

Novel vancomycin and macrolide-lincosamide-streptogramin B resistance plasmids from a set of multi-drug-resistant *Enterococcus* isolated from biofilm grown in wastewater

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Abstract

Antibiotic resistance among Gram-positive bacteria is a serious global health threat. Vancomycin-resistant enterococci (VRE) are especially concerning since they are susceptible to few if any antibiotics, and can serve as a reservoir for mobile genetic elements containing diverse resistance genes that could potentially be transmitted to other Gram-positive bacteria. The plasmids that confer resistance in Gram-positive bacteria, however, are poorly understood. In this project, a set of eight VRE strain was isolated from the influent of a wastewater treatment plant in San Diego County. Antibiotic susceptibilities were determined by disc diffusion methods, revealing resistance most commonly to vancomycin, penicillin, linezolid, and erythromycin. Two novel plasmids were isolated from the strains, and the complete nucleotide sequences were determined by MinION sequencing technology. The plasmids contained a non-standard vanA operon and a novel macrolide-lincosamide-streptogramin B composite transposon.

Introduction

The increasing prevalence of antibiotic resistance is one of the 21st century's largest public health issues.^[1] This growing public health crisis carries with it tremendous human and economic costs. It is estimated that 33,000 people in the EU die from antibiotic-resistant infections annually^[2], and it is estimated that the healthcare costs associated with drug-resistant infections in the USA alone has doubled since 2002.^[3] This issue will continue to grow in magnitude unless proactive work is done to understand the dissemination and diversity of antibiotic resistant bacteria.^[4] In particular, wastewater treatment plants (WWTPs) have been shown to be reservoirs of antibiotic resistance genes^[5] and the release of wastewater into the environment can lead to an increase of abundance of antibiotic resistance genes in said environment,^[6] where the bacteria can continue to evolve and recombine, leading to the development of new resistance phenotypes.^[7]

The microbiome of WWTPs are home to large amounts by bacteria native to the human gastrointestinal tract.^[8-9] These bacteria are frequent causes of infection in humans, and of particular concern for development of antibiotic resistance. *Enterococcus* is a genus of Gram-positive bacteria highly common in the GI tract of mammals, and two species are commonly found in the human GI tract, *E. faecalis* and *E. faecium*.^[9] Though typically

commensal, these species can cause infection, particularly in immunocompromised patients and in the formation of biofilms on medical devices.^[10-12] *Enterococcus* infections are highly common; they are 2nd leading cause of hospital-associated infections in the United States.^[14]

One of the most common clinical treatments for *Enterococcus* infections is a combination of an aminoglycoside and a β -lactam, particularly amino-penicillins.^[10] However, in the case of resistance to one or both of those classes, vancomycin is a standard treatment option.

Vancomycin is a glycopeptide antibiotic with strong activity against Gram-positive bacteria. It binds the precursors to peptidoglycan, preventing the crosslinking of D-ala and L-ala in the creation of the cell wall. Notably, vancomycin is also commonly used in the treatment of *C. difficile* and *diphtheroid*, *Staphylococcus*, and *Streptococcus* species.^[15] However, resistance to vancomycin in *Enterococcus* infections is becoming highly common; nearly one in three isolates of *Enterococcus* species now displays vancomycin resistance.^[13-14] The prevalence of *Enterococcus* infections, few alternate treatment options,^[10] and wide range of vancomycin usage make vancomycin-resistant *Enterococcus* (VRE) a major concern for antibiotic resistance.^[16]

VRE was first observed in the clinical setting in the 1980s,^[17] and the genetic basis was quickly traced to a plasmid harbored in the isolate.^[18] Vancomycin resistance is a complex, polygenic trait. It primarily involves the alteration of peptidoglycan precursors, preventing effective binding of vancomycin.^[19] Frequently the C-terminal D-ala is replaced with D-lac or D-ser. Genotypes are most often grouped by their ligase; there are currently nine described vancomycin resistance ligase genes^[20] each with its own corresponding resistance operon. The location of the resistance varies, with some operons located on the bacterial chromosome, and some on a plasmid. The most common genotypes, *vanA* and *vanB*, are both plasmid-mediated conferring high- and variable-level resistances, respectively, and more commonly found in *E. faecium* and *E. faecalis*, respectively.^[21-23] The *vanA* operon is typically located on a transposon, *Tn1546* being the first and most widely reported. The standard *Tn1546* *vanA* operon is displayed in Figure 1.

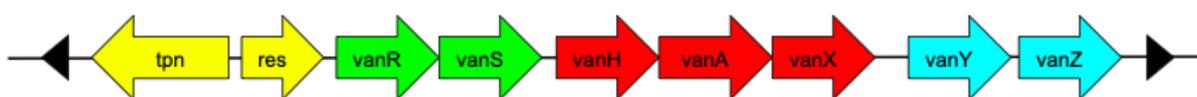


Figure 1. VanA operon: *Tpn* and *res* code for the transposase and resolvase necessary for transposition. The black triangles represent the left and right inverted repeats. *VanS* codes for a membrane-bound signaling protein, which upon stimulation activates *vanR*, a transcriptional activator which activates the expression of the downstream proteins. *VanH* codes for a dehydrogenase which converts pyruvate to D-lac, *vanA* codes for a ligase which joins D-ala to D-lac, and *vanX* codes for a dipeptidase which breaks the bonds between D-ala and D-ala, preventing the inclusion of vancomycin-binding peptidoglycan precursors. *VanH*, *vanA*, and *vanX* are the essential genes for vancomycin resistance. *VanY* removes the terminal D-ala from

any peptidoglycan precursors which did not utilize the modified dipeptide. The role of vanZ is not fully understood, but is believed to be linked to teicoplanin resistance.^[24]

In this study, we isolated VRE from a WWTP grown into biofilm on a stainless-steel coupon filled with sterile sand. The resistance profile of the isolates was determined, with a variety of drugs from multiple clinically relevant antibiotic classes represented. The full plasmid genome of one isolate was isolated, sequenced, and characterized.

Materials & Methods

Sample Isolation

A 55-gallon drum was filled with untreated inflow from Padre Dam Municipal Wastewater Treatment Plant in Santee, California and left on site. A sterile steel mesh biofilm coupon filled with sterile coarse grain sand was suspended in the inflow from the drum covering. The coupon was left in the drum for 6 weeks before it was removed and placed in a sterile 0.85% saline solution for transportation. A 25-mL sample of the inflow was also taken to be tested. The coupon containing the sand was washed by 3x immersion and inversion in a sterile saline solution. The sand was then removed and placed in a flask containing 25 mL of a 0.1% tetra-sodium pyrophosphate solution, and shaken at 150 rpm for 1 hour. 10 mL of the solution was then taken and centrifuged at 10,000 x g for 10 minutes, and the pellet resuspended in a sterile saline solution. 10 mL of the inflow was also centrifuged and resuspended in saline. 100 uL of the sand biofilm extraction and the inflow resuspension were then spread onto Bile Esculin Agar plates supplemented with 20 ug/mL vancomycin and 25 ug/mL cycloheximide. Single colonies which generated the indicative black zone on the agar were picked using sterilized toothpicks, placed in LB broth supplemented with 20 ug/mL vancomycin, and grown overnight. The overnight culture was then centrifuged and resuspended in LB supplemented with 20% (v/v) glycerol and stored at -80°C. Total cellular DNA was extracted from the isolates using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer's instructions and clonal groups were created using randomly amplified polymorphic DNA (RAPD) PCR using primers 208 and 272.^[40] Isolates were screened for plasmids by purification of plasmid DNA from cellular DNA using Qiagen Miniprep (see Appendix "protocol purification of plasmid DNA prepared by other methods" and gel electrophoresis)

Antimicrobial Susceptibility Testing

Kirby-Bauer disk diffusion tests were used to determine the resistance profile of one isolate from each clonal group. From frozen stocks isolates were grown overnight in LB with 20 ug/mL vancomycin as a selective pressure. The overnight culture was spun down and resuspended in saline to achieve turbidity comparable to McFarland 0.5 standard, and then spread onto Mueller Hinton agar. The disks were then applied using a stamper, inoculated at 37°C overnight, and analyzed the following day. Susceptibility was determined according to CLSI breakpoint standards. The antibiotics used are listed in Table 1:

Drug	Abbreviation	Class
Ciprofloxacin	CIP	Fluoroquinolone
Levofloxacin	LVX	Fluoroquinolone
Tetracycline	TET	Tetracycline
Tigecycline	TGE	Glycylcycline
Vancomycin	VAN	Glycopeptide
Gentamicin	GM	Aminoglycoside
Streptomycin	SM	Aminoglycoside
Erythromycin	ER	Macrolide
Penicillin	PCN	Beta-Lactam
Linezolid	LZD	Oxazolidinone

Table 1: Antibiotics whose activity was measured, as well as their abbreviation and class. All drugs tested are relevant to treatment of *Enterococcus* infections^[10] and had CLSI breakpoints available.

Plasmid Genome Sequencing, Annotation and Analysis

A representative isolate was selected and its entire plasmid genome sequenced. Plasmid DNA was extracted using a modified Qiagen Midiprep protocol obtained from their website, and stored at -20 °C until it was ready to be sequenced. The plasmid DNA was prepared and sequenced using an Oxford Nanopore minION using the rapid004 prep kit and protocol. The reads were taken and assembled using a custom Canu with over X coverage, and many individual reads spanning the full length of the plasmid. Initial plasmid annotation was performed using Prokka^[39], coding sequences which were not identified by Prokka were identified by protein homology using NCBI BLAST alignment with a minimum confidence of 50% coverage and 50% identity. Their identity was further confirmed by nucleotide BLAST of the coding sequence, and cross referenced. Any coding sequence that was not identified and was under 90 amino acids in length was not included in the plasmid maps in figures 2 and 3. Plasmid

incompatibility groups were determined using Plasmidfinder 2.0, and insertion sequences were identified using ISfinder.^[38] Plasmid maps were constructed using Geneious 11.1.4. When reporting sequence similarity, % coverage will be reported first followed by % identity.

Results

Antimicrobial Susceptibility

A total of 17 isolates were obtained, of which 2 were isolated directly from the sewage, and 15 from the biofilm grown on the sand coupon. RAPD PCR analysis sorted these 17 isolates into 8 clonal groups. Clonal groups 1-7 were isolated from the biofilm, and group 8 was directly from the liquid sewage. *E. faecalis* OG1RF was included as a control of known resistance profile, containing resistance only to rifampin and fusidic acid. The profile of each individual isolate is listed in table 2.

Drug	OG1RF	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
CIP	S	S	S	S	S	S	R	S	S
LVX	S	S	S	S	S	S	R	S	S
TET	S	S	R	S	R	I	R	S	S
TGE	S	S	S	S	S	R	S	S	S
VAN	S	R	R	R	R	R	R	R	R
GM	S	R	S	S	S	R	R	S	S
SM	S	I	R	S	R	S	R	R	S
ER	I	R	R	R	R	R	R	R	S
PCN	S	R	R	R	R	R	R	R	R
LZD	S	R	R	R	R	R	S	R	R

Table 2. Isolate Resistance Profiles: The high susceptibility of OG1RF and universal isolate resistance of vancomycin matched known resistance profiles. There was a high degree of variability between clonal groups, with only groups 2 and 4 displaying the same resistance pattern.

Besides vancomycin, the most common resistances displayed were to penicillin (8/8), linezolid (7/8), and erythromycin (7/8). Tigecycline and the fluoroquinolones remained active against most isolates. The proportion of resistance by drug is listed in table 3.

Drug	Class	% clinically resistant	% decreased susceptibility	% fully susceptible
Ciprofloxacin	Fluoroquinolone	12.5%	0%	87.5%

Levofloxacin	Fluoroquinolone	12.5%	0%	87.5%
Tetracycline	Tetracycline	37.5%	12.5%	50%
Tigecycline	Glycylglycine	12.5%	0%	87.5%
Gentamicin	Aminoglycoside	37.5%	0%	62.5%
Streptomycin	Aminoglycoside	50%	12.5%	37.5%
Erythromycin	Macrolide	87.5%	0%	12.5%
Penicillin	Beta-Lactam	100%	0%	0%
Linezolid	Oxazolidinone	87.5%	0%	12.5%

Table 3. Drug Resistance Proportions: Resistance to penicillin, erythromycin, and linezolid were highly prevalent. Aminoglycoside and tetracycline resistance was present but not ubiquitous among our isolates. Among the aminoglycosides, gentamicin maintained more activity than streptomycin. Tigecycline and the fluoroquinolones maintained activity against all but one isolate group each.

pADRE 7003V

The plasmid genome of clonal group 3 was isolated and sequenced. It yielded 2 distinct plasmids, which were confirmed to confer the *vanA* operon and macrolide resistance, respectively. They were named pADRE_7003V and pADRE_7003E (plasmid Antimicrobial Drug-Resistant *Enterococcus*). The full maps of pADRE_7003V and pADRE_7003E can be found in figures 2 and 3, respectively.

pADRE_7003V contained a *vanA* operon. The plasmid is a 33,181 base pair plasmid, which was assembled with 281x coverage. Plasmidfinder 2.0 assigned the backbone to rep17 at a very low similarity (49% / 88%). The 5003-base pair backbone (from start codon of gene *dinB* to the stop codon of *repA* N-terminal-containing gene) had 17 hits in BLAST with 100% coverage and 96% identity, primarily uncharacterized plasmid sequences. However none of these plasmids was described with a putative *inc/rep* type. It is 9,762-base pairs long, and contains all of the standard *Tn1546* resistance genes, plus an insertion sequence element *IS1251* which has inserted itself between *vanS* and *vanH*. The entire operon shared extremely high sequence similarity (100% coverage 97% identity) with previously characterized VRE plasmids pPEC286^[25], pISMMS_VRE2^[26], and pHvH-V24^[27] sourced from clinical isolates.

In addition to the backbone and *vanA* operon, the plasmid contained three toxin/antitoxin (TA) systems: RelE/RelBEf, *axe/txe*, and Fic/Doc. ISfinder located identical *IS1216* elements flanking a recombinase family protein and the Fic/Doc TA system, and appears to be a composite transposon. BLASTN failed to find a contiguous sequence with >80% coverage. There were also several other transposase and recombinase family genes throughout the plasmid. There was a cobalt transport system *corA*. Lastly, there was a set of three genes that were determined by protein homology to be similar to type-I endonuclease subunits. The subunit M- and subunit S-like genes had amino acid similarities of 98% / 53% and 99% / 77% respectively.

The subunit S gene appears to be partial, as the gene is only 786 base pairs and the reference amino acid sequence contains 421 amino acids. The final gene carried some similarity (50% for both, 58% for both) to endonuclease subunits M and S, though it carried the greatest similarity (41% / 80%) to subunit R. Like subunit S, it must be partial, as it is only 928 base pairs, and the reference sequence for subunit R is 841 amino acids long. The most similar plasmid found with BLAST was a previously uncharacterized plasmid and covered 83% of pADRE_7003V at 97% identity. There were several other plasmids in the 75-80% coverage range at lower identity..

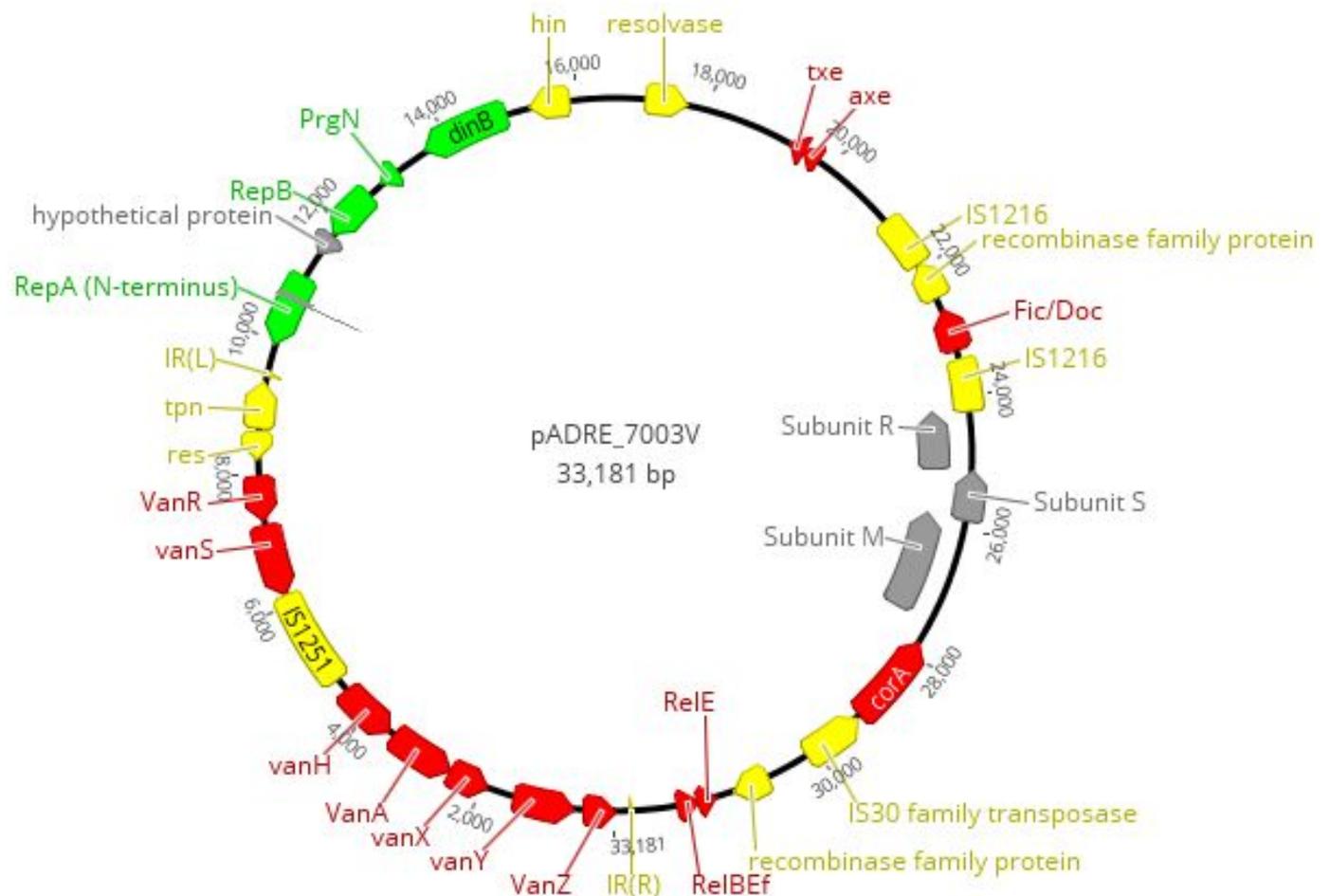


Figure 2. Full map of pADRE_7003V: Backbone (maintenance, replication, partitioning, and stability) genes are colored in green, mobile elements and mobile element protein-encoding genes are colored in yellow, and accessory (antibiotic resistance, metal transport, TA systems) in red.

pADRE 7003E

pADRE_7003E (Figure 4) is a 28,246-base pair Rep17 (99% / 92%) plasmid, which was assembled with 485x coverage. The backbone of the plasmid is 4,909 base pairs long (start codon of DinB to stop codon of RepA) and contains four well known replication and

maintenance proteins. Notably, the backbone, has 41% coverage and 93% identity with the backbone of pADRE_7003V, primarily in the *dinB* to *prgN* region and the N-terminal region of *RepA*. It also contained 3 TA systems, including the *Fic/Doc* transposon found in pADRE_7003V and *axe/txe*. It also contains a *HicA/B* TA system. Lastly, it also has the type-I endonuclease-like genes found in pADRE_7003V. The most closely related plasmid had 77% coverage with 93% identity.

The only previously identified resistance gene present in the plasmid is *erm(B)*, which confers resistance to macrolide-lincosamide-streptogramin B (MLS_B) antibiotics. It also contains several IS elements. There are identical *IS1216* elements surrounding *erm(B)*, as well as two other IS elements (*IS1182*, *ISEfa11*) and a handful of coding sequences which code for an 80-amino acid hypothetical protein containing a group 1413 domain of unknown function (DUF1413), as well as two genes coding for a putative nucleotidyltransferase with amino acid sequence similarity of 88% / 54% compared to the reference, and a putative ubiquinone biosynthesis methyltransferase (100% / 61%). This appears to form a resistance transposon. BLASTN failed to find a continuous result above 80% coverage. There is also a lone *IS256*, a few recombinases, and a *Cro/CI* family transcriptional regulator of unknown function.

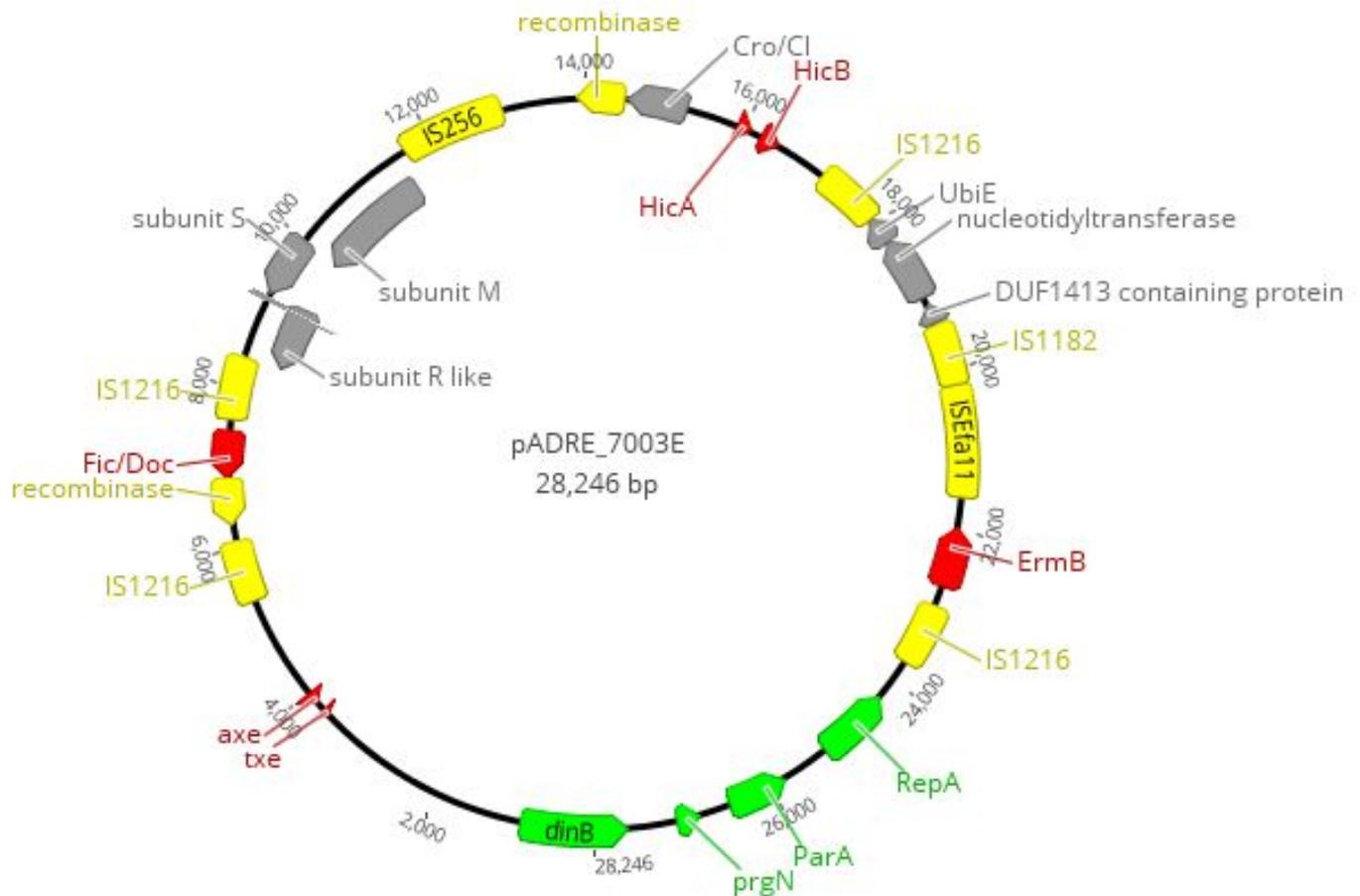


Figure 3. Full map of pADRE_7003E: Backbone (maintenance, replication, partitioning, and stability) genes are colored in green, mobile elements and mobile element protein-encoding genes are colored in yellow, and accessory (antibiotic resistance, metal transport, TA systems) in red.

Discussion

8 strains of vancomycin-resistant *Enterococcus* species were isolated from a wastewater treatment plant in Santee, California. Each isolate was multi-drug-resistant, containing resistance to vancomycin and penicillin, as well as at least one other drug. The plasmid genome of one strain was isolated, sequenced and characterized. It yielded two novel plasmids, one conferring a modified *Tn1546* vanA resistance operon and another erm(B) MLS_B resistance located on a novel putative transposon. They both contain multiple toxin/antitoxin systems, including a novel putative Fic/Doc TA transposon. They both also contain a large region coding for putative partial endonuclease subunits. The translated amino acid sequence low agreement with the references make it likely that the true function of those proteins is not accurately known.

Clonal group 3 possessed resistance to vancomycin, erythromycin, penicillin, and linezolid. The genetic basis for erythromycin and vancomycin resistance were uncovered, but the basis for the penicillin and linezolid resistances were not. It is likely that the penicillin resistance is chromosomally mediated. Chromosomal resistance to penicillin is common among *Enterococcus*,^[41] as it has been discovered that their penicillin binding proteins have low affinity for penicillin.^[42] The genetic basis of the linezolid remains unknown. Chromosomal linezolid resistance genes are not common, as the 23S rRNA subunit is highly conserved, and many mutations could lead to a devastating loss of function.

The resistance profile of our set of isolates is a particularly lethal combination. The high prevalence of aminoglycoside, β -lactam, and linezolid resistance paired with the vancomycin resistance wipe out most preferred treatment options.^[10] The extreme prevalence of linezolid resistance is particularly troubling, as it is not widely reported.^[28] Linezolid acts to disrupt protein synthesis by binding to the A site of the 23S rRNA subunit.^[29] Resistance to linezolid is mediated either by chromosomal mutation of the 23S rRNA or from 3 distinct plasmid mediated genes, *cfr*, *cfr(B)*, and *optrA*.^[30] Spontaneous chromosomal mutation of the linezolid binding site is not common due to the extreme deleterious effect of most mutations at that site. Plasmid mediated *cfr* codes for a methyltransferase conferring resistance to linezolid by methylating adenine 2503 in the subunit, which hinders linezolid binding.^[31-33] Though translated nucleotide / amino acid alignment did not find significant similarity in sequence (13% / 55%), the location of a UbiE methyltransferase along with other unknown function coding sequences on a transposon with another protein synthesis disrupting methyltransferase is suspicious, especially as UbiE is traditionally a chromosomal gene^[34] and our translated sequence only had 61% agreement with UbiE. If pADRE_7003E could be transformed into a linezolid-susceptible host, then differences

between the transconjugants and the host could be observed. If linezolid resistance transfers with the plasmid, then it is likely that it originates on the plasmid on the erm(B)-containing transposon.

The presence of biofilm-forming VRE in wastewater reflects a public health concern, as the sample source makes it highly likely that the bacteria originally entered through human feces.^[6] Wastewater overflow has been linked in the past to the release of resistance genes.^[35] Plasmid-mediated resistance poses a unique threat, as transferable resistance increases the spread of population resistance.^[36] In particular, resistances associated with mobile elements such as transposons pose a threat, as they can move from plasmid to chromosome of their host, and possibly onto plasmids of a broader host-range that can transfer into different species and genera of bacteria.^[37] These threats necessitate the continued monitoring and study of antibiotic resistance genes, resistance mechanisms, and resistance evolution.

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