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Characterization of *poxtA*, a novel phenicol–oxazolidinone–tetracycline resistance gene from an MRSA of clinical origin

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Objectives: To characterize a novel phenicol–oxazolidinone–tetracycline resistance gene, named *poxtA*, identified in a previously described MRSA strain that was highly resistant to linezolid and also carried the *cfr* gene.

Methods: The *poxtA* gene was identified by bioinformatic analysis of the whole genome sequence of *Staphylococcus aureus* AOUC-0915. The *poxtA* gene was cloned in a shuttle plasmid vector and expressed in *Escherichia coli*, *S. aureus* and *Enterococcus faecalis* to investigate the protein function. Comparative sequence analyses at the protein and genetic levels were carried out using standard procedures.

Results: The *poxtA* gene encodes a protein that is 32% identical to OptrA and exhibits structural features typical of the F lineage of the ATP-binding cassette (ABC) protein superfamily that cause antibiotic resistance by ribosomal protection. Expression of *poxtA* in *E. coli*, *S. aureus* and *E. faecalis* was able to decrease susceptibility to phenicols, oxazolidinones and tetracyclines. A database search identified the presence of *poxtA* in *E. faecalis*, *Enterococcus faecium* and *Pediococcus acidilactici* strains, mostly of animal origin, and revealed the presence of *poxtA* homologues in the genomes of some Clostridiales. Analysis of the genetic context revealed that *poxtA* was located in a composite transposon-like structure containing two IS*1216* elements.

Conclusions: A novel resistance gene, named *poxtA*, encoding a protein of the antibiotic resistance (ARE) ABC-F lineage, was identified in the genome of an MRSA of clinical origin. PoxtA can confer decreased susceptibility to phenicols, oxazolidinones and tetracyclines and is associated with a putative mobile element that could contribute to its horizontal dissemination.

Introduction

The ATP-binding cassette (ABC) superfamily includes a large, diverse and ubiquitous group of proteins detected in all kingdoms of life. Some of these proteins are able to confer resistance to various classes of antibiotics in prokaryotes. The ABC proteins associated with antibiotic resistance (ARE) belong to the F lineage of the ABC superfamily (ARE ABC-F) and include single polypeptides containing two conserved nucleotide binding domains (NBDs) separated by a linker of ~80 amino acids of variable composition, with no transmembrane domains (TMDs).[1,2] The mechanism by which the ARE ABC-F proteins mediate resistance has been debated for a long time and attributed to either efflux or ribosomal protection.[3,4] Recently, the resistance mechanism of ARE ABC-F proteins has been clarified and shown to be mediated by ribosomal protection.[5]

The ARE ABC-F proteins can mediate resistance to several different classes of anti-ribosomal antibiotics, including tetracyclines, macrolides, ketolides, lincosamides, phenicols, pleuromutilins, oxazolidinones and streptogramins.[5] Many of them can actually mediate resistance to multiple antibiotic classes, e.g. Msr(A), conferring resistance to macrolides and group B streptogramins, or the recently described OptrA protein, conferring resistance to phenicols and oxazolidinones.[6]

The ARE ABC-F proteins are often encoded by genes carried on mobile genetic elements, which can disseminate these resistance

determinants horizontally among bacterial pathogens, although some are encoded by resident genes in a number of antibiotic-producing *Streptomyces* spp.[6–8]

We recently reported an MRSA strain that exhibited high-level resistance to linezolid (32 mg/L).[9] The strain had been isolated from a cystic fibrosis patient after linezolid treatment and was found to carry at least two different linezolid resistance mechanisms including a G152D substitution in the L3 ribosomal protein[9] and the *cfr* gene, an acquired gene encoding a ribosomal rRNA methylase, which can mediate resistance to oxazolidinones and several other anti-ribosomal drugs.[9–13]

In this study, we describe the identification and characterization of a novel acquired resistance gene, named *poxtA*, found in the same MRSA strain. The *poxtA* gene encodes a protein of the ARE ABC-F family, which is distantly related to OptrA and able to confer reduced susceptibility to phenicols, oxazolidinones and tetracyclines.

Materials and methods

Bacterial strains

AOUC-0915 is an ST5-MRSA-II strain isolated in September 2015 from the respiratory tract of a cystic fibrosis patient at Florence Careggi University Hospital (Florence, Italy).[9] Antimicrobial susceptibility and resistome of this strain, in terms of known resistance genes, have previously been reported.[9] *Escherichia coli* Mach1^{TM} T1^{R} (Thermo Fisher Scientific, Waltham, MA, USA), *Staphylococcus aureus* RN4220[14] and *Enterococcus faecalis* JH2-2[15] were used as hosts for cloning of the *poxtA* gene.

Recombinant DNA methodology

The *poxtA* gene, with its flanking regions, was cloned in the pMU1328 vector[16] using the PIPE (Polymerase Incomplete Primer Extension) cloning method[17] and *E. coli* Mach1^{TM} T1^{R} cells, to obtain the recombinant plasmid pMU-*poxtA*. Cloning was designed to replace the chloramphenicol acetyl transferase (*cat*) gene of the plasmid with the *poxtA* locus, using primers targeting the amplification of a region spanning from 863 bp upstream of the *poxtA* start codon to 60 bp downstream of the stop codon (Figure S1, available as Supplementary data at *JAC* Online). A plasmid derived from the pMU1328 vector, named pMU-E, and carrying a deletion of the *cat* gene, was also constructed using the PIPE cloning method and specific primers (Figure S1) to be used as a control. *E. coli* transformants, obtained by a heat-shock method,[18] were selected on Mueller–Hinton agar (MHA) supplemented with erythromycin (100 mg/L). The authenticity of the cloned DNA fragment in pMU-*poxtA* and of the deletion in plasmid pMU-E were confirmed by Sanger sequencing both strands of plasmid DNA extracted from the Mach1^{TM} T1^{R} transformants. The recombinant plasmids were introduced into *S. aureus* RN4220 and *E. faecalis* JH2-2 by electrotransformation (2.5 kV, 200 Ω, 25 μF). Transformants were selected on MHA containing erythromycin (2 mg/L).

Antimicrobial susceptibility testing

MICs were determined by broth microdilution according to the M7-A10 standard of the CLSI,[19] except for that of tedizolid, which was measured by MIC Test Strip (Liofilchem s.r.l., Roseto degli Abruzzi, Italy). Antimicrobial susceptibility testing was carried out in triplicate. The results were interpreted according to the EUCAST clinical breakpoint tables.[20] Reference strains *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922 were used as quality controls for antimicrobial susceptibility testing.

Bioinformatics and sequence analysis

The presence of TMDs and of a signal peptide in PoxtA was predicted using the TMHMM Server v. 2.0 web service[21] and the SignalP 4.1 server,[22] respectively. The search for *poxtA* homologues was carried out using the BLAST web server, selecting alternatively the wgs or nr databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acid sequence alignments of PoxtA with other ARE ABC-F proteins and the phylogenetic tree, generated using the maximum likelihood method with 1000 bootstrap replicates, were generated with the MEGA 6.0.6 software package.[23] The genetic context of *poxtA* in *S. aureus* AOUC-0915 was investigated by PCR mapping and Sanger sequencing experiments on the regions unresolved by WGS assembly.[9] A graphical comparison of the genetic context of *poxtA* homologues was generated using EasyFig.[24]

The nucleotide sequence of the *poxtA* gene and flanking regions has been deposited in the GenBank database under the accession number MF095097.

Results

Identification of a new ARE ABC-F resistance determinant in S. aureus AOUC-0915

Analysis of the genome sequence of *S. aureus* AOUC-0915 revealed the presence of an ORF encoding a protein of 542 amino acids that exhibited similarity (32% identical amino acid residues) with OptrA over most of its length (coverage of 95%) (Figure 1). This protein showed conserved features with members of the ARE ABC-F family,[5,25] including two NBDs and the lack of detectable TMDs. Moreover, no leader peptide was detected by the two bioinformatics tools used for this analysis (Figure 1).

To confirm its role in antibiotic resistance, the gene, with some flanking regions, was cloned in the pMU1328 shuttle plasmid vector, which can replicate in *E. coli*, in *S. aureus* and in *E. faecalis*. The antimicrobial susceptibilities of the *E. coli*, *S. aureus* and *E. faecalis* strains carrying the cloned gene were investigated and compared with those of strains carrying the empty pMU-E vector. The expression of the gene in *S. aureus* and *E. faecalis* demonstrated that it was associated with decreased susceptibility to phenicols (chloramphenicol and florfenicol), oxazolidinones (linezolid and tedizolid) and tetracyclines (tetracycline and doxycycline). Expression of the gene in *E. coli* was associated with decreased susceptibility to the same antibiotics (tedizolid was not tested) and also to tigecycline (Table 1). No differences in susceptibility to amikacin, gentamicin, ampicillin, ciprofloxacin, rifampicin and trimethoprim/sulfamethoxazole were detected following expression of the gene in the *E. coli* and *S. aureus* hosts (data not shown). Given the antibiotic resistance phenotype, the new resistance gene was designated *poxtA* (after phenicols, oxazolidinones and tetracyclines).

Relatedness of PoxtA with other ARE ABC-F proteins

Comparative sequence analysis of PoxtA with other members of the ARE ABC-F family confirmed that PoxtA is a new member of this protein family. The closest homologues were putative ARE ABC-F proteins encoded by the genomes of *Enterococcus faecium* 9-F-6 and *Azospirillum* sp. 51_20, (73% and 70% amino acid identity, respectively). Other homologues (66%–68% amino acid identity) were detected in the genomes of some Clostridiales, including *Clostridium bolteae*, *Clostridium clostridioforme*, *Hungatella hathewayi*

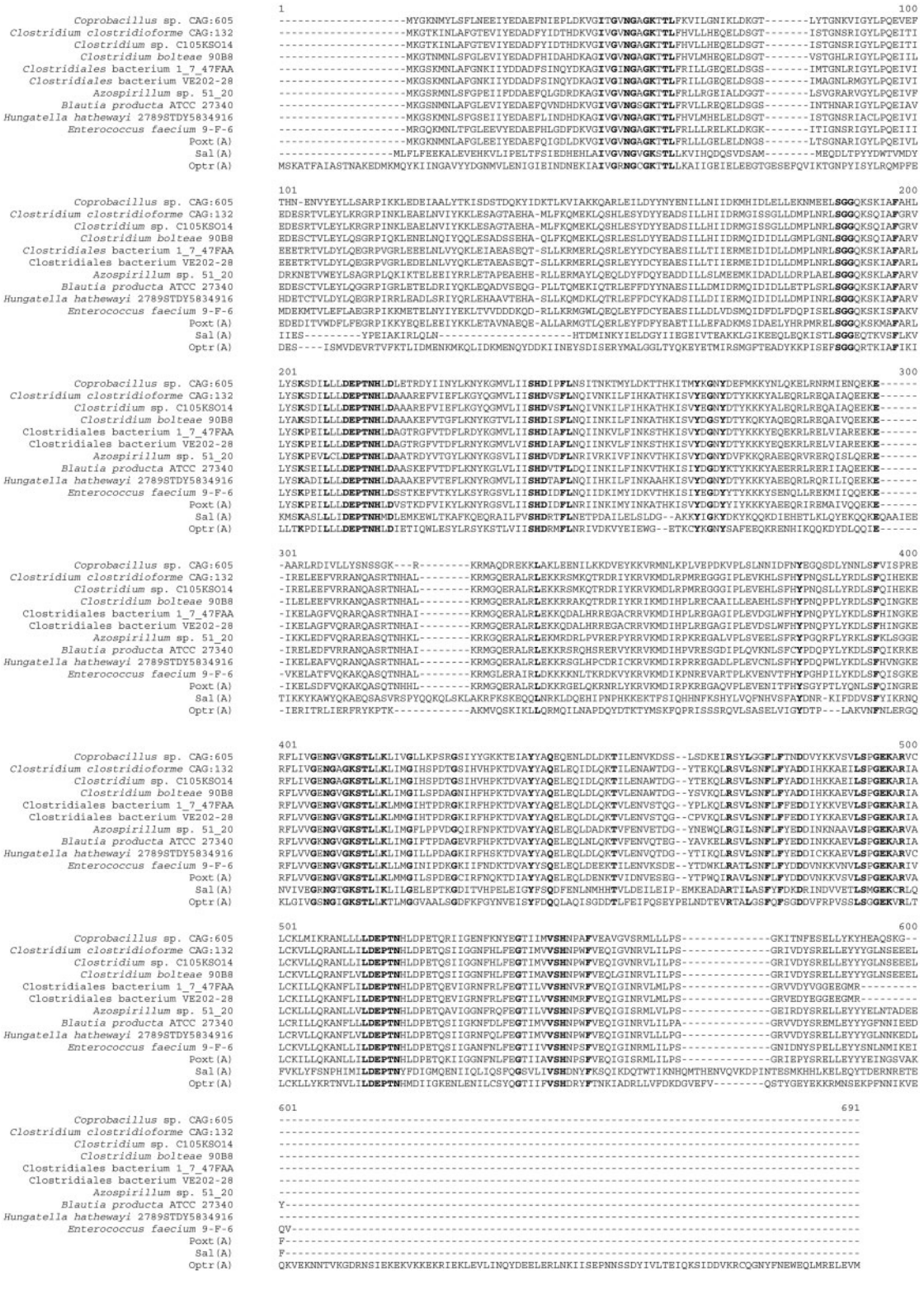

Figure 1. Alignment of PoxtA and homologous proteins. The conserved regions of ABC-F proteins, including the Walker A and B motifs, the signature and the switch motifs, involved in ATP binding and hydrolysis are indicated. Residues conserved in all sequences are shown in bold. Protein accession numbers are as follows: *Coprobacillus* sp. CAG:605, CCZ88872.1; *C. clostridioforme* CAG:132, CDB61438.1; *Clostridium* sp. C105KSO14, CUX74470.1; *C. bolteae* 90B8, ENZ34498.1; Clostridiales bacterium 1_7_47FAA, EEQ60488.1; Clostridiales bacterium VE202-28, WP_025484560.1; *Azospirillum* sp. 51_20, OLA82622.1; *B. producta* ATCC 27340, WP_026255743.1; *H. hathewayi* 2789STDY5834916, WP_055650089.1; *E. faecium* 9-F-6, OZN12776.1; Sal(A), AGN74946.1; and OptrA, AKA86814.1.

Table 1. Antimicrobial susceptibilities of *S. aureus*, *E. faecalis* and *E. coli* strains carrying a cloned copy of the *poxtA* gene

	MIC (mg/L)					
	S. aureus[a]		*E. faecalis*[b]		*E. coli*[c]	
Antibiotic	RN4220 (pMU-*poxtA*)	RN4220 (pMU-E)	JH2-2 (pMU-*poxtA*)	JH2-2 (pMU-E)	Mach1^{TM} T1^{R} (pMU-*poxtA*)	Mach1^{TM} T1^{R} (pMU-E)
Linezolid	2	1	4	1	1024	256
Tedizolid	0.5	0.25	0.5	0.25	—	—
Chloramphenicol	8	4	8	4	32	8
Florfenicol	16	2	16	2	128	8
Tigecycline	0.25	0.25	0.25	0.25	1	0.5
Tetracycline	0.25	0.125	0.25	0.125	2	0.5
Doxycycline	0.25	0.125	0.125	≤0.06	8	2

Susceptibilities of the same strains carrying the empty plasmid vector are also reported for comparison. The MIC measurements were performed in triplicate and results were fully reproducible, with no discrepancies.
[a]The MICs were evaluated in medium supplemented with 50 mg/L erythromycin for plasmid maintenance.
[b]The MICs were evaluated in medium supplemented with 2 mg/L erythromycin for plasmid maintenance.
[c]The MICs were evaluated in medium supplemented with 100 mg/L erythromycin for plasmid maintenance.

(formerly *Clostridium hathewayi*) and *Blautia producta*, and of *Coprobacillus* sp. CAG:605 (Figure 1).

Among known ARE ABC-F proteins, PoxtA belongs to a sublineage that also includes OptrA and Sal(A)[26] (Figure 2).

Detection of the poxtA gene in other strains and genetic context of poxtA

A BLAST search in sequence databases using *poxtA* as a query revealed the presence of identical genes in a number of Gram-positive strains including *E. faecalis* 599 (accession no. EJU87034.1), *E. faecalis* 12 (accession no. KII46686.1), *E. faecium* P36 (accession no. KP834591.1) and *Pediococcus acidilactici* BCC1 (accession no. CP018763.1). This finding confirmed the mobile nature of *poxtA*, which is apparently able to spread among different Gram-positive cocci. Interestingly, these strains were of animal origin (except for *E. faecalis* 599, whose origin was not reported).

The genetic context of *poxtA* in *S. aureus* AOUC-0915 was further investigated by PCR mapping and sequencing experiments. This analysis revealed that *poxtA* was flanked by two IS*1216*-like ISs (hereinafter referred to as IS*1216*_U and IS*1216*_D) in the same orientation (Figure 3a), suggesting that the resistance gene had been mobilized via a composite transposon constituted by the two IS*1216*-like elements. The IR_L of the IS*1216*_D that is present at the 3′-end of the *poxtA* gene is actually part of the coding sequence (CDS) constituting the final 21 nucleotides of the CDS and including the *poxtA* stop codon (Figure 3b). This condition is conserved also in all the available sequences containing *poxtA* (e.g. in *P. acidilactici* BCC1 and in *E. faecium* P36) (Figure 3a), suggesting that the partial deletion of the *poxtA* gene may be an ancestral event that originated from the mobilization of this resistance gene by the IS*1216*-like elements. As a consequence a hybrid gene results, encoding a protein with a modified C-terminus. The IR_R of the IS*1216*_D was disrupted by the insertion of an IS*1252*-like IS that apparently generated a duplicated region of three nucleotides abutting the left and right boundaries of the element. Remnants of the IS*1216*_D IR_R are present downstream of the IS*1252*-like element, suggesting that the insertion of this IS was likely an independent event following *poxtA* mobilization (Figure 3b).

Comparison between the genetic context of *poxtA* in *S. aureus* AOUC-0915, *E. faecium* P36 and *P. acidilactici* BCC1, for which some flanking sequences are available, showed an overall conserved genetic context, but with some differences. In particular, in *P. acidilactici* BCC1 the IS*1216* present upstream of the *poxtA* gene was in the opposite orientation, while in *E. faecium* P36 a copy of IS*1216* was present downstream of the gene (Figure 3a).

Discussion

A novel resistance determinant belonging to the ARE ABC-F family of proteins, named PoxtA, was identified in this work. PoxtA was initially detected by bioinformatic analysis of the genome of *S. aureus* AOUC-0915, a linezolid-resistant MRSA strain isolated from a cystic fibrosis patient, based on sequence homology with OptrA, a recently described member of the ARE ABC-F family of proteins that confers resistance to phenicols and oxazolidinones by means of ribosomal protection.[5,6] Cloning and expression experiments revealed that the *poxtA* gene was functional in decreasing susceptibility to at least three classes of anti-ribosomal antibiotic classes, including phenicols, oxazolidinones and tetracyclines. Altogether, these results confirmed that PoxtA, which carries two recognizable NBDs and is apparently lacking TMDs and a signal peptide, is a new member of the ARE ABC-F family of proteins that can confer reduced susceptibility to phenicols, oxazolidinones and tetracyclines. To the best of our knowledge, no other members of the ARE ABC-F family show a comparable broad spectrum of activity for these three antibiotic classes.

The decreased antimicrobial susceptibility conferred by PoxtA was observed in three different hosts, including *S. aureus*, *E. faecalis* and *E. coli*, with an overall similar pattern. Unexpectedly, when expressed in *E. coli*, *poxtA* conferred a more marked decrease in susceptibility to antibiotics than when expressed in the Gram-positive

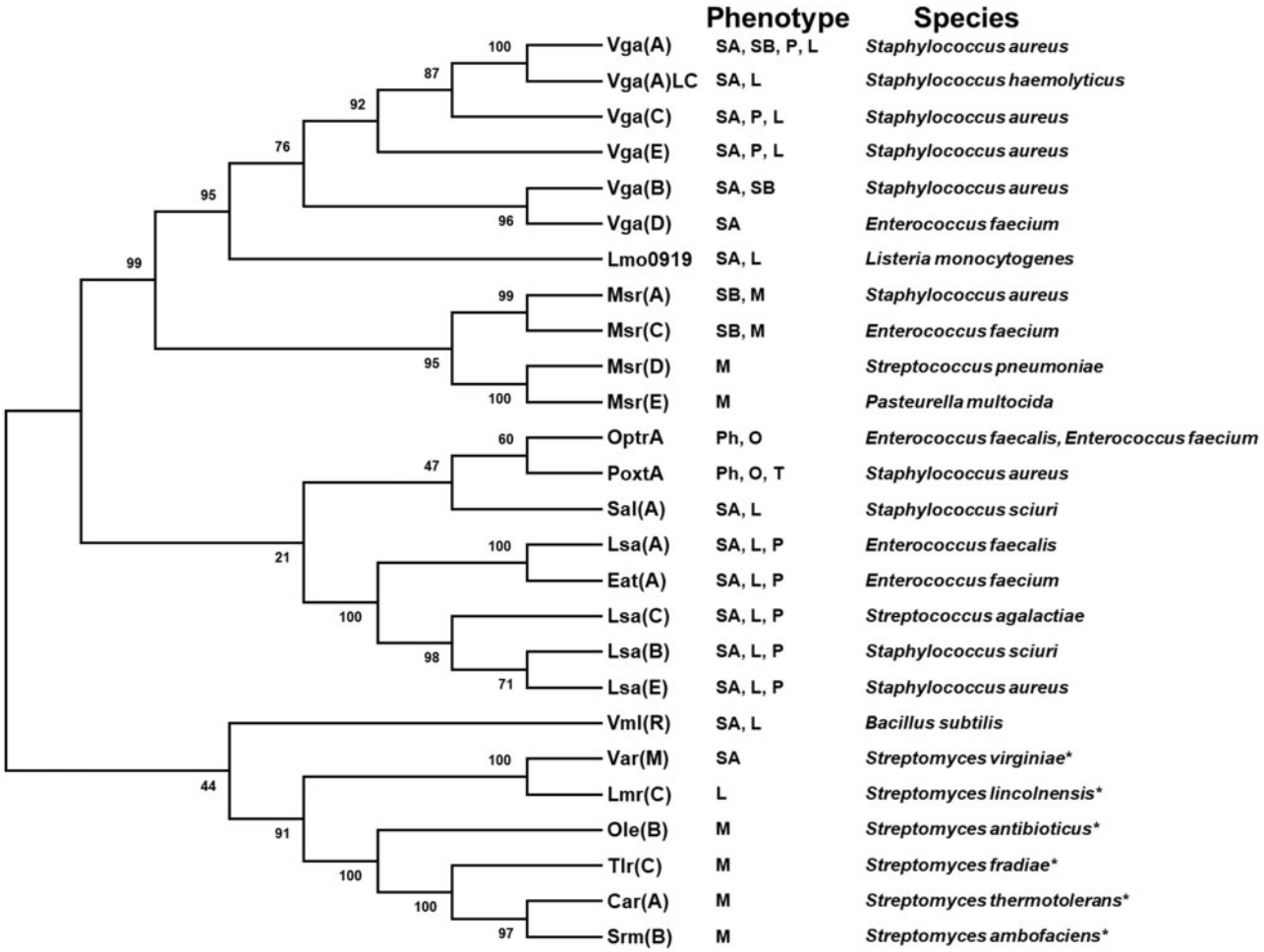

Figure 2. Phylogenetic tree of ARE ABC-F proteins constructed using the maximum likelihood method. The tree represents the consensus obtained after 1000 replicates and bootstrap values are indicated next to the branches. Amino acid sequences of the ARE ABC-F proteins were extracted from the 'tetracycline and MLS nomenclature' web site (https://faculty.washington.edu/marilynr/ermweb2.pdf), except for Lmo0919 (CAC98997.1), Var(M) (BAA96297.1), Vml(R) (WP_003234144.1) and LmrC (CAA55774.1). Antibiotics affected by the different proteins are indicated and the species in which the various ARE ABC-F have been described for the first time are also indicated.

SA, streptogramin A; SB, streptogramin B; P, pleuromutilins; L, lincosamides; M, macrolides; Ph, phenicols; O, oxazolidinones; T, tetracyclines. Antibiotic-producing species are indicated by an asterisk.

cocci. This could be due to differences in the gene expression level or to differences in the interaction of PoxtA with the ribosomal target in the different species.

The genetic context and the scattered distribution among a few strains of different species (*S. aureus*, *Enterococcus* spp. and *P. acidilactici*) strongly support a mobile nature of *poxtA*. Indeed, the gene was found to be associated with ISs, in a structure resembling a composite transposon, which were likely responsible for its capture and mobilization as a hybrid form, due to an IS*1216* insertion at the C-terminus, which nevertheless has conserved a ribosomal protection function.

Since *poxtA* homologues were detected in the genomes of several clostridia, some members of Clostridiales could be the original source of *poxtA*-like genes. Notably, in the genomes of clostridia, the *poxtA* homologues are located between genes encoding putative MATE efflux proteins and antibiotic acetyltransferases. In particular, these antibiotic acetyltransferase homologues exhibit a significant similarity (amino acid identity >49%) with known acetyltransferases, including streptogramin A[27] and chloramphenicol acetyltransferases,[28] suggesting that ABC-F might act synergistically with these enzymes to protect the host from antibiotics.

Since most of the other strains in which the gene was detected originated from animal sources, this suggests that selection of the *poxtA* gene could have occurred in the animal setting, as reported for other genes mediating resistance to phenicols and other antiribosomal drugs that are broadly used in veterinary medicine.[29,30]

The apparently limited distribution in the clinical setting and the relatively low impact on oxazolidinone susceptibility point to an overall limited clinical impact of the *poxtA* gene. However, the discovery of a novel transferable mechanism able to reduce the susceptibility to both linezolid and tedizolid among Gram-positive cocci is a matter of concern. In fact, the *poxtA* gene could also act synergistically with other oxazolidinone

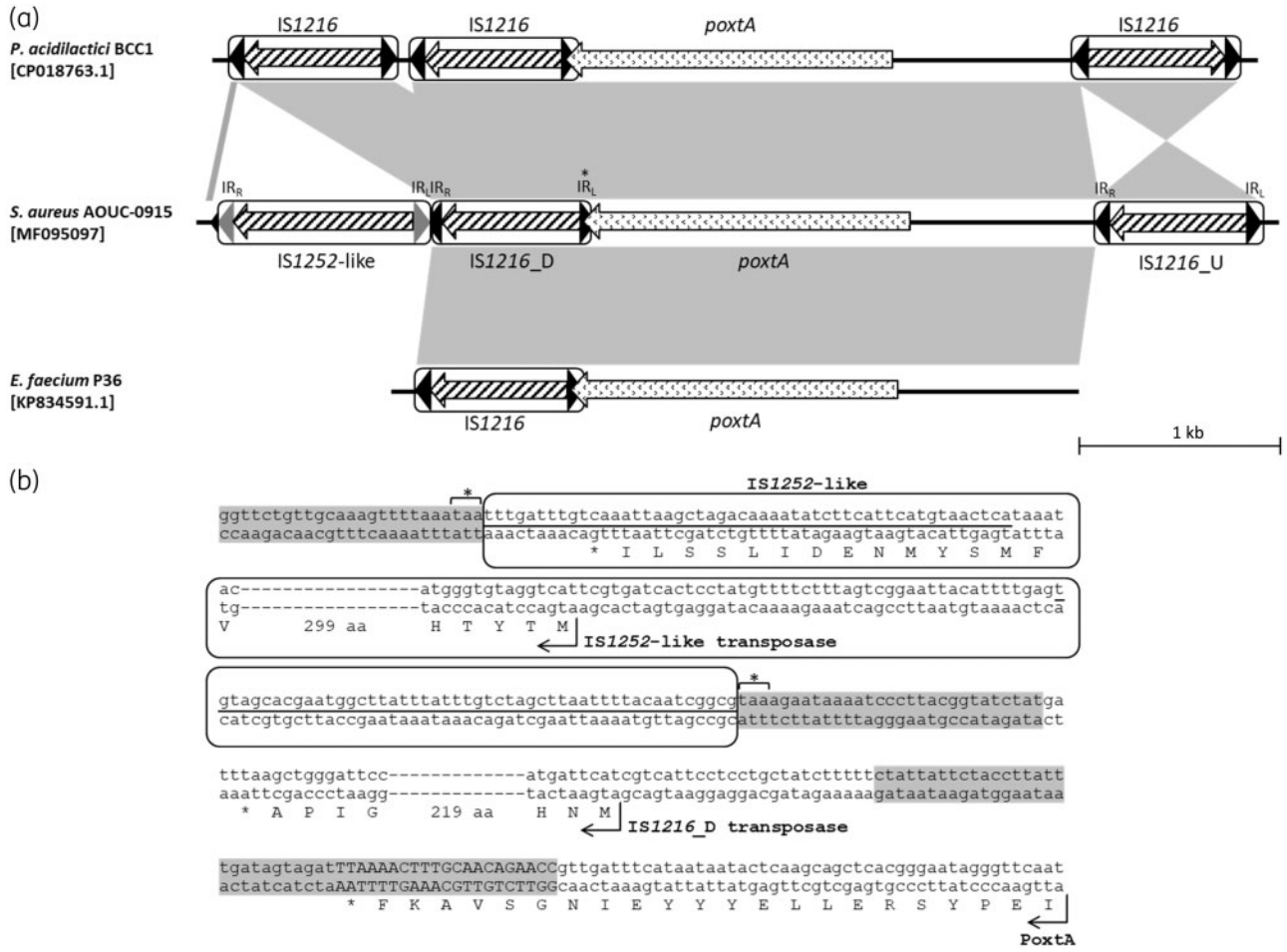

Figure 3. Genetic context of the *poxtA* gene. (a) Comparison of the genetic context of *poxtA* in *P. acidilactici* BCC1 (region 1 547 068–1 552 271 of accession number CP018763.1) and in *E. faecium* P36 (region 1–3421 of accession number KP834591.1). Regions having >99% nucleotide identity are connected by grey zones. ISs are boxed and transposase-encoding genes are indicated by striped arrows, while the *poxtA* gene is represented by a dotted arrow. IS*1216*-like and IS*1252*-like IRs are indicated by black and grey triangles, respectively. In *S. aureus* AOUC-0915, the remnant of IS*1216*_D IR_R is indicated by a black triangle downstream of the insertion point of the IS*1252*-like element. The region of overlap between the IS*1216*_D IR_L and the *poxtA* CDS is indicated by an asterisk. (b) Nucleotide sequence showing the insertion points of IS*1216*_D and of the IS*1252*-like element. The regions encoding the IS*1216*_D and the IS*1252*-like transposases are indicated by arrows and are shown only partially; the numbers of amino acids that are not shown are indicated. The IS*1252*-like element is boxed and its IRs are underlined. An asterisk indicates the three-nucleotide duplication originated by the IS*1252*-like insertion event. IS*1216*_D IRs are highlighted in grey and the region of overlap between the IS*1216*_D IR_L and the *poxtA* CDS is shown in capital letters. The C-terminus PoxtA sequence is shown under the nucleotide sequence.

resistance mechanisms to further increase the level of resistance to these drugs in a stepwise manner. Indeed, the AOUC-0915 strain exhibited high-level resistance to linezolid (due also to the presence of the *cfr* gene and of a G152D substitution in the L3 ribosomal protein) and it will be interesting to ascertain the contribution of *poxtA* and *cfr* to this resistance phenotype by separate and sequential gene inactivation experiments. On the other hand, screening for the presence of the *poxtA* gene in collections of clinical and veterinary isolates would be of interest to assess the real prevalence of this gene.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online.

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