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Vancomycin resistance plasmids affect persistence of *Enterococcus faecium* in water

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ABSTRACT

Vancomycin resistant enterococci (VRE) cause 20,000 infections annually in the United States, most of which are nosocomial. Recent findings of VRE in sewage-contaminated surface waters demonstrate an alternate route of human exposure, and a possible setting for horizontal gene exchange facilitated by plasmids and other mobile genetic elements. Maintenance of antibiotic resistance genes and proteins may, however, present a fitness cost in the absence of selective pressure, particularly in habitats such as environmental waters that are not optimal for gut-associated bacteria. Nutrient levels, which are transiently elevated following sewage spills, may also affect survival. We tested the hypotheses that nutrients and/or plasmids conferring vancomycin resistance affect *Enterococcus faecium* survival in river water by measuring decay of strains that differed only by their plasmid, under natural and augmented nutrient conditions. In natural river water, decay rate (log_{10} reduction) correlated directly with plasmid size; however, plasmid presence and size had no effect on decay rate when nutrients levels were augmented. Under natural nutrient levels, the vancomycin-resistant strain with the largest plasmid (200 kb) decayed significantly more rapidly than the plasmid-less, susceptible parent strain, in contrast to similar decay rates among strains under augmented nutrient conditions. This work is among the first to show that plasmids conferring antibiotic resistance affect fitness of *Enterococcus* species in secondary habitats such as surface water. The nutrient-dependent nature of the fitness cost suggests that conveyance of VRE to environmental waters in nutrient-rich sewage may prolong survival of these pathogens, providing greater opportunity for host infection and/or horizontal gene transfer.

1. Introduction

Vancomycin resistant enterococci (VRE), which are categorized as a serious public health threat (CDC, 2013) cause 20,000 hospital-acquired infections and 1300 deaths annually in the United States and reported incidence is steady or increasing in both the U.S and Europe (Adams et al., 2016; Chiang et al., 2017; Kullar et al., 2016; Laxminarayan et al., 2007; Mendes et al., 2016; Sader et al., 2018). VRE and the mobile, high-level resistance gene *vanA* have been detected in aquatic environments, wildlife feces and human sewage (Ateba et al., 2013; Haenni et al., 2009; Narciso-da-Rocha et al., 2014; Oravcova et al., 2014, 2016; 2017; Roberts et al., 2009; Young et al., 2016). VRE infections are commonly monitored through surveillance programs in healthcare systems and often studied in these contexts, but conditions that contribute to VRE survival in environmental habitats are poorly understood.

Enterococcus spp. can carry mobile genetic elements that confer antibiotic resistance and transfer between bacteria (Paulsen et al., 2003). Nine different gene clusters conferring vancomycin resistance have been identified (Miller et al., 2014) but not all are relevant from a human health perspective. For example, the *vanC* gene confers intrinsic, low-level resistance (2–32 $\mu g\,mL^{-1}$) and is found in certain *Enterococcus* spp. such as *E. gallinarum* but rarely associated with infections in human (Leclercq et al., 1992). Thus, detection of *vanC* in the aquatic environment is not an effective indicator of risk to human health. The most clinically relevant VRE are the high-level resistant *E. faecium* carrying the *vanA* gene (Cetinkaya et al., 2000), with a minimum inhibitory concentration of $\geq 32\,\mu g\,mL^{-1}$ vancomycin (Cetinkaya et al., 2000; Courvalin, 2006). The *vanA* operon is generally located on a plasmid,

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including transposons or gene cassettes that facilitate mobility (Arthur et al., 1993; Guardabassi and Agerso, 2006; Leclercq et al., 1988). The potential for spread of high-level resistance to other pathogens such as *Staphylococcus aureus,* and/or environmental bacteria, make the environmental persistence of bacteria bearing genetic elements carrying *vanA* a relevant public health concern (McGuinness et al., 2017; Stryjewski and Corey, 2014).

Many studies have examined the role of bacterial plasmids in virulence and biological fitness using competitive culture or mouse model experiments (Bingle and Thomas, 2001; Fisher and Phillips, 2009; Süßmuth et al., 2000). Maintenance of plasmids that confer antibiotic resistance can present a fitness cost and large plasmids are usually found in low copy numbers (Andersson and Levin, 1999; Johnsen et al., 2009, 2011; Melnyk et al., 2015; Ramadhan and Hegedus, 2005; San Millan et al., 2015; Sengupta and Austin, 2011; Smith and Bidochka, 1998a; Starikova et al., 2013; Vogwill and MacLean, 2015; Zhang et al., 2017). The cost of resistance can be too great to sustain in the absence of selective pressure, and some bacteria lose plasmids when under stress in order to reduce metabolic expense (Smith and Bidochka, 1998a). However, fitness experiments in the laboratory have shown that the cost of plasmid-associated resistance is host- and plasmid-dependent (Starikova et al., 2013). In some cases, plasmids can be maintained in the absence of selective pressure (Johnsen et al., 2002; Werner et al., 2011) and in starvation states (Ramadhan and Hegedus, 2005).

Bacterial persistence and interactions in aquatic habitats are strongly driven by nutrient availability (Byappanahalli et al., 2012; Cloutier et al., 2015; Staley et al., 2014). Few studies have investigated the influence of nutrients on maintenance of antibiotic resistance plasmids outside of nutrient-rich culture experiments. The aquatic environment provides relatively oligotrophic conditions compared to culture media or intestinal environments (Pereira and Berry, 2017) and the role of nutrients in the survival of bacteria carrying antibiotic resistance plasmids need further study in oligotrophic settings. In this study, chromosomally identical strains of *E. faecium* with vancomycin resistance plasmids of differing sizes and genetic makeup were compared in microcosms containing filtered river water maintained in an outdoor environment. Nutrient levels were natural, or augmented with trace vitamins, minerals and organic carbon sources glucose, pyruvate and acetate. Investigating bacterial persistence associated with plasmids and environmental nutrient levels contributes to understanding antibiotic resistance and its potential to impact human health in a dynamic, ecologically relevant context.

2. Methods

2.1. Enterococcus faecium strains

E. faecium strains were obtained from Dr. Guido Werner (Robert Koch Institute, Wernigerode, Germany). In a previous study, clinically-derived strains carrying plasmids with the *vanA* gene were filter-mated with the vancomycin-sensitive American Type Culture Collection (ATCC) strain 64/3 (Werner et al., 2011). Each VRE strain in Table 1 represents a transconjugant of 64/3 (recipient strain) whose plasmids ranged from 50 to 200 kb. Previous testing showed stable maintenance of plasmids on media without antibiotics for approximately 100 generations (Werner et al., 2011). Additional testing confirmed that strains maintained plasmids and survived for up to two weeks in phosphate-buffered water and filtered river water in microcosms maintained in the laboratory (data not shown). The four *E. faecium* strains used here all had similar growth rates in brain heart infusion (BHI) broth.

2.2. Microcosm set-up

Water was collected from the Hillsborough River in Tampa, FL (28.069992, −82.377558) on March 31, 2017 in sterile, 50 L carboys. The Hillsborough River is a typical tannic Florida River, originating in the Green Swamp and passing through natural and rural watersheds before it reaches the sampling site. The water was filtered on that date using dialysis filters (REXEED-25S, Asahi Kasei) to remove particles and microorganisms. Filtered water was tested for endogenous enterococci and VRE on mEI agar and mEI agar amended with 32 μg mL^{-1} vancomycin to confirm absence of culturable enterococci prior to the start of the experiment (EPA, 2006).

Four strains were inoculated in separate microcosms. Each microcosm contained 1 L filtered river water in a 2 L beaker. The nutrient treatment included unamended (control) conditions, and added nutrients (see below). Five replicates were created for each strain representing both control and nutrient conditions. Microcosms were covered with plastic wrap to allow light penetration and prevent contamination. A 1000-gallon capacity tank functioned as a water bath to modulate temperature fluctuations in the greenhouse at the USF Botanical Gardens. The greenhouse is protected from rainfall and covered and fenced to prevent animal intrusion, but is exposed to UV light and susceptible to environmental temperature changes. Microcosms were secured to shelves and partially submerged in the tank three days prior to inoculation in order to stabilize.

Carbon sources, vitamins and minerals were added as nutritional supplements in augmented nutrient conditions and compared to a control treatment with no nutrients added. Nutrient additions were as follows: hydrated sodium salts of pyruvate (8.3 mg L $C_3H_3NaO_3$), acetate (6.2 mg L $C_2H_3NaO_2$) and glucose (18 mg L $C_6H_{12}O_6$) for final concentrations of 75 μM, 75 μM and 100 μM respectively; 1 mL of pre-mixed additives for trace vitamins and 1 mL of pre-mixed minerals were added to each microcosm (ATCC, Manassas, VA) (Wanjugi et al., 2016). Two HOBO data loggers (H08-004-02, Onset Computer Corporation, Bourne, MA) were placed in identical beakers with the same source water inside the water bath, where measurements for temperature, humidity and light intensity were logged hourly over eight days. Light wavelength and intensity were recorded on each sampling date using a spectroradiometer (Model ILT950, International Light Technologies, Peabody, MA) at the time of collection, confirming that natural UV light was reaching the microcosms though not as strong as direct sunlight.

Inoculum preparation and inoculation: Overnight cultures of each *E. faecium* strain were prepared in 10 mL BHI broth (Becton, Dickinson and Company, Sparks, MD, USA) using isolated colonies obtained from frozen stock cultures of VRE transconjugants E0292, H182, H74, and the parent strain, vancomycin sensitive 64/3. Cultures were incubated for 12 h at 37 °C, then transferred to a sterile 15 mL tube and centrifuged at 5000 rpm for 3 min. Supernatant broth was removed and 6 mL of sterile phosphate-buffered solution was added to each tube and vortexed to create stock inoculant for each strain of approximately 9 $\log_{10}$ CFU mL^{-1} based on mEI agar quantification. Individual strains were added separately to each of five replicate beakers containing 1L each of river water, such that each mesocosm contained only one *E. faecium* strain. One mL of the stock inoculant was added to make an initial concentration in the mesocosms of approximately 6 $\log_{10}$ CFU mL^{-1} (equivalent to 8 $\log_{10}$ CFU• 100 mL^{-1}).

2.3. Sample collection and processing

Water samples were collected at the initial inoculation time (Day 0), and on Day 2, Day 4 and Day 7. Samples were collected

Table 1
E. faecium strain origin and plasmid characteristics, adapted from Werner et al., (2011).

E. faecium strain	Origin	Country of origin	Year	Plasmid characteristics	Traits/mechanisms
64/3	**ATCC**			None; recipient strain	N/A
H74	hepatic fluid	Portugal	2001	50 kb vanA plasmid (rep-Inc18,rep-pRUM; PSK: ω-ε-ζ)	• MDR gene cassette (Rosvoll et al., 2010) • inhibit growth (Grady and Hayes, 2003) • enhance fitness • support plasmid maintenance • HGT* (Palmer et al., 2010)
H182	hepatic fluid	Portugal	2002	92 kb vanA plasmid (rep-pRUM)	• MDR gene cassette (Rosvoll et al., 2010) • inhibit growth (Grady and Hayes, 2003)
E0292	urine	USA	1992	200 kb vanA plasmid (rep-Inc18)	• HGT* (Palmer et al., 2010)

*HGT = horizontal gene transfer.

from each microcosm (n = 40) in 10 mL volumes using sterile pipets, after stirring to mix using the collection pipets. Samples were transported on ice to the laboratory in sterile 15 mL centrifuge tubes, held on ice, and processed within 6 h. Water samples were processed by membrane filtration for enterococci in duplicate, on both mEI and on mEI +32 μg mL^{-1} vancomycin, except for the non-resistant strain 64/3, which was only processed on unamended mEI (EPA, 2006). Dilutions were established to ensure a countable number of colonies (20–100 per plate). Estimates for dilution series were based on preliminary studies and earlier sampling results when processing samples at later time points. VRE strains were cultured on both mEI and mEI amended with vancomycin on Day 0 and Day 4. Data from Day 7 are shown in Figs. 1 and 2 but were excluded from analyses because some strains were reduced to undetectable concentrations by that date (<1 CFU •100 mL^{-1}).

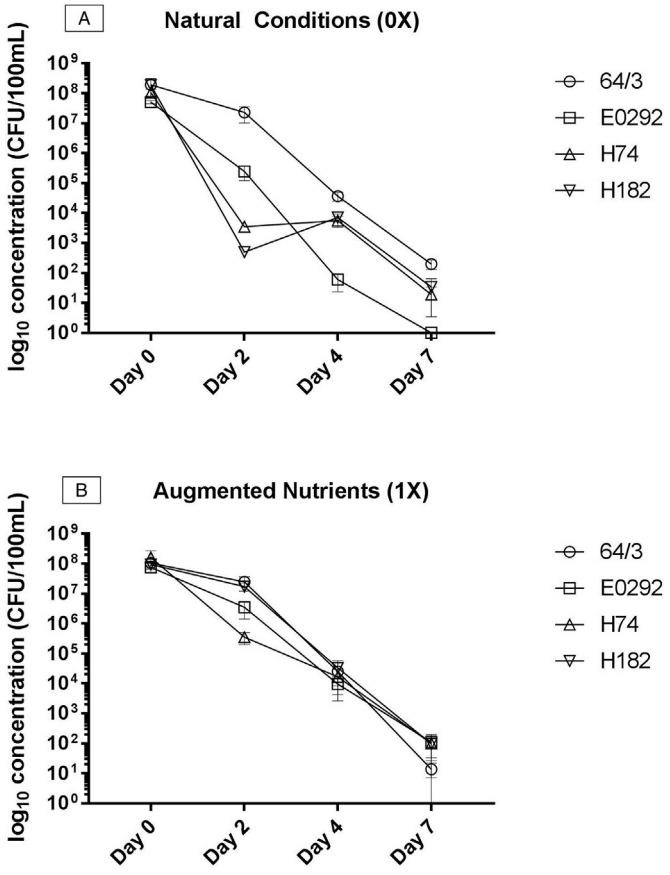

Fig. 1. Decay of vancomycin-resistant E. faecium strains E0292 (200 kb plasmid), H74 (50 kb plasmid), and H182 (92 kb plasmid) and vancomycin-sensitive E. faecium strain 64/3 (no plasmid) over time in (A) natural and (B) augmented nutrient conditions, shown as log10 CFU 100mL-1. Error bars are SEM.

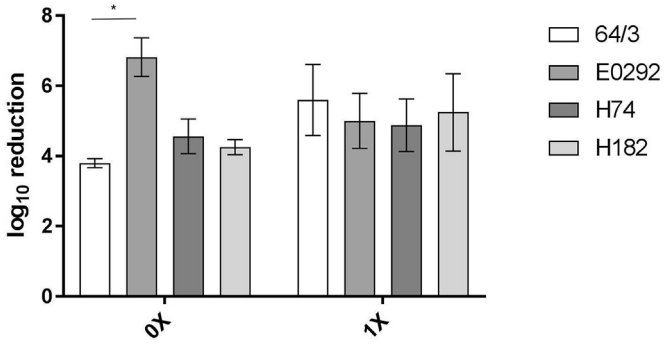

Fig. 2. Decay (log_{10} reduction after 4 days) for each *E. faecium* strain under natural (0X) and augmented (1X) nutrient treatments. Error bars are SEM. Star indicates significant difference ($P < 0.05$) within the treatment.

2.4. Data analysis

Culturable enterococci concentrations were recorded as CFU•100mL^{-1}. Log_{10} reduction, which represents the log decrease in cell concentration from Day 0 to Day X, was calculated for each replicate based on the final concentration at Day 4 and the initial concentration at Day 0, using an equation as previously described (Wanjugi and Harwood, 2013). Greater log_{10} reduction corresponds to a more rapid decay rate and reduced survival. Day 4 was chosen based the most appropriate time point for capturing the decay rate before enterococci reached undetectable levels. GraphPad Prism was used to perform 2-way ANOVA with Tukey's multiple comparison and linear correlation assuming Gaussian distribution with Pearson's r (Version 6.07, GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Plasmid characteristics

Natural nutrient concentrations of ammonium, nitrate and phosphorus in filtered river water (0.165, 0.536 and 0.047 ppm, respectively) were consistent with Florida water quality guidelines for unpolluted waters and measurements from similar freshwater systems in the region (EPA, 2015). Plasmids in each transconjugant strain differed in their sizes and other characteristics as described previously (Werner et al., 2011) and are summarized in Table 1. Plasmid sizes ranged from 50 kb to 200 kb and were characterized by different genetic content, including toxin-antitoxin systems and replicon typing. Each transconjugant contained a plasmid from a clinical isolate; one from a hospital in the United States (E0292) and two from hospitals in Portugal (H74 and H182) (Table 1).

3.2. Plasmid maintenance

E. faecium strains were cultured on media with and without antibiotics added to confirm maintenance of plasmids throughout the experiment, as retention of plasmids through the experiment was necessary to test the hypothesis that plasmid size affects survival. No significant differences in concentrations of transconjugant *E. faecium* strains cultured on mEI compared to vancomycin-amended mEI were detected in natural or augmented nutrient conditions (2-way ANOVA; $P = 0.66$ for and $P = 0.73$, respectively), showing plasmid stability in the absence of selective pressure.

3.3. Decay of E. faecium strains

The relative decay of plasmid-bearing and wild type *E. faecium* strains was compared in natural river water and with added nutrients. The mean initial concentration of all four strains inoculated in natural and augmented microcosms on Day 0 was 7.9 $\log_{10}$•100 mL^{-1} (standard deviation = 0.3 $\log_{10}$•100 mL^{-1}). All strains declined 1–4 $\log_{10}$ CFU•100 mL^{-1} after four days (Fig. 1). The only significant difference observed among strains ($\alpha < 0.05$) was that transconjugant E0292 experienced significantly greater $\log_{10}$ reduction than the parent strain under natural nutrient conditions (Tukey's multiple comparisons, $P = 0.0245$, Fig. 2). This difference was not observed under augmented nutrient conditions ($P = 0.934$). Nutrient levels did not significantly affect $\log_{10}$ reduction of any strain (two-way ANOVA; $P = 0.523$), although E0292 reduction was about a log greater under low nutrient compared to augmented conditions. The significant difference in decay between E0292 and the parent strain under natural nutrient conditions does suggest that the nutrients are a biologically relevant factor in survival.

3.4. Reduction associated with plasmid size

The $\log_{10}$ reduction of the *E. faecium* strains directly correlated with plasmid size in the natural nutrient treatment (Fig. 3A; $P = 0.027$, $r = 0.2436$), but not in the augmented nutrient treatment (Fig. 3B; $P = 0.5963$, $r = -0.1261$). Larger plasmids were associated with greater reduction in the natural nutrient treatment but not in the augmented nutrient treatment.

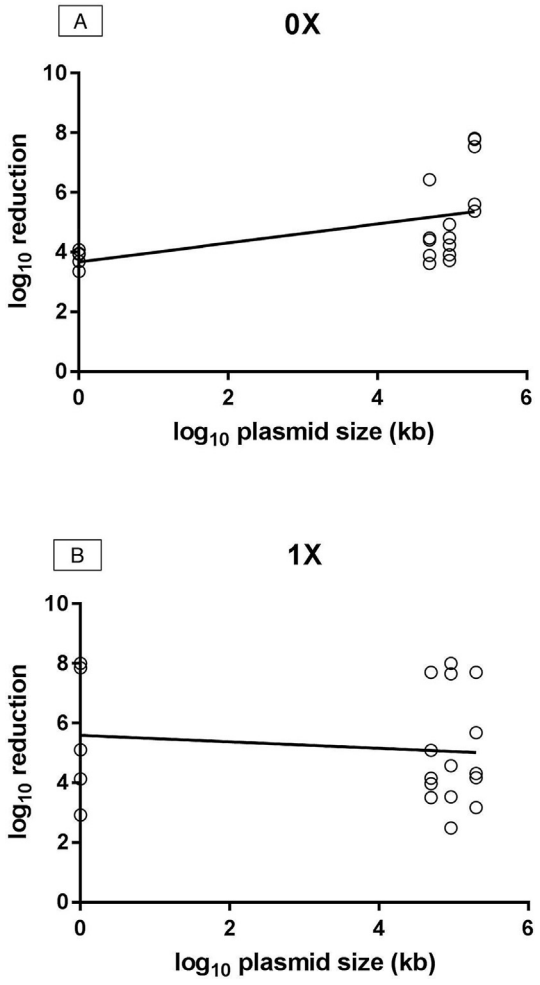

Fig. 3. Correlation of decay ($\log_{10}$ reduction after 4 days) and $\log_{10}$ plasmid size of *E. faecium* strains under (A) natural and (B) augmented nutrient conditions.

4. Discussion

The fitness cost of plasmids in general (Smalla et al., 2015) and antibiotic resistance plasmids in particular (San Millan et al., 2015; Schrag et al., 1997; Smith and Bidochka, 1998b) has been the subject of study for decades. The general conclusion drawn from such studies is that plasmids incur a fitness cost in the absence of selective pressure under laboratory conditions. Because growing emphasis is placed on the importance of environmental reservoirs of antibiotic resistance (McLain et al., 2016) we designed this study to assess the fitness cost of mobile plasmids carrying the *vanA* operon for *E. faecium* in a simulated aquatic environment. We hypothesized that organic nutrient levels could influence the burden of plasmid maintenance, a linkage that has not been well-explored. The most obvious mechanism for such an influence would be that increased nutrient levels offset the metabolic costs of plasmid DNA and protein synthesis (Carroll and Wong, 2018).

In this study, effects of nutrients on survival were statistically significant only when analyzed based on a relationship to a continuous scale of plasmid size; increasing plasmid size was associated with decreased survival under natural nutrient conditions, but not when river water was augmented with nutrients. Augmented nutrient conditions designed to reflect organic carbon levels in a eutrophic surface water (Wanjugi et al., 2016) were expected to enhance survival of all strains in this study, given that nutrient availability is a key influence on bacterial survival in aquatic ecosystems (Anderson et al., 2005; Barcina et al., 1997; Craig et al., 2004). This is particularly true of organic carbon for *Enterococcus* spp. (Zhang et al., 2017) and other heterotrophic bacteria. However, added nutrients did not have a significant within-strain effect on survival, which may be explained by relatively low nutrient concentration amendments used in the treatments. The lack of within-strain effect could also be due low cellular metabolic activity. A previous study (Wanjugi et al., 2016) using the same river water and organic carbon sources (glucose, pyruvate, and acetate) as this study determined that nutrient concentrations at five times the augmented level used here enhanced survival of *E. coli* compared to natural and 1X amended conditions. Those results suggest that using more concentrated nutrient treatments in future studies may reveal a relationship or help to disentangle the differences between strains and nutrient levels, and could better assess the impacts of raw sewage containing VRE released into aquatic ecosystems.

Greater reduction of one plasmid-bearing *E. faecium* strain (E0292) compared to the parent strain, which lacked plasmids, confirms previous findings of a fitness cost of plasmids in environments devoid of selective pressure (Ramadhan and Hegedus, 2005, San Millan et al., 2015; Starikova et al., 2013; Vogwill and

MacLean, 2015; Zhang et al., 2017). Two of the transconjugant strains used in this study, E0292 and H182, were previously studied to assess fitness cost in competitive mixed culture experiments under laboratory conditions (Starikova et al., 2013). The largest plasmid had a detrimental effect (E0292; relative fitness = 0.73) and the smallest was beneficial (H182; relative fitness = 1.1). While the microcosms in this study confirm that reduced fitness is associated with the large plasmid, there was no observed benefit to smaller plasmids in our experiments, which included environmental stressors such as sunlight, and ambient nutrient levels. Results from the previous study with the same transconjugants (Starikova et al., 2013) support the hypothesis that large plasmids exert fitness costs to their hosts, although the conditions in the competitive culture experiments contrasted sharply from those in this study, which simulated a natural oligotrophic aquatic environment rather than nutrient-rich culture media.

The largest vancomycin resistance plasmid significantly reduced survival in *E. faecium* transconjugant E0292 compared to the vancomycin-susceptible counterpart under natural nutrient conditions in this study. Log_{10} reduction of the other transconjugant VRE strains was not significantly different than that of the parent strain, suggesting that there may be a cut-off or threshold size for larger plasmids to negatively impact survival under conditions used here. While plasmid copy numbers were not assessed here, their size and previously characterized replicon types indicate that they are large, low-copy number plasmids, with less than 5–10 copies per cell (San Millan et al., 2014; Werner et al., 2011). While the precise genes or gene cassettes responsible for decreased fitness have not been elucidated, the difference between the large plasmid and the parent strain was only determined in the natural (unaugmented) nutrient conditions, which suggests that the oligotrophic environment affects the relative success of bacteria bearing large plasmids, and that eutrophic nutrient levels may reduce fitness cost. The fact that smaller plasmids that confer clinically-relevant VRE characteristics did not exert a cost of fitness in the environmental conditions studies here is cautionary, as it suggests that smaller plasmids may not hamper survival of these pathogens.

Various genes within conjugative plasmids can mediate virulence and survival (Elwell and Shipley, 1980; Groisman and Ochman, 1996; Guilloteau et al., 1996; Ramirez et al., 2014; Trevors et al., 1989), but known genes on the plasmids studied here were not associated with survival advantages or inhibition. All three conjugative plasmids of the VRE strains used in this study are classified as repINC18, repINC-pRUM, or both (Shintani et al., 2015; Werner et al., 2011). E0292 and H74 contained a repINC18 plasmid, which is associated with horizontal gene transfer and no known survival advantages (Palmer et al., 2010), while H74 and H182 both contained the repINC-pRUM-type plasmid (see Table 1). Toxin-antitoxin systems of repINC-pRUM plasmids have been shown to inhibit survival and also support plasmid maintenance in *E. coli* (Grady and Hayes, 2003) but did not appear to affect survival in this study with *E. faecium* when compared to parent strains lacking plasmids. Both transconjugants with the repINC-pRUM plasmids (H74 and H182) had lower decay rates compared to E0292. However, this study was not desiged to determine whether the plasmid types contribute to or inhibit persistence of VRE broadly, or how variability in molecular structure of plasmids influenced survival.

The findings presented here are initial steps towards understanding how antibiotic resistance plasmids influence survival in aquatic environments and should lead to further experimental investigation and genetic analyses. While some genetic aspects of the plasmids have been characterized, sequencing of entire plasmids might further clarify the role of resistance determinants in the differential survival demonstrated in this study. Fitness costs shown in the strain carrying the largest plasmid (E0292) could be associated with strong promoters regulating gene expression, including any additional antibiotic resistance genes located on the plasmids. Expression of plasmid genes could also be impacted by nutrient levels or other environmental factors, and could be explored by transcriptomic analysis. Focusing on and isolating the role of the plasmid, outside of the chromosomal genetic background shared by each strain, represents novel research that can inform future studies of fitness costs of antibiotic resistance.

5. Conclusions

This study showed that the transconjugant *E. faecium* strain carrying the largest plasmid decayed faster than its plasmid-less parent strain in an aquatic environment with low available nutrients and in the absence of selective pressure. Strains with smaller plasmids persisted similarly to the parent strain. Clinical VRE strains can enter the environment through sewage spills (Young et al., 2016) and this study investigated the fate of these pathogens in environmental surface water, showing that large plasmids inhibit survival, but that increased levels of organic nutrients can alleviate the fitness costs.

- Vancomycin resistance plasmids that are maintained in the absence of selective pressure can exert fitness costs under natural nutrient conditions
- The size of vancomycin resistance plasmids was correlated with fitness costs under natural nutrient conditions
- Characteristics of antibiotic resistance plasmids can influence the survival of bacterial pathogens released into aquatic ecosystems through runoff, sewage spills, and other wastewater inputs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Paulsen, I.T., Banerjei, L., Myers, G.S.A., Nelson, K.E., Seshadri, R., Read, T.D., Fouts, D.E., Eisen, J.A., Gill, S.R., Heidelberg, J.F., Tettelin, H., Dodson, R.J., Umayam, L., Brinkac, L., Beanan, M., Daugherty, S., DeBoy, R.T., Durkin, S., Kolonay, J., Madupu, R., Nelson, W., Vamathevan, J., Tran, B., Upton, J., Hansen, T., Shetty, J., Khouri, H., Utterback, T., Radune, D., Ketchum, K.A., Dougherty, B.A., Fraser, C.M., 2003. Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis</em&gt. Science 299 (5615), 2071.

Adams, D.J., Eberly, M.D., Goudie, A., Nylund, C.M., 2016. Rising vancomycin-resistant Enterococcus infections in hospitalized children in the United States. Hosp. Pediatr. 6 (7), 404–411.

Anderson, K.L., Whitlock, J.E., Harwood, V.J., 2005. Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. Appl. Environ. Microbiol. 71 (6), 3041–3048.

Andersson, D.I., Levin, B.R., 1999. The biological cost of antibiotic resistance. Curr. Opin. Microbiol. 2 (5), 489–493.

Arthur, M., Molinas, C., Depardieu, F., Courvalin, P., 1993. Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in Enterococcus faecium BM4147. J. Bacteriol. 175 (1), 117–127.

Ateba, C.N., Lekoma, K.P., Kawadza, D.T., 2013. Detection of vanA and vanB genes in vancomycin-resistant enterococci (VRE) from groundwater using multiplex PCR

analysis. J. Water Health 11 (4), 684–691.
Barcina, I., Lebaron, P., Vives-Rego, J., 1997. Survival of allochthonous bacteria in aquatic systems: a biological approach. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 23 (1), 1–9.
Bingle, L.E., Thomas, C.M., 2001. Regulatory circuits for plasmid survival. Curr. Opin. Microbiol. 4 (2), 194–200.
Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R., Harwood, V.J., 2012. Enterococci in the environment. Microbiol. Mol. Biol. Rev. 76 (4), 685–706.
Carroll, A.C., Wong, A., 2018. Plasmid persistence: costs, benefits, and the plasmid paradox. Can. J. Microbiol. 64 (5), 293–304.
CDC, 2013. Antibiotic Resistance Threats in the United States, 2013. Online.
Cetinkaya, Y., Falk, P., Mayhall, C.G., 2000. Vancomycin-resistant enterococci. Clin. Microbiol. Rev. 13 (4), 686–707.
Chiang, H.-Y., Perencevich, E.N., Nair, R., Nelson, R.E., Samore, M., Khader, K., Chorazy, M.L., Herwaldt, L.A., Blevins, A., Ward, M.A., 2017. Incidence and outcomes associated with infections caused by vancomycin-resistant enterococci in the United States: systematic literature review and meta-analysis. Infect. Control Hosp. Epidemiol. 38 (2), 203–215.
Cloutier, D.D., Alm, E.W., McLellan, S.L., 2015. Influence of land use, nutrients, and geography on microbial communities and fecal indicator abundance at lake Michigan beaches. Appl. Environ. Microbiol. 81 (15), 4904–4913.
Courvalin, P., 2006. Vancomycin resistance in gram-positive cocci. Clin. Infect. Dis. 42, S25–S34.
Craig, D.L., Fallowfield, H., Cromar, N.J., 2004. Use of microcosms to determine persistence of Escherichia coli in recreational coastal water and sediment and validation with in situ measurements. J. Appl. Microbiol. 96 (5), 922–930.
Elwell, L.P., Shipley, P.L., 1980. Plasmid-mediated factors associated with virulence of bacteria to animals. Annu. Rev. Microbiol. 34 (1), 465–496.
EPA, 2006. Method 1600: Enterococci in Water by Membrane Filtration Using Membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI).
EPA, 2015. Chapter 6-302: Surface Water Quality Standards. Online.
Fisher, K., Phillips, C., 2009. The ecology, epidemiology and virulence of Enterococcus. Microbiology 155 (6), 1749–1757.
Grady, R., Hayes, F., 2003. Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrug-resistant, clinical isolate of Enterococcus faecium. Mol. Microbiol. 47 (5), 1419–1432.
Groisman, E.A., Ochman, H., 1996. Pathogenicity islands: bacterial evolution in quantum leaps. Cell 87 (5), 791–794.
Guardabassi, L., Agerso, Y., 2006. Genes homologous to glycopeptide resistance vanA are widespread in soil microbial communities. FEMS Microbiol. Lett. 259 (2), 221–225.
Guilloteau, L.A., Wallis, T.S., Gautier, A.V., MacIntyre, S., Platt, D.J., Lax, A.J., 1996. The Salmonella virulence plasmid enhances Salmonella-induced lysis of macrophages and influences inflammatory responses. Infect. Immun. 64 (8), 3385–3393.
Haenni, M., Saras, E., Chatre, P., Meunier, D., Martin, S., Lepage, G., Menard, M.F., Lebreton, P., Rambaud, T., Madec, J.Y., 2009. vanA in Enterococcus faecium, Enterococcus faecalis, and Enterococcus casseliflavus detected in French cattle. Foodb. Pathog. Dis. 6 (9), 1107–1111.
Johnsen, P.J., Simonsen, G.S., Olsvik, Ø., Midtvedt, T., Sundsfjord, A., 2002. Stability, persistence, and evolution of plasmid-encoded VanA glycopeptide resistance in enterococci in the absence of antibiotic selection in vitro and in gnotobiotic mice. Microb. Drug Resist. 8 (3), 161–170.
Johnsen, P.J., Townsend, J.P., Bohn, T., Simonsen, G.S., Sundsfjord, A., Nielsen, K.M., 2009. Factors affecting the reversal of antimicrobial-drug resistance. Lancet Infect. Dis. 9 (6), 357–364.
Johnsen, P.J., Townsend, J.P., Bohn, T., Simonsen, G.S., Sundsfjord, A., Nielsen, K.M., 2011. Retrospective evidence for a biological cost of vancomycin resistance determinants in the absence of glycopeptide selective pressures. J. Antimicrob. Chemother. 66 (3), 608–610.
Kullar, R., Merchant, S., Tabak, Y.P., Deryke, C.A., Johannes, R.S., Sarpong, E.M., Gupta, V., 2016. Regional and Source Variations in Vancomycin-Resistant Enterococci Rates in United States Hospitals 2015. Oxford University Press.
Laxminarayan, R., Malani, Anup, Howard, David, Smith, David L., 2007. In: Future, R.f.t (Ed.), Extending the Cure: Policy Responses to the Growing Threat of Antibiotic Resistance (Washington, D.C.
Leclercq, R., Derlot, E., Duval, J., Courvalin, P., 1988. Plasmid-Mediated resistance to vancomycin and teicoplanin in Enterococcus faecium. N. Engl. J. Med. 319 (3), 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 (9), 2005–2008.
McGuinness, W.A., Malachowa, N., DeLeo, F.R., 2017. Focus: infectious diseases: vancomycin resistance in Staphylococcus aureus. Yale J. Biol. Med. 90 (2), 269.
McLain, J.E., Cytryn, E., Durso, L.M., Young, S., 2016. Culture-based methods for detection of antibiotic resistance in agroecosystems: advantages, challenges, and gaps in knowledge. J. Environ. Qual. 45 (2), 432–440.
Melnyk, A.H., Wong, A., Kassen, R., 2015. The fitness costs of antibiotic resistance mutations. Evol. Appl. 8 (3), 273–283.
Mendes, R.E., Castanheira, M., Farrell, D.J., Flamm, R.K., Sader, H.S., Jones, R.N., 2016. Longitudinal (2001–14) analysis of enterococci and VRE causing invasive infections in European and US hospitals, including a contemporary (2010–13) analysis of oritavancin in vitro potency. J. Antimicrob. Chemother. 71 (12), 3453–3458.
Miller, W.R., Munita, J.M., Arias, C.A., 2014. Mechanisms of antibiotic resistance in enterococci. Expert Rev. Anti Infect. Ther. 12 (10), 1221–1236.
Narciso-da-Rocha, C., Varela, A.R., Schwartz, T., Nunes, O.C., Manaia, C.M., 2014. blaTEM and vanA as indicator genes of antibiotic resistance contamination in a hospital-urban wastewater treatment plant system. J. Glob. Antimicrob. Resist. 2 (4), 309–315.
Oravcova, V., Zurek, L., Townsend, A., Clark, A.B., Ellis, J.C., Cizek, A., Literak, I., 2014. American crows as carriers of vancomycin-resistant enterococci with vanA gene. Environ. Microbiol. 16 (4), 939–949.
Oravcova, V., Hadelova, D., Literak, I., 2016. Vancomycin-resistant Enterococcus faecium with vanA gene isolated for the first time from wildlife in Slovakia. Vet. Microbiol. 194, 43–47.
Oravcova, V., Svec, P., Literak, I., 2017. Vancomycin-resistant enterococci with vanA and vanB genes in Australian gulls. Environ. Microbiol. Rep. 9 (3), 316–318.
Palmer, K.L., Kos, V.N., Gilmore, M.S., 2010. Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. Curr. Opin. Microbiol. 13 (5), 632–639.
Pereira, F.C., Berry, D., 2017. Microbial nutrient niches in the gut. Environ. Microbiol. 19 (4), 1366–1378.
Ramadhan, A., Hegedus, E., 2005. Survivability of vancomycin resistant enterococci and fitness cost of vancomycin resistance acquisition. J. Clin. Pathol. 58 (7), 744–746.
Ramirez, M.S., Traglia, G.M., Lin, D.L., Tran, T., Tolmasky, M.E., 2014. Plasmid-Mediated antibiotic resistance and virulence in gram-negatives: the Klebsiella pneumoniae paradigm. Microbiol. Spectr. 2 (5), 1–15.
Roberts, M.C., Soge, O.O., Giardino, M.A., Mazengia, E., Ma, G., Meschke, J.S., 2009. Vancomycin-resistant Enterococcus spp. in marine environments from the west coast of the USA. J. Appl. Microbiol. 107 (1), 300–307.
Rosvoll, T.C.S., Pedersen, T., Sletvold, H., Johnsen, P.J., Sollid, J.E., Simonsen, G.S., Jensen, L.B., Nielsen, K.M., Sundsfjord, A., 2010. PCR-based plasmid typing in *Enterococcus faecium* strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTbeta-related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems. FEMS Immunol. Med. Microbiol. 58, 254–268.
Sader, H.S., Castanheira, M., Flamm, R.K., 2018. Distribution of main Gram-positive pathogens causing bloodstream infections in United States and European hospitals during the SENTRY Antimicrobial Surveillance Program (2010–2016): concomitant analysis of oritavancin in vitro activity AU - Mendes, Rodrigo E. J. Chemother. 30 (5), 280–289.
San Millan, A., Heilbron, K., MacLean, R.C., 2014. Positive epistasis between co-infecting plasmids promotes plasmid survival in bacterial populations. ISME J. 8 (3), 601.
San Millan, A., Santos-Lopez, A., Ortega-Huedo, R., Bernabe-Balas, C., Kennedy, S.P., Gonzalez-Zorn, B., 2015. Small-plasmid-Mediated antibiotic resistance is enhanced by increases in plasmid copy number and bacterial fitness. Antimicrob. Agents Chemother. 59 (6), 3335–3341.
Schrag, S.J., Perrot, V., Levin, B.R., 1997. Adaptation to the fitness costs of antibiotic resistance in Escherichia coli. Proc. R. Soc. Lond. B Biol. Sci. 264 (1386), 1287–1291.
Sengupta, M., Austin, S., 2011. Prevalence and significance of plasmid maintenance functions in the virulence plasmids of pathogenic bacteria. Infect. Immun. 79 (7), 2502–2509.
Shintani, M., Sanchez, Z.K., Kimbara, K., 2015. Genomics of microbial plasmids: classification and identification based on replication and transfer systems and host taxonomy. Front. Microbiol. 6.
Smalla, K., Jechalke, S., Top, E.M., 2015. Plasmid detection, characterization, and ecology. Microbiol. Spectr. 3 (1). PLAS-0038-2014.
Smith, M.A., Bidochka, M.J., 1998a. Bacterial fitness and plasmid loss: the importance of culture conditions and plasmid size. Can. J. Microbiol. 44 (4), 351–355.
Smith, M.A., Bidochka, M.J., 1998b. Bacterial fitness and plasmid loss: the importance of culture conditions and plasmid size. Can. J. Microbiol. 44 (4), 351–355.
Staley, C., Dunny, G.M., Sadowsky, M.J., 2014. Environmental and animal-associated enterococci. Adv. Appl. Microbiol. 87, 147–186.
Starikova, I., Al-Haroni, M., Werner, G., Roberts, A.P., Sorum, V., Nielsen, K.M., Johnsen, P.J., 2013. Fitness costs of various mobile genetic elements in Enterococcus faecium and Enterococcus faecalis. J. Antimicrob. Chemother. 68 (12), 2755–2765.
Stryjewski, M.E., Corey, G.R., 2014. Methicillin-resistant Staphylococcus aureus: an evolving pathogen. Clin. Infect. Dis. 58 (Suppl. l_1), S10–S19.
Süßmuth, S.D., Muscholl-Silberhorn, A., Wirth, R., Susa, M., Marre, R., Rozdzinski, E., 2000. Aggregation substance promotes adherence, phagocytosis, and intracellular survival of Enterococcus faecalis within human macrophages and suppresses respiratory burst. Infect. Immun. 68 (9), 4900–4906.
Trevors, J.T., Elsas, J.D.V., Starodub, M.E., Overbeek, L.S.V., 1989. Survival of and plasmid stability in Pseudomonas and Klebsiella spp. introduced into agricultural drainage water. Can. J. Microbiol. 35 (7), 675–680.
Vogwill, T., MacLean, R.C., 2015. The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. Evol. Appl. 8 (3), 284–295.
Wanjugi, P., Harwood, V.J., 2013. The influence of predation and competition on the survival of commensal and pathogenic fecal bacteria in aquatic habitats. Environ. Microbiol. 15 (2), 517–526.
Wanjugi, P., Fox, G.A., Harwood, V.J., 2016. The interplay between predation, competition, and nutrient levels influences the survival of Escherichia coli in aquatic environments. Microb. Ecol. 72 (3), 526–537.
Werner, G., Freitas, A.R., Coque, T.M., Sollid, J.E., Lester, C., Hammerum, A.M., Garcia-Migura, L., Jensen, L.B., Francia, M.V., Witte, W., Willems, R.J., Sundsfjord, A.,

2011. Host range of enterococcal vanA plasmids among Gram-positive intestinal bacteria. J. Antimicrob. Chemother. 66 (2), 273–282.
Young, S., Nayak, B., Sun, S., Badgley, B.D., Rohr, J.R., Harwood, V.J., 2016. Vancomycin-resistant enterococci and bacterial community structure following a sewage spill into an aquatic environment. Appl. Environ. Microbiol. 82 (18), 5653–5660.
Zhang, X., Guzman Prieto, A.M., de Maat, V., Prajsnar, T.K., Bayjanov, J.R., de Been, M., Rogers, M.R.C., Bonten, M.J.M., Mesnage, S., Willems, R.J.L., van Schaik, W., 2017. Fitness Determinants of Vancomycin-Resistant Enterococcus Faecium during Growth in Human Serum (bioRxiv).