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Novel linezolid resistance plasmids in *Enterococcus* from food animals in the USA

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Objectives: To sequence the genomes and determine the genetic mechanisms for linezolid resistance identified in three strains of *Enterococcus* isolated from cattle and swine caecal contents as part of the US National Antimicrobial Resistance Monitoring System (NARMS) surveillance programme.

Methods: Broth microdilution was used for *in vitro* antimicrobial susceptibility testing to assess linezolid resistance. Resistance mechanisms and plasmid types were identified from data generated by WGS on Illumina® and PacBio® platforms. Conjugation experiments were performed to determine whether identified mechanisms were transmissible.

Results: Linezolid resistance plasmids containing *optrA* were identified in two *Enterococcus faecalis* isolates and one *Enterococcus faecium*. The *E. faecium* isolate also carried the linezolid resistance gene *cfr* on the same plasmid as *optrA*. The linezolid resistance plasmids had various combinations of additional resistance genes conferring resistance to phenicols (*fexA*), aminoglycosides [*spc* and *aph(3′)-III*] and macrolides [*erm*(A) and *erm*(B)]. One of the plasmids was confirmed to be transmissible by conjugation, resulting in linezolid resistance in the transconjugant.

Conclusions: To the best of our knowledge, this is the first identification of linezolid resistance in the USA in bacteria isolated from food animals. The oxazolidinone class of antibiotics is not used in food animals in the USA, but the genes responsible for resistance were identified on plasmids with other resistance markers, indicating that there may be co-selection for these plasmids due to the use of different antimicrobials. The transmissibility of one of the plasmids demonstrated the potential for linezolid resistance to spread horizontally. Additional surveillance is necessary to determine whether similar plasmids are present in human strains of *Enterococcus*.

Introduction

Enterococci, in particular *Enterococcus faecium* and *Enterococcus faecalis*, are significant pathogens of immunocompromised and hospitalized patients.[1] Their resistance to several commonly used antimicrobial agents can make infections caused by enterococci difficult to treat, especially infections caused by VRE.[2] The advent of newer antimicrobials such as daptomycin and linezolid has provided more therapeutic options for VRE infections.[3] Linezolid, which was approved by the FDA in 2000, is used to treat infections caused by VRE and antimicrobial-resistant staphylococci and streptococci.[4] Linezolid is an oxazolidinone antibiotic, a class of drugs that inhibits the growth of bacteria by disrupting protein synthesis. This drug class is not approved for use in food animals in the USA.

To date, reports of linezolid resistance have been rare, with surveillance from US hospitals finding that only 0.2% of enterococci, staphylococci and streptococci were resistant.[5] Most resistance to linezolid is associated with mutations in 23S rRNA, which disrupts the binding of oxazolidinones and other protein synthesis inhibitors. Recent work has also identified two transmissible linezolid resistance mechanisms. The *cfr* gene product, which methylates 23S rRNA, confers resistance to linezolid, phenicols and clindamycin.[6] Findings of *cfr* in the USA have been sporadic, indicating a generally

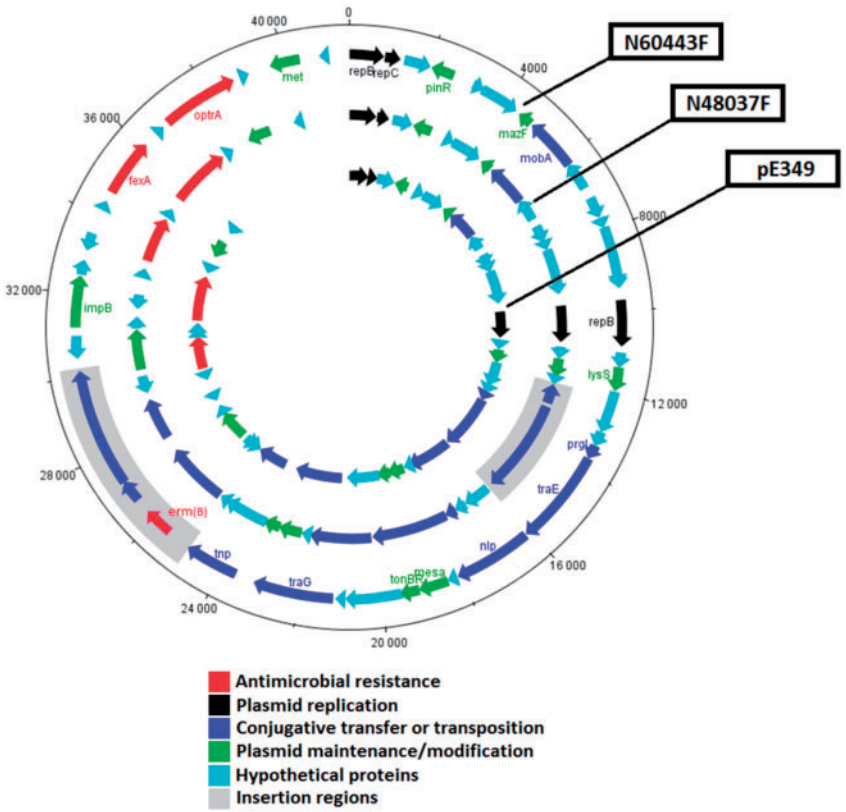

Figure 1. Comparison of *optrA*-containing plasmids from *E. faecalis* strains with a previously known plasmid (pE349). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

low prevalence of this resistance mechanism. In 2015, an additional linezolid resistance gene, *optrA*, was identified, encoding an ABC transporter that exports oxazolidinones, in addition to phenicols;[7] this gene has only previously been identified in a single human isolate of *Enterococcus* in the USA.[5] An additional gene, *poxtA*, was identified in *Staphylococcus aureus* in 2018.[8] These resistance genes have the potential for horizontal transfer of linezolid resistance among a largely susceptible population of enterococci and may also result in transmission to other linezolid-susceptible Gram-positive bacteria, including staphylococci and streptococci.

In this report, to the best of our knowledge, we describe the first identification of plasmid-mediated linezolid resistance in bacteria from food animal caecal contents in the USA. The National Antimicrobial Resistance Monitoring System (NARMS) partners will perform continued surveillance to determine whether these or similar plasmids are identified in *Enterococcus* and/or other Gram-positive organisms causing human infections.

Materials and methods

Enterococcus isolation

Bacteria were collected as part of nationwide surveillance of NARMS. The US Department of Agriculture's Food Safety and Inspection Service collected caecal contents of swine, poultry and cattle at slaughter facilities, isolating *Enterococcus* as previously described.[9]

Sequencing and analysis

Illumina MiSeq® technology was used to sequence *Enterococcus*, with v2 and v3 chemistry, as previously described.[10] Assembly was performed using CLC Genomics Workbench version 10.0 (QIAGEN; Hilden, Germany) using automated assembly parameters. Isolates were also sequenced on the Pacific Biosciences® (Menlo Park, CA, USA) RS II Sequencer as previously described.[11] The library was sequenced using two single-molecule real-time (SMRT) cells, analysing sequence reads using PacBio® SMRT Analysis 2.3.0 and performing *de novo* assembly with HGAP3.0. Isolate-level coverage information is listed in Table S1 (available as Supplementary data at *JAC* Online). The presence of resistance genes was analysed by performing BLAST analysis of the ResFinder database, with 90% sequence identity thresholds.[12] RAST was used for plasmid annotation.[13] Sequence accessions for each of the chromosomes and plasmids, along with coverage information, are listed in Table S1.

In vitro antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using broth microdilution, as previously described.[9] Non-susceptibility to linezolid (≥4 mg/L) was determined in accordance with standards of the CLSI.[14] Resistance to tylosin (≥32 mg/L) and kanamycin (≥1024 mg/L) was determined using NARMS breakpoints.

Conjugation

Conjugation assays were performed as previously described.[15] Briefly, equal amounts of donor and recipient strains were mixed and plated onto non-

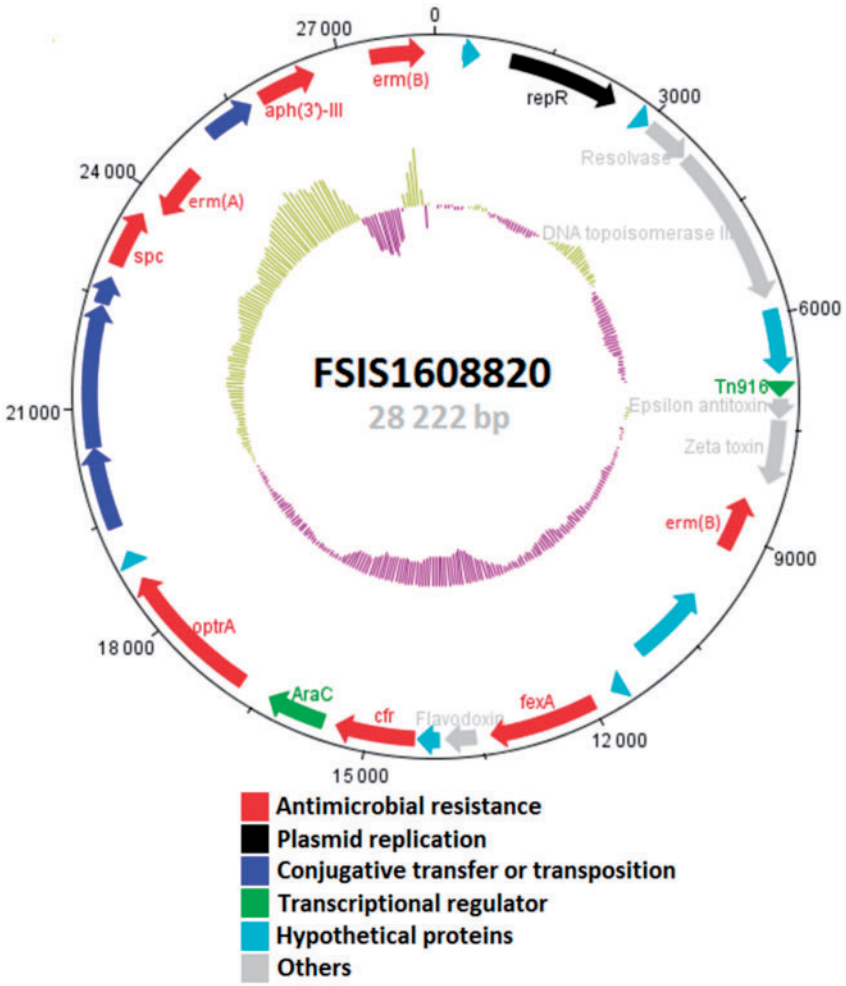

Figure 2. Depiction of the genetic structure of a novel *cfr/optrA*-containing plasmid. Lines in the interior circle represent the GC content along the plasmid relative to that of the overall plasmid, with tan lines being above average and purple lines being below. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

selective blood agar plates and incubated overnight. Isolates were subsequently plated onto selective LB plates containing 500 mg/L streptomycin and 8 mg/L florfenicol to select for transconjugants. Identification of the plasmid in the transconjugant was confirmed by WGS.

Results

As part of NARMS retail meat surveillance from 2002 to present, the presence and resistance of *Enterococcus* has been monitored in retail meat products. Consistent with the lack of use of oxazolidinones in food animals in the USA, linezolid resistance has never been detected among over 20 000 tested isolates.[9]

In 2013, NARMS began sampling caecal contents of food animals at slaughter for the presence of various bacteria, including *Enterococcus*, with over 5000 isolates having been collected from 2013 to 2016. From antimicrobial susceptibility testing of these isolates, three strains were identified as non-susceptible to linezolid. Two were from cattle caecal samples, including one each of *E. faecium* and *E. faecalis*, and one *E. faecalis* was from a swine caecal sample (Table S1).

To identify the mechanisms underlying the resistant phenotypes, we performed WGS. Using BLAST searches for known resistance genes and resistance-associated mutations, we found that all three isolates possessed the linezolid resistance gene *optrA*, with one also carrying *cfr* (Table S1). None of the isolates possessed resistance-associated mutations in their 23S rRNA-encoding genes. Long-read sequencing was then employed to obtain complete circular chromosomes and plasmids.

As expected, the linezolid resistance genes were present on plasmids in all three isolates. The *optrA*-containing plasmids of the *E. faecalis* isolates are not identical to any previously sequenced plasmids, but shared significant homology with the first *optrA* plasmid, identified in China.[7] As shown in Figure 1, the US plasmids each had ~4 kb insertions not found in the Chinese strain. The plasmid of N60443F *E. faecalis* carried not only *optrA*, but also the phenicol resistance gene *fexA* and the macrolide resistance gene

erm(B), with the latter localized within the 4 kb insertion. The N48037F *E. faecalis* plasmid was similar, with the IS located in a distinct region and lacking the *erm*(B) gene (Figure 1). N60443F had one additional plasmid that also had *erm*(B), which was also present on the chromosome (Table S1). Each of these three copies was located on the same transposable element Tn*551* that has been previously described in *Staphylococcus*.[16]

The *E. faecium* isolate that possessed both *cfr* and *optrA* had them both on the same plasmid; to the best of our knowledge, this is the first identification of such a plasmid in any strain of *Enterococcus* in the USA (Figure 2). These two linezolid resistance genes have only twice been reported on the same plasmid, in a *Staphylococcus sciuri* isolate from China[17] and an *E. faecium* isolate from Ireland.[18] This *E. faecium* plasmid differs from the previously reported plasmids in its structure and shares no more than 10 kb of homology with any sequence in GenBank, with PlasmidFinder[19] characterizing it as a *rep1*-type plasmid. Our *E. faecium* plasmid contained several additional resistance genes, including those conferring resistance to macrolides [*erm*(A) and two copies of *erm*(B)], aminoglycosides [*spc* and *aph(3′)-III*] and phenicols (*fexA*). These were reflected in phenotypic resistance to chloramphenicol, kanamycin, erythromycin and tylosin (Table S2).

To understand the transmissibility of resistance that may be associated with these plasmids, conjugations were performed with susceptible recipient strains of *Enterococcus*. Only one of the three conjugations was successful and it was unclear why the other two conjugations failed despite repeated attempts. In the case of the successful conjugation, the linezolid MIC increased from 1 mg/L in the original recipient strain to >8 mg/L in the transconjugant (Table S2).

Discussion

Linezolid resistance is rare in the USA and the identification of resistance plasmids in *Enterococcus* from food animal sources, where oxazolidinones are not used, is surprising. This study is also among the first to report *optrA* in the USA and, to the best of our knowledge, the first example of *optrA* and *cfr* co-located in the same plasmid in the USA. Both *optrA* and *cfr* also confer resistance to phenicols, including florfenicol, which is used in animal medicine. Furthermore, the presence of additional resistance genes on these plasmids implies that use of other antimicrobials, such as macrolides, may lead to increased linezolid resistance.

Although only three linezolid-resistant strains of *Enterococcus* were identified in this study, they were from different sources and were genetically diverse. This finding strongly suggests that linezolid resistance plasmids are not limited to a distinct lineage of *Enterococcus* and that resistant strains might independently emerge in different environments. How these plasmids reached bacteria in the food animal environment is unknown, but this finding is important since oxazolidinones are critically important antimicrobials in human medicine for the treatment of infections caused by various Gram-positive organisms. Furthermore, a study in China found that isolates from animal and human sources can have similar mobile elements associated with *optrA*.[20]

One potential concern with drug-resistant enterococci is the possibility for horizontal transfer of the resistance genes via plasmids to other pathogenic bacteria. Although the presence of linezolid-resistant strains in food animals may not directly increase the risk of limiting the efficacy of oxazolidinones in human therapy, people may become exposed to these bacteria through animal contact or contamination of foods. Now that we have identified linezolid resistance genes on plasmids that also carry other resistance genes, it remains to be seen whether these genes spread further through co-selection, clonal expansion or other mechanisms. NARMS will continue to perform surveillance of linezolid resistance among enterococci from animals and foods to identify any notable trends.

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Transparency declarations

None to declare.

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Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online.

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