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A novel gene, *optrA*, that confers transferable resistance to oxazolidinones and phenicols and its presence in *Enterococcus faecalis* and *Enterococcus faecium* of human and animal origin

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Objectives: The oxazolidinone-resistant *Enterococcus faecalis* E349 from a human patient tested negative for the *cfr* gene and 23S rRNA mutations. Here we report the identification of a novel oxazolidinone resistance gene, *optrA*, and a first investigation of the extent to which this gene was present in *E. faecalis* and *Enterococcus faecium* from humans and food-producing animals.

Methods: The resistance gene *optrA* was identified by whole-plasmid sequencing and subsequent cloning and expression in a susceptible *Enterococcus* host. Transformation and conjugation assays served to investigate the transferability of *optrA*. All *optrA*-positive *E. faecalis* and *E. faecium* isolates of human and animal origin were analysed for their MICs and their genotype, as well as the location of *optrA*.

Results: The novel plasmid-borne ABC transporter gene *optrA* from *E. faecalis* E349 conferred combined resistance or elevated MICs (when no clinical breakpoints were available) to oxazolidinones (linezolid and tedizolid) and phenicols (chloramphenicol and florfenicol). The corresponding conjugative plasmid pE349, on which *optrA* was located, had a size of 36 331 bp and also carried the phenicol exporter gene *fexA*. The *optrA* gene was functionally expressed in *E. faecalis*, *E. faecium* and *Staphylococcus aureus*. It was detected more frequently in *E. faecalis* and *E. faecium* from food-producing animals (20.3% and 5.7%, respectively) than from humans (4.2% and 0.6%, respectively).

Conclusions: Enterococci with elevated MICs of linezolid and tedizolid should be tested not only for 23S rRNA mutations and the gene *cfr*, but also for the novel resistance gene *optrA*.

Keywords: linezolid, tedizolid, florfenicol, plasmids, interspecies transfer, enterococci

Introduction

Enterococcal infections have become one of the most challenging nosocomial problems, with two species, *Enterococcus faecium* and *Enterococcus faecalis*, ranking among the leading causes of hospital-acquired infections.[1,2] The ability of enterococci to acquire and exchange plasmids and transposons that carry antimicrobial resistance and virulence genes has contributed to their role as multiresistant pathogens.[3] The emergence and rapid spread of VRE represents a particular challenge, as there are few remaining options for antimicrobial treatment of infections caused by VRE.[4,5] Linezolid, the first oxazolidinone introduced into clinical use in the USA in 2000 and in China in 2007, is a most promising agent against infections caused by VRE, MRSA and penicillin-resistant pneumococci.[6]

However, oxazolidinone resistance among enterococci has been reported during recent years. Mutations in the central loop of domain V of the 23S rRNA represented the most common mechanism of oxazolidinone resistance in enterococci, with G2576T (*Escherichia coli* numbering) as the predominantly found

mutation.[5] In general, oxazolidinone resistance in enterococci conferred by mutations has commonly been associated with prolonged courses of linezolid administration,[7] and sporadic cases of nosocomial transmission of oxazolidinone-resistant enterococci between patients have been documented.[8,9] Transferable oxazolidinone resistance due to the multiresistance gene *cfr* has been reported in enterococci of both human and animal origin.[10] The gene *cfr* encodes an rRNA methyltransferase that modifies the adenine residue at position 2503 in domain V of the 23S rRNA and thereby confers resistance to oxazolidinones, phenicols, lincosamides, pleuromutilins and streptogramin A ($PhLOPS_A$) antibiotics.[11] A novel oxazolidinone, tedizolid, was approved in June 2014 by the FDA for the management of acute bacterial skin and skin structure infections in adult humans. Tedizolid has increased activity against linezolid-resistant isolates that harbour the *cfr* gene, possibly due to the structural differences between these two oxazolidinones in the C5 substituents of the A ring.[12]

In this study we report a novel gene, designated *optrA*, which confers transferable resistance to oxazolidinones (linezolid and tedizolid) and phenicols (chloramphenicol and florfenicol) in *E. faecalis* and *E. faecium* isolates from humans and food-producing animals.

Materials and methods

Bacterial isolates

Initially, three oxazolidinone-resistant *E. faecalis* isolates, E349, E363 and E399, of human origin were collected in 2009 from Tianjin Medical University General Hospital, which participated in the Ministry of Health National Antimicrobial Resistance Investigation Net programme in China. To screen a larger collection of *Enterococcus* isolates for the presence of the novel oxazolidinone resistance gene, 595 clinical non-duplicate *Enterococcus* spp. isolates (*E. faecalis* $n=236$; *E. faecium* $n=359$) were collected from the Second Affiliated Hospital of Zhejiang University (SAHZU) during 1998–2014 in Hangzhou, China (Table S1, available as Supplementary data at *JAC* Online). In addition, 290 non-duplicate *Enterococcus* spp. isolates (*E. faecalis* $n=202$; *E. faecium* $n=88$) of animal origin, including 149 isolates from porcine faecal samples and 141 isolates from cloacal swabs of chickens, were collected during 2009–13 from farms and abattoirs in five provinces/cities, including Shandong, Henan, Tibet, Guangdong and Shanghai (Table S2). The vancomycin-resistant *E. faecium* A4 was selected from the SAHZU collection for mating assays.

Bacterial species identification and molecular typing

Species identification of enterococci of human and animal origin was conducted by 16S rDNA sequencing and MALDI-TOF MS (BrukerDaltonik GmbH, Bremen, Germany). PCR-directed detection of the *cfr* gene and 23S rRNA mutations was performed following previously described procedures.[13,14] Isolates exhibiting oxazolidinone resistance, but negative for *cfr* and 23S rRNA mutations, were genotyped by PFGE and MLST, as described previously.[13] Since the clinical *E. faecalis* isolates E349, E363 and E399 exhibited an indistinguishable PFGE pattern (Figure S1), only isolate E349 was selected for further analysis.

Plasmid analysis, transfer and cloning experiments

The plasmids of *E. faecalis* E349 were extracted using the Qiagen Plasmid Midi Kit (Qiagen, Germany) and transferred into *E. faecalis* JH2-2 via electrotransformation. The transferability of resistance to linezolid was also assessed by filter mating using *E. faecalis* E349 as donor and *E. faecalis* FA2-2 and the vancomycin-resistant *E. faecium* A4 as recipients. Both the transformants and transconjugants were selected on brain heart infusion (BHI) plates containing 3 mg/L linezolid and 25 mg/L rifampicin. The approximate size of the transferred plasmid was estimated by S1 nuclease PFGE.[13] The conjugation frequency was determined as described previously.[15]

The plasmid of the transformants was extracted and then subjected to whole-plasmid sequencing by constructing a shotgun library using Illumina Hiseq 2500, which produced 100 bp paired-end reads (Berry Genomics Company, Beijing, China). A draft assembly of the plasmid was conducted using CLC Genomics Workbench 5 (CLC Bio, Aarhus, Denmark), which produced six contigs that had average coverage of >1000-fold. Gap closure of the plasmid was done by PCR using a modified random primer walking strategy.[13] Sequence analysis was conducted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and the BLAST functions (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

To confirm the role of the gene in question in oxazolidinone resistance, a 2582 bp DNA segment was amplified using primers opR-F (5′-GCTCTAGATTTCTCACCCAGATATGCC-3′) and opR-R (5′-CGGGATCCCGGCAAACTCAAAAGGTC-3′). This segment included the putative oxazolidinone resistance gene, as well as 491 bp of its upstream region and 123 bp of its downstream region. The amplicon was inserted into the shuttle plasmid pAM401 and then transferred into *E. faecalis* JH2-2 and *Staphylococcus aureus* RN4220 by electrotransformation. Transformants were selected on BHI plates containing 2 mg/L linezolid.

Antimicrobial susceptibility testing

The MICs for all original isolates, transconjugants and transformants were determined by broth microdilution following the recommendations given in CLSI documents VET01-S2 and M100-S25.[16,17] *E. faecalis* ATCC 29212 served as a quality control strain. An internal PCR was designed to detect the *optrA* gene among *Enterococcus* isolates from humans and animals using the primers A-F (5′-AGGTGGTCAGCGAACTAA-3′) and A-R (5′-ATCAACTGTTCCCATTCA-3′). The size of the expected PCR product was 1395 bp. All isolates carrying the gene in question were investigated for their MICs of chloramphenicol, florfenicol, linezolid, tedizolid, vancomycin, gentamicin, ampicillin and daptomycin, as well as for their PFGE patterns and their MLST types. S1-PFGE and Southern blotting were conducted to confirm the location of the novel resistance gene in each of these isolates.[13] In addition, these isolates were analysed by PCR for the presence of the phenicol exporter genes *fexA* and *fexB* as described previously.[13]

Results

Characterization of the cfr- and 23S rRNA mutation-negative E. faecalis E349

Neither the *cfr* gene nor mutations in 23S rRNA were observed in *E. faecalis* E349. Both the transformant (designated JH2-2/pE349) and the transconjugant (designated FA2-2-E349) exhibited 4- to 8-fold increases in the MICs of linezolid and tedizolid, compared with recipient strains JH2-2 and FA2-2 (Table 1). S1-PFGE revealed the presence of a plasmid of ~36 kb, designated pE349, in both the original E349 and the transformant *E. faecalis* JH2-2/pE349 (Figure 1a). Oxazolidinone-resistant transconjugants were obtained with a frequency of 2.21×10^{-6} per donor cell, suggesting that pE349 is a conjugative plasmid. These results strongly suggested the existence of a novel plasmid-borne oxazolidinone resistance gene in *E. faecalis* E349.

Characterization of the plasmid pE349 and identification of the gene optrA

To identify this gene, plasmid pE349 obtained from *E. faecalis* JH2-2/pE349 was sequenced completely. It had a size of

Table 1. MICs for *E. faecalis* E349, *E. faecium* A4, their transformants and transconjugants, and the recipient strains

Bacterial isolate	MIC (mg/L)				
	CHL	FFC	LZD	TZD	VAN
Clinical *E. faecalis* E349 (with *optrA*-carrying pE349)	64	64	8	2	1
E. faecalis FA2-2	4	2	2	0.5	1
Transconjugant *E. faecalis* FA2-2-E349	32	64	8	2	1
E. faecalis JH2-2	4	4	2	0.5	1
Transformant *E. faecalis* JH2-2/pE349	64	64	16	2	1
Transformant *E. faecalis* JH2-2/pAM401	≥128[a]	4	2	0.5	1
Transformant *E. faecalis* JH2-2/pAM401+*optrA*	≥128[a]	64	16	2	1
S. aureus RN4220	8	4	2	0.25	1
Transformant *S. aureus* RN4220/pAM401	≥128[a]	4	2	0.25	1
Transformant *S. aureus* RN4220/pAM401+*optrA*	≥128[a]	64	8	1	1
Clinical *E. faecium* A4 (with *vanA*-carrying pA4)	4	2	2	0.25	>128
Transconjugant *E. faecium* A4-E349	32	128	8	2	>128
Transconjugant *E. faecalis* E349-A4	64	64	8	2	128

CHL, chloramphenicol; FFC, florfenicol; LZD, linezolid; TZD, tedizolid; VAN, vancomycin.
[a]The shuttle vector pAM401 carries a pIP501-analogous *cat* gene that confers high-level resistance to chloramphenicol, but not to florfenicol.

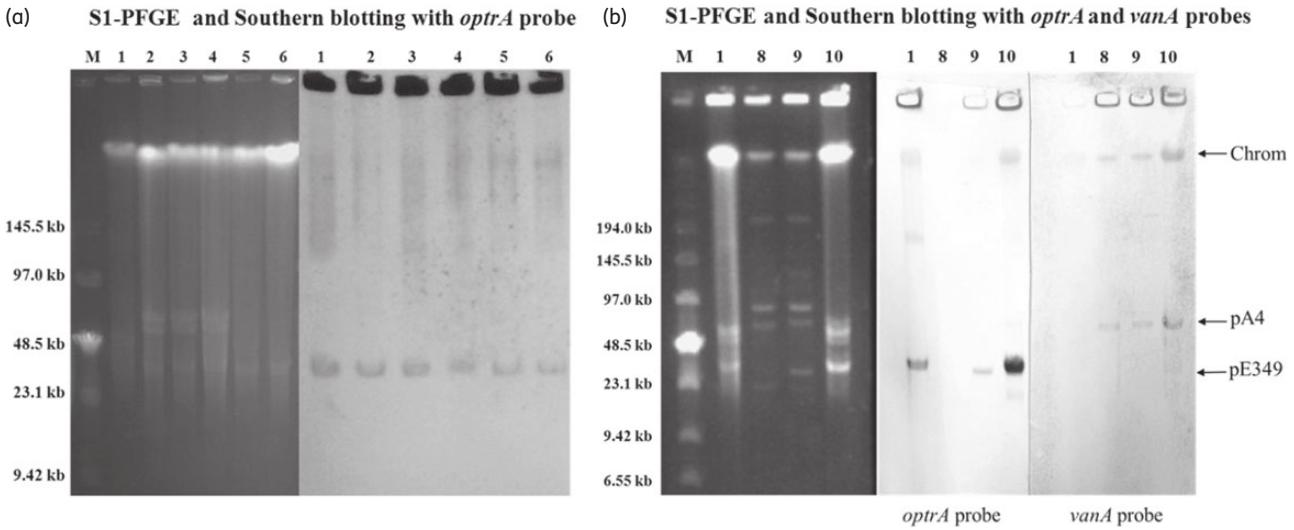

Figure 1. Results of S1-PFGE and Southern blotting. (a) Location of the *optrA*-carrying plasmid pE349 in *Enterococcus* spp. by S1-PFGE (left panel) and Southern blot hybridization (right panel). Lane M, low-range pulsed-field gel marker (New England BioLabs, Beverly, MA, USA); lane 1, *E. faecalis* En6; lane 2, *E. faecalis* E399; lane 3, *E. faecalis* E363; lane 4, *E. faecalis* E349; lanes 5 and 6, transformants 1 and 2 of JH2-2/pE349. (b) Location of the *optrA*-carrying plasmid pE349 and the *vanA*-carrying plasmid pA4 in *Enterococcus* spp. by S1-PFGE (left panel) and Southern blot hybridization (middle and right panels). Lane 1, *E. faecalis* E349; lane 8, *E. faecium* A4; lane 9, transconjugant *E. faecium* A4-E349; lane 10, transconjugant *E. faecalis* E349-A4.

36 331 bp (GenBank accession number KP399637). A total of 39 ORFs coding for proteins of >50 amino acids were identified (Figure 2 and Table S3). When searching for the potential oxazolidinone resistance gene in pE349, a 1968 bp gene encoding a 655 amino acid protein that exhibited 99.4% (651/655 amino acids) identity to a putative ABC transporter from *E. faecalis* 599 (GenBank accession number EJU90935.1) and *E. faecium* C1904 (GenBank accession number EJY24646.1) was detected, both of which represent reference genomes for the Human Microbiome Project. *E. faecalis* JH2-2, carrying plasmid pAM401 with the cloned ABC transporter gene, exhibited 16-, 8- and 4-fold increases in the MICs of florfenicol, linezolid and tedizolid, respectively, when compared with the MICs of *E. faecalis* JH2-2 carrying only the shuttle vector pAM401 (Table 1), while no MIC changes were found for ciprofloxacin, erythromycin, tetracycline and amikacin (data not shown). In addition, *S. aureus* RN4220 carrying pAM401 with the cloned ABC transporter gene exhibited 16-fold, 4-fold and 4-fold increases in the MICs of florfenicol, linezolid and

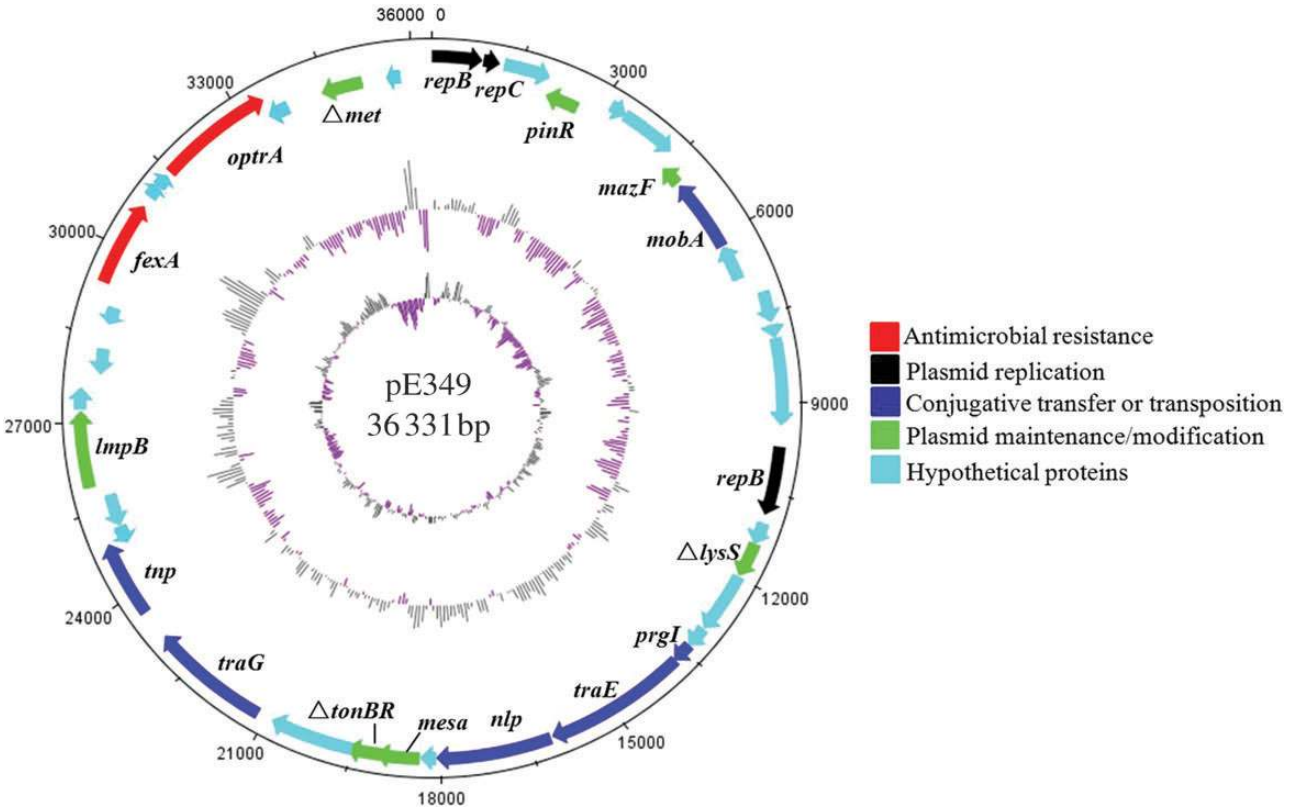

Figure 2. Annotation of plasmid pE349. The circles display (from the outside to inside): (i) size in bp; (ii) positions of predicted coding sequences transcribed in the clockwise orientation; (iii) position of predicted coding sequences transcribed in the counterclockwise orientation; (iv) GC content plotted against 50%, with light grey indicating >50% and pink/purple indicating <50%; and (v) GC skew [(G+C)/(G−C)] in a 300 bp window.

tedizolid, respectively, when compared with the MICs of *S. aureus* RN4220 carrying only pAM401 (Table 1), while no MIC changes were found for clindamycin, valnemulin, virginiamycin M1 (data not shown) and chloramphenicol. The high chloramphenicol MICs of ≥128 mg/L (Table 1), which did not change in the presence of *optrA*, were due to the presence of a pIP501-analogous *cat* gene that confers high-level resistance to chloramphenicol, but not to florfenicol. These results strongly suggested that the ABC transporter gene mediated combined resistance to oxazolidinones and phenicols in *E. faecalis* and *S. aureus*. Therefore, it was designated *optrA* (oxazolidinone phenicol transferable resistance). Detailed analysis of this ABC transporter revealed two ATP-binding domains, each containing the ABC family characteristic motifs, including the conserved ABC signature and the Walker A and Walker B motifs, but lacked transmembrane domains (Figure S2).[18] A phylogenetic tree based on a multi-sequence alignment (http://www.pasteur.fr/recherche/unites/pmtg/abc/advanced.html) indicated that OptrA clustered together with the proteins Vga(A), Vga(C), Vga(E) and Lsa(E), which confer resistance to lincosamides, pleuromutilins and streptogramin A antibiotics (Figure 3).

Among the 39 ORFs identified in plasmid pE349, 21 encoded hypothetical proteins with no defined function, while the products of the remaining 18 exhibited divergent similarities (23%–99%) to proteins with known functions, including antimicrobial resistance (*optrA* and *fexA*), plasmid replication (*repB* and *repC*), plasmid maintenance (*mazF* and *lysS*) and conjugative transfer (*mobA*, *prgI*, *traE*, *tnp* and *traG*) (Table S3). The average GC content of plasmid pE394 (34.4%) was in the range of that of the *E. faecalis* genomes (33.3%–37.5%) available in the GenBank database.

Transferability of pE349 into vancomycin-resistant E. faecium A4

Conjugation was not only successful using *E. faecalis* E349 (with pE349) as donor and the clinical vancomycin-resistant *E. faecium* A4 with the *vanA*-carrying plasmid pA4 as recipient, but also with *E. faecium* A4 as donor and *E. faecalis* E349 as recipient. Two transconjugants, designated *E. faecium* A4-E349 and *E. faecalis* E349-A4, respectively, were obtained. S1-PFGE and Southern blot analysis using probes for *optrA* and *vanA* revealed that the ~60 kb *vanA*-carrying plasmid pA4 and the 36 kb *optrA*-carrying plasmid pE349 can co-exist (Figure 1b) and that resistance to oxazolidinones, phenicols and vancomycin is expressed in both transconjugants (Table 1).

Screening for the presence of optrA among E. faecalis and E. faecium of human and animal origin

Among the additional 885 *Enterococcus* spp. isolates (*E. faecalis* *n*=438; *E. faecium* *n*=447) examined in this study, 58 (*E. faecalis* *n*=51; *E. faecium* *n*=7) were positive for the *optrA* gene, including 12 (12/595, 2.0%) from humans and 46 (46/290, 15.9%) from animals (pigs *n*=37; chickens *n*=9). Among the 12 human

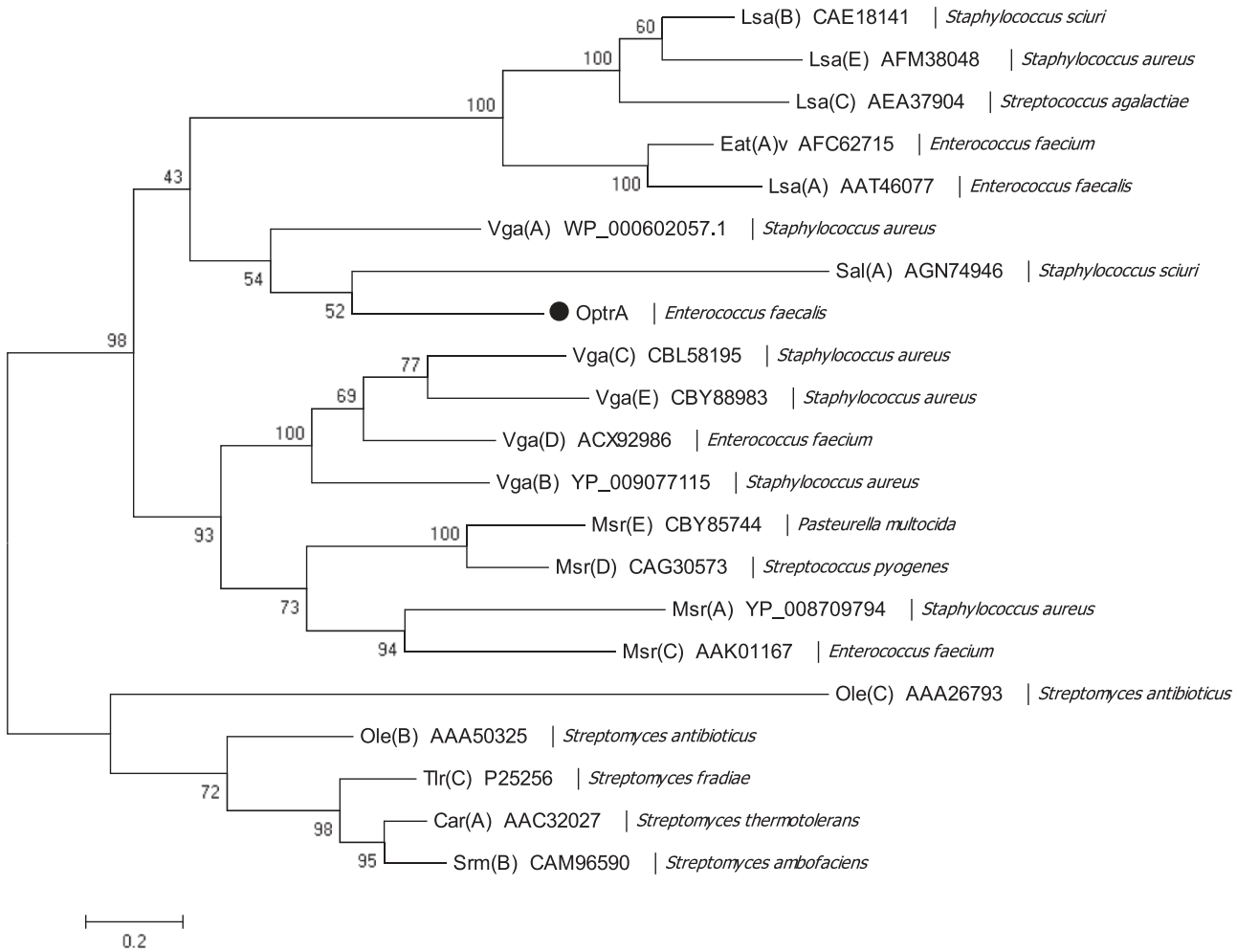

Figure 3. Phylogenetic analysis of OptrA and other ABC transporters involved in antimicrobial resistance. The phylogenetic tree was generated using the maximum likelihood method. The GenBank accession numbers of proteins are indicated. Numbers next to the branching points indicate the relative support from 500 replications. Analysis was performed using the MEGA 6.0.6 software package (Tempe, AZ, USA).

Enterococcus spp., 10 were *E. faecalis* and 2 were *E. faecium* (Table S1). The 46 *optrA*-positive *Enterococcus* isolates from animals included 41 *E. faecalis* and 5 *E. faecium* (Table S2). The gene *optrA* was detected more frequently in isolates from pigs (37/149, 24.8%, *E. faecalis* $n=33$ and *E. faecium* $n=4$) than in those from chickens (9/141, 6.4%, *E. faecalis* $n=8$ and *E. faecium* $n=1$). All 58 *optrA*-positive isolates from the screening study and the three initial *E. faecalis* isolates of human origin from Tianjin were subjected to PFGE analysis. Using a cut-off at 80% pattern similarity, the 54 *E. faecalis* and 7 *E. faecium* clustered into 28 and 7 PFGE subtypes, respectively (Figures S1 and S3). Isolates of the same source that exhibited indistinguishable PFGE patterns were considered as duplicate isolates. All 15 human isolates and 25 non-duplicate isolates of animal origin (in total 33 *E. faecalis* and 7 *E. faecium*) were further analysed by MLST, localization of the *optrA* gene, detection of phenicol exporter genes and antimicrobial susceptibility testing (Table 2). The *optrA* gene was located on plasmids in 16 *E. faecalis* isolates, in the chromosomal DNA of 17 isolates (*E. faecalis* $n=14$; *E. faecium* $n=3$) or on both a plasmid and chromosomal DNA in 7 isolates (*E. faecalis* $n=3$; *E. faecium* $n=4$; Figure S4). Six *E. faecalis* of animal origin belonged to six different STs (ST27, ST116, ST256, ST476, ST480 and ST593), all of which were shared by 10 human isolates (Table 2 and Figure 4). Moreover, the human *E. faecalis* isolate E347 from Ningbo and the porcine isolate 23-1 from Guangdong were in the same PFGE cluster (subtypes R1 and R2) and shared the same MLST profile ST593 (Table 2 and Figure 4). A similar observation was made for the human *E. faecalis* isolate E381 from Taizhou and the porcine isolate XY12 from Henan, which also clustered together by PFGE (Y1 and Y2) and MLST (ST256) (Table 2 and Figure 4).

Discussion

So far, the gene *cfr* has been the only transferable oxazolidinone resistance gene in enterococci.[10] The genes *cfr* and *optrA* differ in their mechanisms of resistance and in their substrate spectrum. While *cfr* codes for a 23S rRNA methyltransferase that confers resistance to $PhLOPS_A$ antibiotics,[11] *optrA* codes for an ABC transporter that confers resistance to oxazolidinones and phenicols. Besides these differences, the two genes are often located on plasmids, which might also harbour additional resistance

Table 2. Characteristics of all 15 human and 25 non-duplicate animal *E. faecalis* and *E. faecium* isolates that carry *optrA*

Isolate	Host	Location (city/province)	Sample	Year	Location of *optrA* (kb)[a]	Other CHL/FFC resistance genes	PFGE pattern	MLST	Clonal complex[b]	MIC (mg/L)							
										CHL	FFC	LZD	TZD	VAN	GEN	AMP	DAP
E. faecalis																	
E349	human	Tianjin	blood	2009	pE349 (36.3)	*fexA*	Z	ST116	CC116	64	128	8	2	2	1000	1	0.5
E363	human	Tianjin	vaginal secretion	2009	pE349 (36.3)	*fexA*	Z	ST116	CC116	64	64	8	2	2	1000	0.5	0.5
E399	human	Tianjin	urine	2009	pE349 (36.3)	*fexA*	Z	ST116	CC116	64	128	8	2	2	1000	1	1
E147	human	Hangzhou/Zhejiang	bile	2011	C	*fexA*	H	ST476	none	>256	64	4	1	2	1000	2	1
E121	human	Shangrao/Jiangxi	urine	2012	P (~80)	*fexA*	F1	ST480	none	>256	128	8	1	2	<500	2	1
E016	human	Hangzhou/Zhejiang	faeces	2013	C	*fexA*	M	ST16	CC16	>256	32	4	1	2	<500	1	1
E079	human	Jinhua/Zhejiang	faeces	2013	C	—	W	ST27	none	128	64	8	1	1	<500	2	0.5
E071	human	Hangzhou/Zhejiang	urine	2013	P (~60)	*fexA*	F2	ST480	none	>256	128	8	2	2	<500	2	1
E347	human	Ningbo/Zhejiang	faeces	2014	C	*fexA*	R2	ST593	none	>256	32	4	1	2	<500	1	1
E419	human	Hangzhou/Zhejiang	faeces	2014	P (~80)	*fexA*	X	ST480	none	>256	128	8	2	1	<500	0.5	1
E161	human	Hangzhou/Zhejiang	urine	2014	P (~60)	*fexA*	AC	ST585	CC4	>256	64	8	1	2	<500	2	1
E383	human	Hangzhou/Zhejiang	faeces	2014	C	*fexA*	V	ST416	CC403	>256	32	4	1	2	<500	2	1
E381	human	Taizhou/Zhejiang	faeces	2014	C	*fexA*	Y2	ST256	CC557	>256	64	8	1	2	>1500	2	1
G22	Tibetan pig	Tibet	faeces	2012	P (~55)	*fexA*	A2	ST116	CC116	128	128	8	2	2	<500	1	1
G20	Tibetan pig	Tibet	faeces	2012	C	*fexA*	B	ST93	CC93	32	32	2	1	2	<500	1	1
G24	Tibetan pig	Tibet	faeces	2012	C	*fexA*	K	ST618	CC59	128	64	4	1	1	<500	1	1
5-7	Tibetan pig	Tibet	faeces	2012	C	*fexA*	C	ST619	CC81	64	64	8	2	1	<500	1	1
XY5	pig	Henan	faeces	2012	C	*fexA*	G1	ST476	none	64	32	2	0.5	0.5	<500	<0.5	1
XY12	pig	Henan	faeces	2012	P (~60)	*fexB*	Y1	ST256	CC557	128	128	4	1	1	1000	1	1
XY16	pig	Henan	faeces	2012	C, P (~33)	*fexA*	I	ST475	none	64	256	4	1	1	<500	1	1
XY17	pig	Henan	faeces	2012	P (~30)	*fexA*	E	ST27	none	128	128	8	1	0.5	<500	1	1
XY18	pig	Henan	faeces	2012	C	*fexA*	L	ST480	none	32	64	8	2	1	1000	1	1
XY21	pig	Henan	faeces	2012	C	—	U	ST623	CC21	128	16	2	0.5	2	<500	2	1
XY29	pig	Henan	faeces	2012	P (~50)	—	N	ST74	CC25	16	16	2	0.5	1	<500	1	1
En6	pig	Henan	faeces	2012	pE349 (36.3)	*fexA*	T	ST21	CC21	128	128	8	2	1	<500	2	1
SF35	chicken	Henan	faeces	2013	P (~65)	*fexA*	J	ST330	none	128	256	8	2	0.5	<500	1	1
SF53	chicken	Henan	faeces	2013	C, P (~55)	*fexA*	O	ST621	none	128	128	8	1	0.5	<500	<0.5	1
LY4	chicken	Shandong	faeces	2009	C	—	S	ST21	CC21	128	16	2	0.5	1	<500	2	1
10–120	pig	Guangdong	faeces	2012	C	*fexA*, *fexB*	Q1	ST403	CC403	128	128	8	2	1	<500	1	1
10–2-2	pig	Guangdong	faeces	2012	P (~60)	*fexA*	D	ST59	CC59	128	128	8	1	1	<500	1	1
85	pig	Guangdong	faeces	2012	P (~65)	*fexB*	AB	ST620	CC59	128	128	4	0.5	1	1000	1	1
23-1	pig	Guangdong	faeces	2012	C, P (~60)	*fexA*	R1	ST593	none	128	128	8	1	0.5	<500	2	1
FX13	pig	Shanghai	faeces	2013	P (~34)	*fexA*	P	ST622	CC26	32	64	4	1	1	<500	1	0.5
E. faecium																	
E019	human	Hangzhou, Zhejiang	urine	2005	C	*fexA*	AI	ST956	CC17	>256	128	8	1	0.5	<500	8	2
E388	human	Hangzhou, Zhejiang	faeces	2014	C	*fexB*	AJ	ST29	CC17	>256	64	4	1	2	>1500	256	2
PF02	chicken	Shandong	faeces	2009	C, P (~250)	—	AH	ST32	CC17	16	32	4	0.5	<0.25	<500	1	2
53	pig	Guangdong	faeces	2012	C	*fexB*	AD	ST184	CC17	128	128	8	1	2	<500	8	2

Continued

Table 2. *Continued*

Isolate	Host	Location (city/province)	Sample	Year	Location of *optrA* (kb)[a]	Other CHL/FFC resistance genes	PFGE pattern	MLST	Clonal complex[b]	MIC (mg/L)							
										CHL	FFC	LZD	TZD	VAN	GEN	AMP	DAP
54	pig	Guangdong	faeces	2012	C, P (~250)	*fexB*	AE	ST957	none	64	128	8	1	<0.25	<500	1	2
63	pig	Guangdong	faeces	2012	C, P (~250)	*fexB*	AF	ST957	none	64	128	8	2	0.5	<500	0.5	2
73	pig	Guangdong	faeces	2012	C, P (~180)	*fexB*	AG	ST29	CC17	32	64	8	1	1	<500	8	2

CHL, chloramphenicol; FFC, florfenicol; LZD, linezolid; TZD, tedizolid; VAN, vancomycin; GEN, gentamicin; AMP, ampicillin; DAP, daptomycin.
[a]P, plasmid; C, chromosomal DNA.
[b]None means that the ST could not be attributed to one of the recognized clonal complexes.

genes.[10] This fact is of particular relevance when considering the dissemination of *optrA*-carrying plasmids under the selective pressure imposed by the use of antimicrobial agents other than oxazolidinones and phenicols. Moreover, both genes proved to be functionally active not only in *Enterococcus* spp., but also in *S. aureus*. The observation that *in vitro* transfer experiments resulted in transformants and transconjugants, in which the *optrA*-carrying plasmid was stably maintained and in which the *optrA* gene was functionally expressed, point to the likelihood of *in vivo* transfer of *optrA*-carrying plasmids between different enterococcal species, but also between enterococci and other Gram-positive bacteria, such as staphylococci. Examples of resistance gene flux between enterococci and staphylococci are the Tn*916*-borne tetracycline resistance gene *tet*(M),[19] the Tn*917*/Tn*551*-borne macrolide–lincosamide–streptogramin B resistance gene *erm*(B),[20] the Tn*1546*-borne *vanA* gene cluster for glycopeptide resistance[21] and, most recently, the transfer of a multiresistance gene cluster carrying the resistance genes *aadE* (streptomycin resistance), *spw* (spectinomycin resistance), *lsa*(E) (resistance to pleuromutilins, lincosamides and streptogramin A) and *lnu*(B) (lincosamide resistance).[22–25] The OptrA protein exhibited >99% identity to putative ABC transporters from enterococci of human origin. However, it is not known whether the enterococci that carry these *optrA*-related genes are resistant to oxazolidinones and phenicols. If not, the few amino acid substitutions observed between OptrA and the aforementioned ABC transporters might account for a different substrate specificity.

One important question refers to the potential role of food-producing animals in the epidemiology of human *E. faecalis* and *E. faecium* carrying the *optrA* gene. Although the data presented in this study do not provide direct evidence for the transmission of *optrA*-positive isolates between animals and humans, the finding of *E. faecalis* isolates from humans and pigs with similar PFGE patterns and the same MLST profile points to an exchange of such isolates. However, the direction of exchange remains to be clarified. For *E. faecium*, the majority of the *optrA*-positive isolates from humans, pigs and chickens belonged to CC17, a distinct high-risk clonal complex, which was found to be associated with the majority of hospital outbreaks and clinical infections in five continents.[26] A recent review suggested that *E. faecium* isolates of animal origin might not constitute a human health hazard, but could act as donors of antimicrobial resistance genes for other pathogenic enterococci.[27] In contrast, *E. faecalis* of animal origin seemed to be a human health hazard, as the same types of *E. faecalis* could be detected in samples from animals, meat, faecal samples from humans in the community and patients with bloodstream infections.[27] The observation that the *optrA*-carrying plasmid pE349 could easily be transferred between *E. faecalis* and *E. faecium* via conjugation and that it could stably replicate and express its resistance genes in the presence of a plasmid that carried the *vanA* gene cluster is alarming. Such transfer events—when occurring *in vivo*—might seriously limit the therapeutic options for infections caused by VRE.

The first *optrA*-carrying *E. faecium* isolate of human origin was detected in 2005. This happened 2 years before linezolid, the sole commercially available oxazolidinone in China, was approved for use in human medicine in 2007. Moreover, all 15 patients from whom *optrA*-positive *E. faecalis* or *E. faecium* was isolated did not receive linezolid or chloramphenicol, both of which could directly select for the *optrA* gene. Linezolid has not been approved for

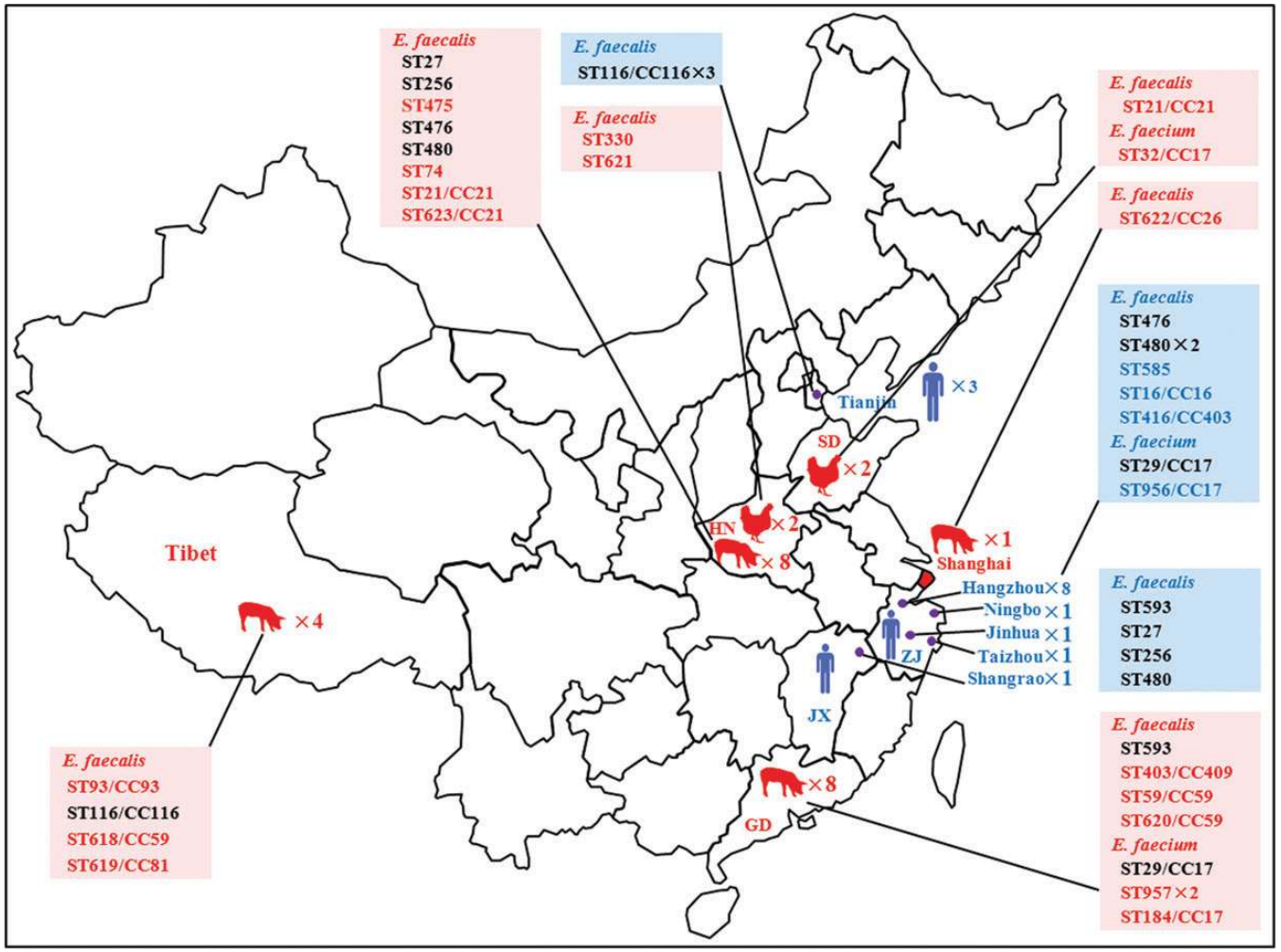

Figure 4. Geographical distribution of 40 *optrA*-carrying *E. faecium* and *E. faecalis* isolates in China, including all 15 isolates of human origin and 25 non-duplicate isolates of animal origin. The symbols for animals and humans are coloured red and blue, respectively. The STs shared by isolates from humans and animals are presented in black. Provinces are abbreviated as follows: GD, Guangdong; HN, Henan; JX, Jiangxi; SD, Shandong; and ZJ, Zhejiang.

veterinary applications worldwide, while florfenicol—approved in 1999—is widely used in food-producing animals. Since the banning of chloramphenicol from use in food-producing animals in China in 2002, florfenicol appears to be the only antimicrobial agent that directly selects for *optrA* in isolates of food animal origin. The observation that the *optrA* gene was more frequently detected in enterococci of animal origin (15.9%) than in enterococci of human origin (2.0%) might suggest an animal reservoir. However, another scenario could be that the *optrA* gene might have developed in human enterococci and—after transfer to animal enterococci—has disseminated more rapidly in enterococci of animal origin, possibly under the selective pressure imposed by the use of florfenicol.

In China, florfenicol-containing antimicrobial agents are available for the treatment of enteric, respiratory tract or uterine infections in swine, cattle or poultry. When excreted, florfenicol remained bioactive in soils and might exert a selective pressure on bacteria in the environment.[28] This selective pressure might support the evolution of novel genes that confer resistance to florfenicol (and possibly also to other antimicrobial agents).[29]

Bearing in mind that antimicrobial agents such as florfenicol can select for genes, such as *cfr* and *optrA*, that also confer resistance to critically important antimicrobial agents in human medicine, such as the oxazolidinones linezolid and tedizolid,[30] the prudent use of florfenicol in food-producing animals is urgently warranted. The same applies to the use of antimicrobial agents of last resort, such as linezolid and tedizolid, in human medicine.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 to S3 and Figures S1 to S4 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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