFinder version 2.0.1 database in ABRicate v0.8 tool [13].

2.5. Illumina reads trimming and mapping reads

Quality trimming, adapter removal, and quality assessment of raw reads generated from Illumina sequencing were performed as previously described [14]. To validate the PacBio assemblies, trimmed Illumina reads were aligned using BWA mem (v07.17) [14] and sorted with SAMtools (v1.10) [15].

2.6. Data availability

Genome sequences generated in this study are published under BioProject number PRJNA858283.

2.7. Phylogenetic analysis and mobile genetic content

The phylogenetic relationship of E1 and E2 to all *Efs* closed genomes deposited in NCBI as of August 1st 2022 (n=458) were examined. A global tree based on the core genome SNP was generated using Parsnp v1.2 [16]. Mobile genetic structures in *vanE*-VRE*fs* genomes were located using BLASTn v2.6.0 [17], along with Easyfig for further visualization [18].

2.8. Assessment of vancomycin resistance induction

Changes in the generation time (Tg) were calculated according to growth rate assessment of the isolates with or without vancomycin. Growth rate comparison was performed for isolates cultured in sub-MIC concentrations (4, 8, and 10 mg/L corresponding to 1/4, 1/2, and 5/8 of the MIC) vs. without vancomycin using three biological and technical replicates as described [3]. The optical density was measured every 30 minutes at 600 nm during the 24 hours of incubation in Synergy H1/Biospa microplate reader (Biotek Instruments, Winooski, VT).

2.8.1. Assessment of vanS$_E$ gene expression

Expression of the *vanS*$_E$ gene after growth of the *vanE*-VRE*fs* in BHI medium overnight without and with vancomycin (10 mg/L) was assessed in three biological and technical replicates. RNA was extracted using Qiagen bacterial RNA kit (Warrington, UK) and cDNA was synthesized as described [19] and quantified by qPCR using the method employed in our research group [20]. The *gyrB* was the expression normalization reference. The mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method [21]. The qPCR primers were designed for the 3′ part of the *vanS*$_E$ gene that encodes the histidine kinase domain (forward 5≠AGAAGAAGCTTGAGTGGGATTT-3ánd reverse 5≠TCGTTGTATCATTGAGCGAGTAT-3ʹ).

2.9. Excision and transferability of vanE mobile genetic elements (MGEs)

To explore the ability of the MGE to circularize, a PCR approach with pairs of primers directed outwards from their ends (forward: 5≠TGGATTCCTGCATCAACAGA-3′and reverse 5≠TTGCCAATGATAAACGCTGA-3ʹ) was carried out. Filter-mating

Table 1
Relevant characteristics of the Norwegian *vanE*-VRE*fs* isolates.

Isolate	Species	ST	Source	Year	VAN*	TEC	AMP	GEN	LIN
E1	*Enterococcus faecalis*	34	Urine	2016	16	0.5	1	<32	2
E2	*E. faecalis*	34	Wound	2019	16	<0.5	2	<32	2

* MICs in mg/L for VAN (vancomycin), TEC (teicoplanin), AMP (ampicillin), GEN (gentamicin), and LIN (linezolid).

[22] was performed to determine the transferability of the MGE harbouring the *vanE* gene cluster to a vancomycin-susceptible *Efs* strain, JH2–2 [23].

2.10. Sequence-typing and variant-calling

The minimum spanning tree (MST) was built using SeqSphere+ software v6.0.2 [24] based on 1972 gene targets of the *Efs* cgMLST scheme with default settings [25]. Variant calling between E1 and E2 isolates was done with Snippy v4.4.0 [26] and SNP effects were predicted using SNPeff tool v4.3t [27].

2.11. Determining spontaneous mutation rate

The mutation rates were assessed in seven biological replicates in rifampicin-containing BHI agar as described elsewhere [28]. Additionally, the protein sequences of the *mutS* and *mutL* genes were compared with the reference strain sequences using Clustal Omega online tool [29] and their promoters were predicted using SAPPHIRE [30]. *Efs* ATCC 29212 was chosen as a reference strain for *mutS* and *mutL* genes comparison because its rifampicin MIC (8 mg/L) was similar to that of E1 and E2.

3. Results and discussion

The first Norwegian *vanE*-VRE*fs* isolates are, to our knowledge, also the first in Europe. We compared their genetic relatedness, *vanE* gene cluster, and mobile genetic context to *vanE* (Tn*6202*), as well as their genetic relatedness towards two other *vanE* isolates (N00-410 and BM4405) and a global collection of closed *Efs* genomes.

3.1. van genotype and phenotype

The initial positive *vanE* PCR result was confirmed by WGS. Antimicrobial susceptibility testing revealed low-level resistance to vancomycin (MIC=16 mg/L); susceptibility to teicoplanin, ampicillin, and linezolid; and a lack of high-level gentamicin resistance (Table 1). The glycopeptide phenotype is consistent with earlier reports on *vanE* [7]. Minimum inhibitory concentrations for rifampicin and fusidic acid (16 mg/L and 8 mg/L, respectively, for both E1 and E2) were determined to set higher concentrations for filter mating.

3.2. vanE *gene cluster is harboured on Tn*6202

BLAST searches of the chromosomal region containing the *vanE* gene clusters of E1 and E2 showed their similarity to the putative integrative conjugative element (ICE) Tn*6202* of *Efs* strain N00–410 (FJ872411.1) covering the entire sequence (Fig. 1A).

In the global tree, the Dutch vancomycin-susceptible *Efs* (VSE*fs*) strain 26975_1#118 (GCA_905120835.1) from 2021 showed the evolutionarily closest closed genome to E1 and E2 isolates (supplement Fig. 1). However, this strain contains an uncharacterised MGE precisely at the integration site of Tn*6202*; therefore, the second closest genome, GCA_905123845.1 (strain 28157_4#211, the Netherlands, 2021), was chosen as a reference for genomic comparison and extraction of the MGE (Tn*6202*) sequence from E1 and E2 genomes (Fig. 1B). In both strains, the MGE containing the *vanE* gene cluster was inserted 7 bp before the 3énd of the *guaA* gene (glutamine-hydrolysing GMP synthase) at an 11 bp direct repeat (5≠TATTCCCACTC-3ˆ) (Fig. 1A, Table 2). The only publicly available sequence that contains Tn*6202* harbouring the *vanE* gene cluster (FJ872411.1 from strain N00–410) shows the same insertion site and direct repeat. The *vanE* gene cluster is the only AMR that is encoded on Tn*6202* [9].

The entire *vanE* gene cluster is found in all genomes at NCBI of the human gut commensal *Enterococcus caccae* (n = 4) [31]. The nucleotide sequence identity between the *vanE* gene clusters of *E. caccae* and E1 and E2 is 91% (supplement Fig. 2). We found an integrase (tyrosine-based site-specific recombinase) next to the *vanE* gene cluster in *E. caccae*, suggesting it is present on a putative MGE; however, because there were no available *E. caccae* genome sequences without *vanE*, we were not able to define any putative MGE harbouring the *vanE* gene cluster in this species.

3.3. Inducibility of vancomycin resistance

The regulatory $vanS_E$ gene in both E1 and E2 isolates was truncated. The truncation was due to a premature stop codon caused by a single nucleotide deletion in E1 (362delA) and an insertion of IS*6770* in E2 at position 383, which resulted in two coding sequences (CDSs) in both isolates (Fig. 1A), each containing different functional domains of the $vanS_E$ gene. The first CDS contains a transmembrane-associated sensor domain (123 and 117 amino acids (AAs) in E1 and E2, respectively), and the second contains the histidine kinase domain (211 and 230 AA in E1 and E2, respectively) (Fig. 2). Inducible vancomycin resistance has been achieved in other *vanE*-type isolates with a truncated $vanS_E$ gene and was suggested to occur because of crosstalk with another two-component signal transduction system or by $vanR_E$ acting with a heterologous histidine kinase [4,8,32].

IS*6770* elements can be integrated at different locations of the *Efs* genome, and it is recognised as a common IS element in the enterococcal genomes, usually in more than one copy [33]. This is the first report of insertion of IS*30*-like elements (IS*6770*) in the $vanS_E$ gene cluster, but other IS elements have been reported in the *vanS* gene of the *vanA* gene cluster [34].

Although the normal *vanE* gene cluster encodes inducible vancomycin resistance, *vanE*-VRE*fs* strains may have a truncated $vanS_E$ gene and constitutively expressed resistance to vancomycin [4,7]. qPCR plots for pre-growth in BHI without and with sub-MICs of vancomycin showed a two-fold increase in expression of the histidine kinase part of $vanS_E$ in E1, while expression in the E2 isolate was reduced three-fold. These changes were not statistically significant (Fig. 3). In the *vanE*-type isolates with inducible resistance, the Tg is shorter when pre-cultured in a sub-MIC concentration of vancomycin [3]. Because the CDS that contains the histidine kinase domain in E1 is in the same frame as $vanR_E$, it is possible that this domain is still controlled by the $vanR_E$ promoter [8]. Thus, the slight decrease in Tg and increase in expression of the $vanS_E$ gene after exposure to sub-MICs of vancomycin likely represent low-level inducibility of vancomycin resistance in E1 (data not shown). In E2, the insertion of IS*6770* (between the two CDSs of the *vanS* gene) with a transposase encoded in the opposite direction clearly disturbed the expression of the histidine kinase part

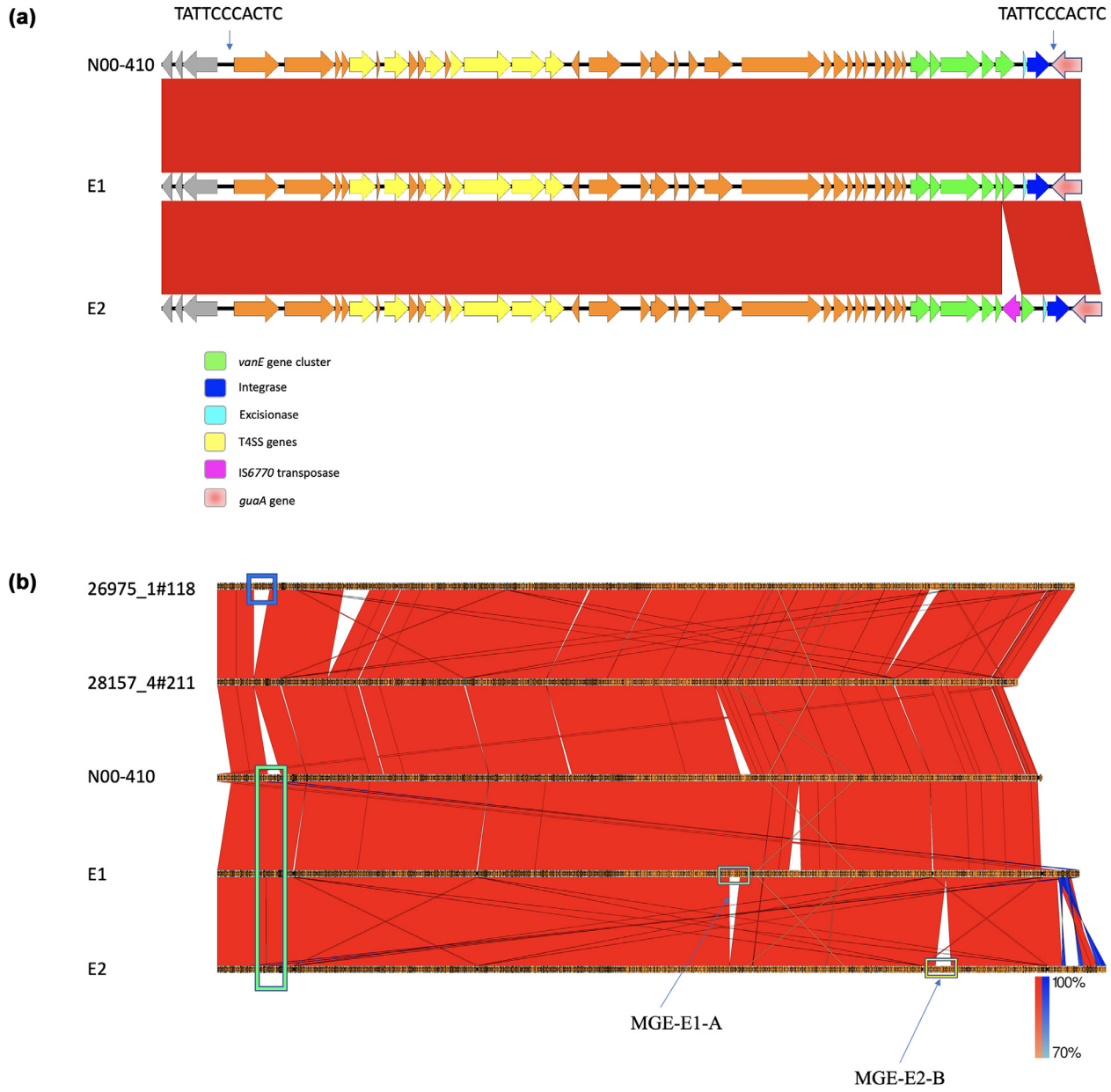

Fig. 1. Comparison of Tn*6202* region and whole genomes of *vanE*-VRE*fs*. Fig. 1A illustrates the comparison between Tn*6202* and its flanking sequences in E1 and E2 to their reference (N00–410). Coding sequences (CDSs) and their directions are shown with arrows. The red bars represent 99.99% identity. The Tn*6202* insertion site and its sequence are marked on the reference N00–410. Fig. 1B shows similarities between the genomes of E1, E2, *vanE*-VRE*fs* strain of N00–410, and the two closest closed genomes of non-*vanE*-type *Efs* isolates retrieved from NCBI. The green rectangle marks the position of Tn*6202* in the genome and the blue square is for the uncharacterized MGE integrated at the exact same location of Tn*6202* on the reference genome 26975_1#118. The yellow rectangles highlight other MGEs found in E1 and E2. The red and blue gradient bars represent normal and inverted matches, respectively.

Table 2
Characteristics of Tn*6202* in E1 and E2.

Strain	Tn*6202* size (bp)	Number of CDSs	Repeat in insertion site (5'-3' strand)	
			L side	R side
E1	43647	39	TATTCCCACTC	TATTCCCACTC
E2	44716	40	TATTCCCACTC	TATTCCCACTC

of the $vanS_E$ gene from the $vanR_E$ promoter. As expected, low level constitutive expression of resistance to vancomycin was seen in E2 (Fig. 3), as has also been observed for other strains with truncated $vanS_E$. Insertion of IS elements carrying promoters or partial promoters can contribute to expression of downstream genes [35] and thus may have contributed to the low-level constitutive expression of the histidine kinase domain of $vanS_E$ in E2.

3.4. Activity and transferability of the Tn6202

Tn*6202* carries seven Type IV secretion system (T4SS) genes [36], and a putative integrase and excisionase. So, the genes needed for excision and integration, as well as some putative genes for transfer, are present in E1 and E2. In the transfer process, many MGEs, including Tn*6202*, form a circular double-stranded interme-

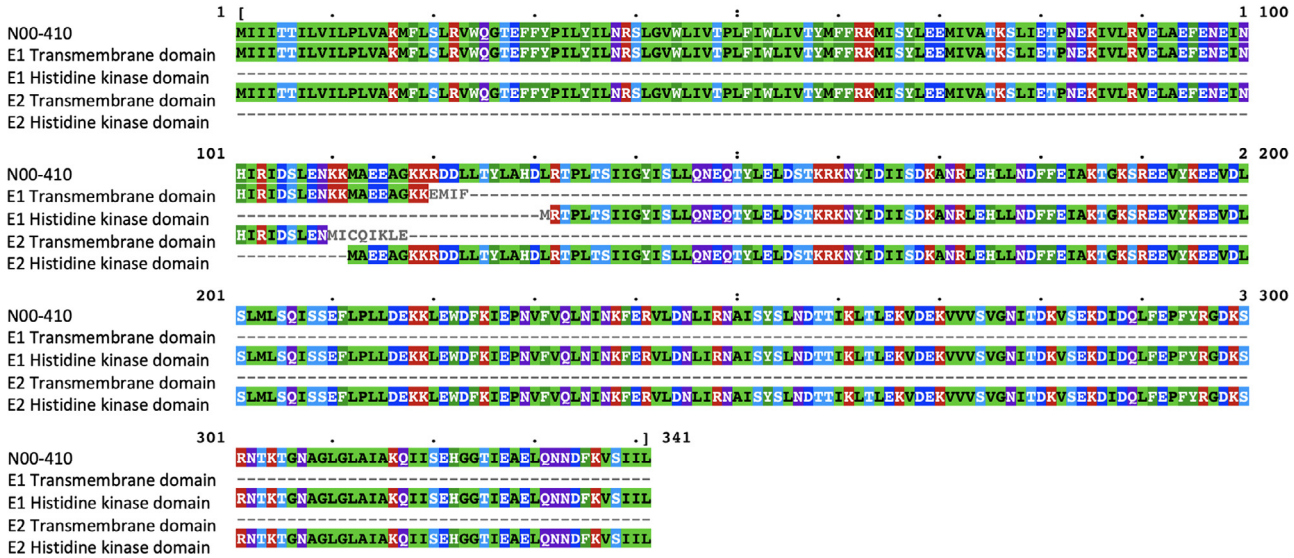

Fig. 2. Amino acid sequence alignment of VanS$_E$ in *vanE*-VRE*fs* isolates using Clustal omega online tool compared to the 341 AA reference sequence (N00–410 AAL27446.1). Because they were in E1 and E2, the two domains of *vanS$_E$* were annotated as functional CDSs in Prokka and we added each of the domains (transmembrane and histidine kinase) as separate sequences in the alignment.

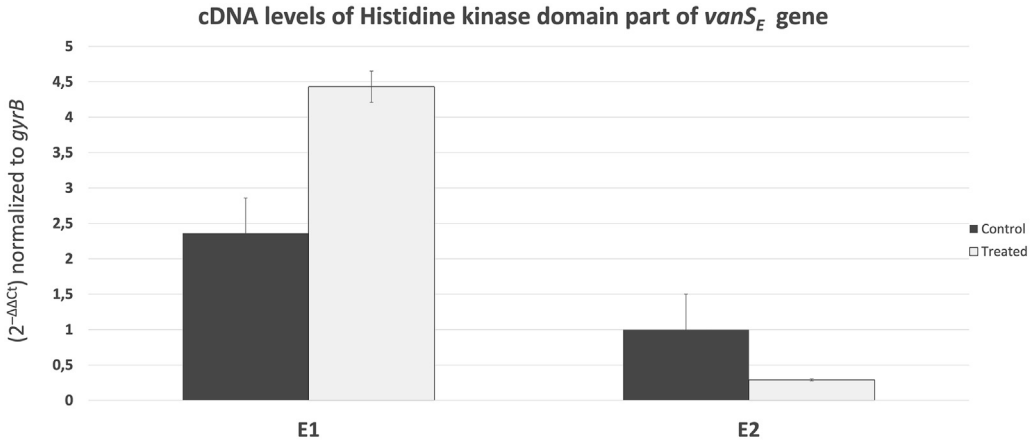

Fig. 3. The level of expression of the *vanS$_E$* histidine kinase part determined by RT-qPCR. The average expression levels of the histidine kinase part of the *vanS$_E$* gene ($2^{-\Delta\Delta Ct}$) of E1 and E2. Data are expressed as the mean ± standard deviation. Two-tailed *t* test showed no significant changes ($P = 0.62$ for E1 and $P = 0.76$ for E2) in expression of the histidine kinase domain in E1 or E2 pre-grown in BHI broth without (control) and with 10 mg/L vancomycin (treated).

diate [9]. Polymerase chain reaction for circularization of Tn*6202* in E1 and E2 was negative, which means that the transferable form of Tn*6202* was not detected. Moreover, transfer of Tn*6202* from E1 and E2 to JH2–2 was not detected (detection limit 10^{-10} to 10^{-7} transconjugants/ donor cell), which is in line with a previous report on Tn*6202* not being transferrable among *Efs* [9]. Thus, the putative ICE Tn*6202* either lacks some necessary genes, a host factor is necessary for transfer, or the transfer frequency was below the detection limit.

3.5. Genetic relatedness and mutation rates

E1 and E2 belong to ST34 but have different cluster types (CTs). According to cgMLST results (supplement Fig. 3), there are 32 allelic differences between E1 and E2, resulting in CT3081 and CT2880, respectively. Variant calling (using E1 assembly as a reference for E2 reads) showed 60 variants between E1 and E2, including 49 SNPs, three insertions, and eight deletions. A total of 51 variants occurred in coding sequences, including a missense mutation in the *vanR$_E$* gene. A total of 12 variants (20%) were predicted to have a high impact on gene function due to gain of a stop codon (n = 4), loss of start codon (n = 1), or frame shift (n = 7). The frame shift variants resulted in premature stop codon (n = 5) or shorter product from the 5énds (n = 2) in the CDSs (supplement File 1).

The genome of E1 shows high similarity and synteny to the N00–410 VRE*fs* strain (Fig. 1B) reported in 2002. N00–410 also belongs to ST34. Variant calling showed 57 variants (supplement File 1) and cgMLST showed only 34 allelic differences between E1 and N00–410, while BM4405, which is ST4, has 1796 allelic differences compared with E1 (supplement Fig. 3). These similarities suggest that E1, E2, and N00–410 may be genetically related, but we do not have any metadata that link N00–410 to the Norwegian isolates. Compared with the closest VSE*fs* reference genome GCA_905120835.1, E1 and E2 showed 454 and 699 chromosome variants, respectively (supplement File 1). This confirms that ST34 *vanE*-VRE*fs* strains (E1, E2, and N00–410) are much more like each other than the reference genome.

Rifampicin exposure is a useful method to calculate the mutation rate in pathogenic bacteria [37]. For E1 and E2, the spontaneous mutation rate was 1×10^{-7} and 8×10^{-10}, respectively, and 3×10^{-10} for the ATCC29212 reference strain. If we then as-

sume the generation time of *E. faecalis* is similar to *E. coli* (1.5–1.7 doubling times per day) in a non-laboratory setting [38,39], 33 to 37 mutations could occur in the 3 Mb genome over 30 months. Because E1 has a 125-fold higher mutation rate, 60 variants between E1 and E2 in 30 months is not an unexpected number. It has been shown that mutation in DNA mismatch repair genes (*mutS* and *mutL*) can cause elevated spontaneous mutation frequencies in *Efs* [28]. However, the difference in mutation rate cannot be explained by this because the DNA sequences of *mutS* and *mutL* genes and their promoters were identical in E1 and E2 isolates and showed 99.5% identity to the *mutS* and *mutL* genes of ATCC29212. Additionally, no differences in the promoter region of *mutS* and *mutL* genes between E1, E2, and the reference genome (GCA_905120835.1) were observed.

E1 and E2 have some differences in their accessory genomes. Plasmid finder identified only one replicon in E1 (rep9c) and two additional replicons in E2 (rep9a and repUS43). Local BLASTn search showed that the common plasmid between the E1 and E2 (rep9c) has 98% identity and 39% coverage. The only plasmid AMR gene is a *tet* gene on rep9c of E2. The isolates have other differences in MGEs integrations. E1 has a 31 kb MGE (MGE-E1-A) integrated at the overlapping sequence of two hypothetical proteins genes, while E2 contains a 45 kb MGE (MGE-E2-B) integrated at the 5' end of putative tRNA sulphur transferase genes. Despite these differences, the genome organization of E1 and E2 is identical (Fig. 1B).

The similarity in genome organization, low allelic differences (n = 32) in cgMLST, and number of variants (n = 60) in the E1 and E2 genomes suggest relatedness. The 30 months that have passed between their isolations have allowed time for evolution diversification. The similarity between E1 and N00–410 Tn*6202* and genomes also support genetic relatedness between the Canadian and Norwegian *vanE*-VRE*fs*, but we lack the epidemiological data to link them.

4. Conclusions

The *vanE*-VRE*fs* isolates (E1 and E2) contained chromosomally Tn*6202*-related *vanE* gene clusters. The $vanS_E$ genes were truncated, resulting in two CDSs with different functional domains. The E1 truncation with the histidine kinase domain in frame with $vanR_E$ was consistent with low-level inducible vancomycin resistance. The IS element insertion in E2 $vanS_E$ likely explains the constitutive expression of vancomycin resistance. A total of 60 variants between E1 and E2 genomes were identified. Their accessory genomes showed different MGE profiles. However, similar chromosomal gene content and synteny suggest that the isolates are genetically related. The two Norwegian isolates show high similarity to the only previously whole-genome sequenced *vanE*-VRE*fs* (N00–410).

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Competing interests: None declared

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2023.12.021.

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