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TetAB(46), a predicted heterodimeric ABC transporter conferring tetracycline resistance in *Streptococcus australis* isolated from the oral cavity

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Objectives: To identify the genes responsible for tetracycline resistance in a strain of *Streptococcus australis* isolated from pooled saliva from healthy volunteers in France. *S. australis* is a viridans *Streptococcus*, originally isolated from the oral cavity of children in Australia, and subsequently reported in the lungs of cystic fibrosis patients and as a cause of invasive disease in an elderly patient.

Methods: Agar containing 2 mg/L tetracycline was used for the isolation of tetracycline-resistant organisms. A genomic library in *Escherichia coli* was used to isolate the tetracycline resistance determinant. In-frame deletions and chromosomal repair were used to confirm function. Antibiotic susceptibility was determined by agar dilution and disc diffusion assay.

Results: The tetracycline resistance determinant from *S. australis* FRStet12 was isolated from a genomic library in *E. coli* and DNA sequencing showed two open reading frames predicted to encode proteins with similarity to multidrug resistance-type ABC transporters. Both genes were required for tetracycline resistance (to both the naturally occurring and semi-synthetic tetracyclines) and they were designated *tetAB(46)*.

Conclusions: This is the first report of a predicted ABC transporter conferring tetracycline resistance in a member of the oral microbiota.

Keywords: antibiotic resistance, oral microflora, oral streptococci

Introduction

Tetracyclines are broad-spectrum antibiotics that are used extensively to combat bacterial infections in humans and animals, and have been used as growth promoters in animals, agriculture and aquaculture.^{1–3} Bacterial resistance to tetracycline is primarily mediated through acquired genes encoding one of three main mechanisms: active efflux, ribosomal protection proteins (RPPs), or enzyme-mediated drug inactivation.⁴ Within the oral cavity, ribosomal protection [e.g. *tet(M)*, *tet(Q)* and *tet(O)*] is the most commonly observed mechanism, whereas tetracycline-inactivating enzymes and efflux mechanisms occur less frequently.^{5,6}

Tetracycline efflux systems have been reported in both Gram-negative and Gram-positive bacteria.^{7,8} The best-studied tetracycline efflux genes [e.g. *tet(A)* and *tet(B)*] encode membrane-associated, energy-dependent proteins belonging to the

major facilitator superfamily (MFS) in which proton-motive force is used to drive efflux.⁴ However, other tetracycline efflux proteins not belonging to the MFS group have been reported: (i) Tet(35) of *Vibrio harveyi*⁹ is a member of the H⁺ antiporter (NhaC) family and confers resistance to tetracycline, oxytetracycline and minocycline; and (ii) OtrC of *Streptomyces rimosus* is predicted to encode, on distinct polypeptides, the nucleotide-binding domain (NBD) and membrane-spanning domain (MSD) typical of members of the ABC transporter family. No functional information is available for OtrC.⁴

In this study, we characterize a novel tetracycline resistance determinant in *Streptococcus australis* FRStet12, isolated from pooled saliva from healthy French subjects as part of a study investigating antibiotic resistance in bacteria colonizing adult humans.¹⁰ We show that two proteins, each encoding predicted ABC transporter subunits, are both required for tetracycline resistance.

Materials and methods

Sample collection and culture

Saliva samples (~5 mL) were collected from 20 healthy adult volunteers who had not received antibiotic therapy in the previous 3 months, from two centres in France (Faculté de Pharmacie, Université Paris Sud and INRA-UEPSD, Domain de Vilvert), as previously described.¹⁰ The samples were pooled in a sterile 200 mL Duran bottle and processed within 48 h of collection. A 10-fold dilution series was prepared from 1 mL of the sample in Luria–Bertani (LB) broth and spread onto Iso-Sensitest agar (Oxoid) supplemented with 5% defibrinated horse blood (E&O Laboratories, Bonnybridge, UK) and 2 mg/L tetracycline. The plates were incubated in air enriched with 5% CO₂ for up to 72 h.¹⁰ Growth at concentrations >2 mg/L is defined as resistant by the BSAC.¹¹

Identification of the resistance genes

Genomic DNA from *S. australis* FRStet12 was hybridized to a macroarray containing 23 known tetracycline resistance genes [9 RPP genes—M, O, B(P), Q, S, T, W, 32 and 36; 12 efflux genes—A, B, C, D, E, G, H, J, A(P), Y, Z and 30; and 2 enzymatic inactivation genes—*tet*(X) and *tet*(34)], as previously described.¹² The genomic DNA was also tested for the presence of RPP-encoding genes by PCR using a set of universal RPP primers.¹³ To clone the resistance genes, *S. australis* FRStet12 genomic DNA was partially digested with HindIII, ligated into HindIII-digested, dephosphorylated pUC19 and transformed into *Escherichia coli* JM109 competent cells, according to the supplier's instructions (Promega). Bacteria were spread on to LB agar supplemented with 100 mg/L ampicillin and 5 mg/L tetracycline and incubated at 37°C in air for up to 36 h. A tetracycline-resistant clone, designated P9, was isolated and the insert sequenced.

Species identification

Amplification and sequencing of a manganese-dependent superoxide dismutase (*sodA*) gene fragment was carried out according to Poyart *et al.*,¹⁴ using the primers listed in Table S1 (available as Supplementary data at JAC Online).

DNA sequencing

To sequence the HindIII genomic DNA fragment of *S. australis* in pUC19 from clone P9 (pP9), a walking strategy was employed using the primers listed in Table S1 (available as Supplementary data at JAC Online).

Mutagenesis

In-frame deletions in *tetA*(46) and *tetB*(46) were created using 'splicing by overlapping extension' (SOEing) PCR.¹⁵ The hybrid fragments were ligated into pGEM-T-Easy (Promega) and verified by DNA sequencing. The Δ *tetA*(46) fragment was created using two sets of primer pairs: ABC1-1F/ABC1-2R and ABC1-3F/ABC1-4R; primers ABC1-2R and ABC1-3F shared 24 nucleotides of complementary sequence to facilitate the ligation of the two amplicons (Table S1 available as Supplementary data at JAC Online). The ligated mutant fragment, when recombined into the genome, resulted in a 51 bp in-frame deletion (bp 1073–1123 inclusive) in *tetA*(46). The Δ *tetB*(46) fragment was created in the same way, using two sets of primer pairs: ABC2-1F/ABC2-2R and ABC2-3F/ABC2-4R; similarly, recombination of this mutagenic fragment resulted in a 51 bp in-frame deletion (bp 1090–1140 inclusive) in *tetB*(46). The Δ *tetA*(46) or Δ *tetB*(46) mutagenic fragments were co-transformed with

pVA838¹⁶ into *S. australis* FRStet12 to provide selection for successful transformation (Erm^R).

Transformation

Genetic competence was induced in *S. australis* FRStet12 using a modified version of the method reported by Hudson and Curtiss.¹⁷ A single colony was inoculated into 10 mL of Todd–Hewitt broth (THB) containing 10% horse serum and incubated at 37°C, in air+5% CO₂, for 18 h. A 1/40 dilution of the overnight culture was grown in THB plus 10% horse serum under the same conditions until the optical density at 600 nm was between 0.1 and 0.2. For co-transformation experiments, 1 µg of either the Δ *tetA*(46) or Δ *tetB*(46) mutant fragments plus ~250 ng of pVA838¹⁶ were added to 1 mL of culture, mixed and incubated for 3 h in the CO₂-enriched atmosphere. pVA838 is a streptococcal shuttle plasmid that carries an erythromycin resistance gene as a selectable marker.¹⁶ Aliquots of 100 µL were then spread on to brain heart infusion (BHI) agar supplemented with 5% defibrinated horse blood plus 10 mg/L erythromycin and incubated at 37°C, in air+5% CO₂, for 24 h. Colonies were then transferred to BHI agar plates supplemented with 5% defibrinated horse blood plus 10 mg/L erythromycin and replica plated on to BHI agar plates supplemented with 5% defibrinated horse blood plus 10 mg/L erythromycin and 5 mg/L tetracycline. All plates were incubated at 37°C, in air+5% CO₂, for 24–48 h.

Construction of plasmids for chromosome repair

The wild-type *tetA*(46) or *tetB*(46) genes were PCR amplified, ligated into pVA838 and transformed into the mutant strains, as described above. The *tetA*(46) gene was amplified using primers ABC1-8F and ABC1-9R (Table S1 available as Supplementary data at JAC Online), each containing XbaI restriction sites. The *tetB*(46) gene was amplified using primers ABC2-7F and ABC2-8R, each containing SphI restriction sites (Table S1 available as Supplementary data at JAC Online). The amplified products were digested with their respective restriction endonucleases and ligated into either the XbaI or SphI sites of pVA838, creating the recombinant plasmids pABC1 and pABC2, respectively. The plasmids were transformed into *E. coli* α -select bronze competent cells (Bioline) and the presence of the wild-type gene confirmed by DNA sequencing. Both *E. coli* strains were grown overnight in LB broth supplemented with 80 mg/L chloramphenicol, at 37°C, aerobically with shaking at 200 rpm. Plasmid DNA was extracted using a HiSpeed Plasmid Midi Kit (Qiagen).

Antibiotic susceptibility testing

The MICs of tetracycline, oxytetracycline, doxycycline, chlortetracycline and tigecycline were determined according to BSAC guidelines.¹⁸ The recommended medium and inoculum (10⁶ cfu/spot) was also used for determination of the acriflavine and ethidium bromide MICs. Triplicate individual colonies of each strain were inoculated into 10 mL BHI broth and incubated at 37°C, in air+5% CO₂, for 18 h. The cells were diluted and then spotted, using a multipoint inoculator, on to Iso-Sensitest agar supplemented with 5% defibrinated horse blood or Iso-Sensitest agar supplemented with 5% defibrinated horse blood plus the appropriate antibiotic at concentrations of 0.25–32 mg/L. The plates were incubated at 37°C, in air+5% CO₂, for up to 48 h.

Agar diffusion assays were carried out according to BSAC guidelines.¹¹ Discs containing the following amounts of antibiotics were laid on to agar: tetracycline (10 µg), ciprofloxacin (1 µg), metronidazole (5 µg), azithromycin (15 µg), ampicillin (2 µg), methicillin (5 µg), oxacillin (1 µg), penicillin (1 µg) and gentamicin (10 µg). Plates were incubated at 37°C, in air+5% CO₂, for 20 h and the zones of inhibition measured.

Results

S. australis FRStet12 was isolated on agar containing 2 mg/L tetracycline.¹⁹ Sequencing of a *sodA* gene fragment showed that the closest relative was the *S. australis* type strain CIP 107167 (DQ132987), with 95.2% identity. A phylogenetic tree showing the relationship between the *sodA* gene fragments of *S. australis* FRStet12 and other streptococcal species is shown in the supplementary data (Figure S1, available as Supplementary data at JAC Online). To determine whether tetracycline resistance was conferred by a previously described mechanism, genomic DNA from *S. australis* FRStet12 was hybridized to a macroarray containing known tetracycline resistance genes and the genomic DNA was also tested for the presence of RPP-encoding genes by PCR.¹³ The genomic DNA failed to hybridize to the macroarray and was negative in the RPP PCR, suggesting the presence of a rare or novel tetracycline resistance determinant. In our study, of a total of 69 Gram-positive, facultatively anaerobic, tetracycline-resistant isolates, *S. australis* FRStet12 was one of only two isolates that failed to hybridize with the array.¹⁹ The other isolate that failed to hybridize has not been investigated.

To identify the gene(s) conferring resistance in *S. australis* FRStet12, a library of HindIII-digested genomic DNA was created in pUC19, selecting transformants on agar containing 100 mg/L ampicillin and 5 mg/L tetracycline. One tetracycline-resistant transformant was selected for further study and designated *E. coli* P9. The insert from the plasmid in P9 (pP9) was completely sequenced (accession number HQ652506) and found to contain five putative open reading frames (orfs), two of which encoded predicted proteins with similarity to multidrug resistance (MDR)-type ABC transporters (Figure 1). These genes, which were both subsequently shown to encode resistance to tetracycline (see below), have $\leq 79\%$ amino acid identity to previously characterized *tet* genes and were assigned *tetA*(46) and

tetB(46) under the current naming standards.²⁰ They are predicted to encode non-identical polypeptides of 574 and 578 amino acids, respectively, each containing an NBD and an MSD characteristic of the ABC transporter superfamily. *TetA*(46) and *TetB*(46) are most closely related to YheI (36% amino acid identity) and YheH (35% identity) of *Bacillus subtilis*, respectively, which, when overexpressed in *E. coli*, form a heterodimeric MDR-type ABC transporter. *TetA*(46) and *TetB*(46) show sequence similarity with other heterodimeric ABC transporters with experimentally proven function, including LmrCD of *Lactococcus lactis* and EfrAB of *Enterococcus faecalis*, as well as related systems in the Gram-negative bacteria, *Serratia marcescens* and *E. coli* (Table 1).

To determine the function of each gene, 51 bp in-frame deletions were created within the sequences predicted to encode the Walker A box of the ATP-binding site of each gene product using SOEing PCR.¹⁵ The mutant gene fragments were co-transformed²⁶ into *S. australis* FRStet12 along with plasmid pVA838,¹⁶ making use of the erythromycin resistance gene on the plasmid for selection. This plasmid was chosen for co-transformation since our experience showed it to be rapidly lost from other *Streptococcus* species in the absence of selection. Of 90 erythromycin-resistant clones examined following transformation with the *tetA*(46) mutant fragment and pVA838, five were unable to grow on 5 mg/L tetracycline and sequence analysis confirmed the presence of the $\Delta tetA$ (46) allele in all five (Figure 1). The tetracycline-resistant transformants contained wild-type *tetA*(46) and were erythromycin resistant, indicating they had only taken up pVA838. Despite repeated subculturing without erythromycin (14 passages in total), pVA838 remained in the mutant strains. Therefore, to allow comparison between wild-type and mutant strains, an isogenic strain was created by transforming the wild-type with pVA838. Of 72 erythromycin-resistant clones examined following transformation of the wild-type with the *tetB*(46) mutant fragment and pVA838, 7

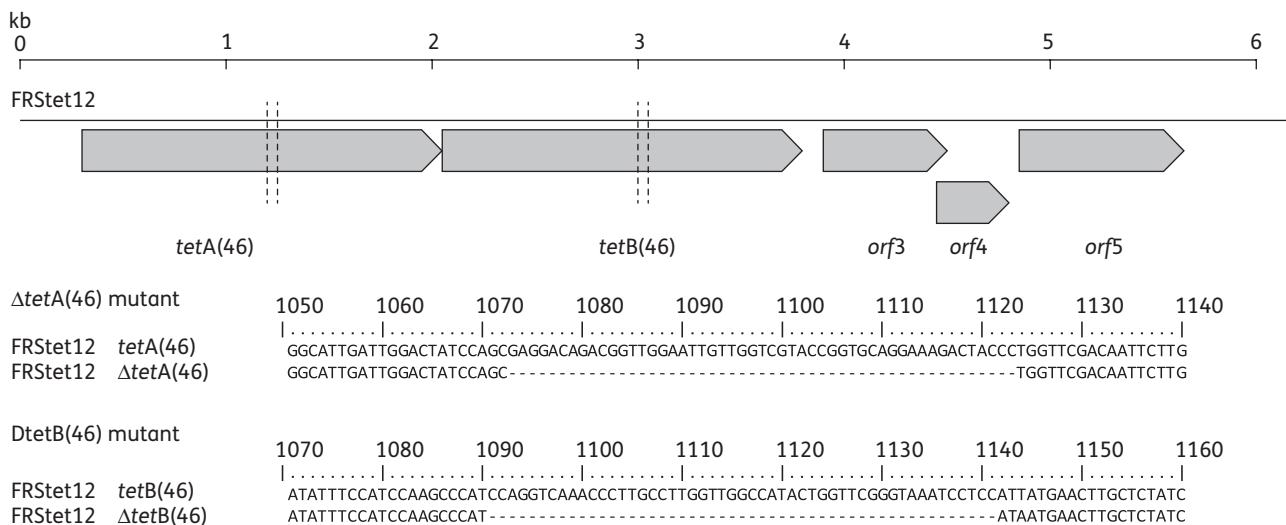


Figure 1. Diagram of the cloned genomic DNA fragment from *S. australis* FRStet12, indicated by the black line. Five putative orfs are indicated in grey [*tetA*(46); *tetB*(46); *orf3*, encodes a putative metalloprotease; *orf4*, encodes a putative diacylglycerol kinase; and *orf5*, encodes a putative GTP-binding protein—accession number HQ652506]. The vertical broken black lines indicate the point of the in-frame deletions in *tetA*(46) and *tetB*(46) and the sequence of the deletion is given beneath.

Table 1. Comparison between *S. australis* TetA(46)/TetB(46) and ABC transporters with experimentally proven function

Organism (reference)	ABC transporter subunit	Percentage identical (similar) residues	
		TetA(46)	TetB(46)
<i>S. australis</i> (this study)	TetA(46)	—	23.3 (52.7)
	TetB(46)	23.3 (52.7)	—
<i>B. subtilis</i> ²¹	YheI	36.4 (65.3)	26.7 (51.1)
	YheH	21.4 (50.1)	34.9 (57.5)
<i>S. marcescens</i> ²²	SmdA	30.3 (58.6)	25.5 (49.6)
	SmdB	23.3 (51.0)	29.1 (54.0)
<i>E. coli</i> ²³	MdIA	30.3 (57.8)	25.0 (50.2)
	MdIB	23.0 (51.6)	28.4 (52.6)
<i>L. lactis</i> ²⁴	LmrC	26.2 (55.1)	23.5 (51.5)
	LmrD	21.4 (47.2)	26.1 (48.5)
<i>E. faecalis</i> ²⁵	EfrA	23.6 (55.0)	25.3 (51.5)
	EfrB	25.3 (53.4)	27.7 (56.6)

GenBank proteins numbers: YheI, NP_388852; YheH, NP_388853; SmdA, BAF79679; SmdB, BAF79680; MdIA, P77265; MdIB, P0AAG5; LmrC, Q9CIP6; LmrD, Q9CIP5. For the other sequences, Swiss-Prot entries (in parentheses) were used: EfrA (Q82ZX7_ENTFA); and EfrB (Q82ZX8_ENTFA). Percentage identity and similarity were obtained by sequence alignment using Clustal W at <http://npsa-pbil.ibcp.fr/>.

were unable to grow on 5 mg/L tetracycline and sequencing confirmed the presence of the Δ tetB(46) allele (Figure 1), while those that remained resistant to tetracycline contained wild-type tetB(46).

To determine whether tetracycline resistance could be restored in the FRStet12 Δ tetA(46) and FRStet12 Δ tetB(46) mutants, a chromosome repair was carried out. The wild-type tetA(46) and tetB(46) genes were cloned into pVA838 to create the recombinant plasmids pABC1 and pABC2, respectively. In three independent experiments, transformation of pABC1 into FRStet12 Δ tetA(46) resulted in colonies on agar containing 5 mg/L tetracycline, whereas transformation with pVA838 alone did not. Sequence analysis of two tetracycline-resistant transformants from each of the three independent experiments, using primers flanking tetA(46) in the chromosome, revealed that in each case the wild-type tetA(46) had replaced the mutant allele within the chromosome, demonstrating that wild-type tetA(46) is required for tetracycline resistance. The transformants contained empty pVA838 and the mutant Δ tetA(46) allele was not detectable by PCR. Transformation of pABC2 into FRStet12 Δ tetB(46) restored the ability of the mutant strain to grow on 5 mg/L tetracycline, whereas transformation with pVA838 did not. Sequence analysis of these transformants confirmed that wild-type tetB(46) had replaced the mutant allele within the chromosome, demonstrating that wild-type tetB(46) is also essential for tetracycline resistance.

The MIC of tetracycline was determined for *S. australis* FRStet12, *S. australis* FRStet12 containing pVA838, the Δ tetA(46) and Δ tetB(46) isogenic mutants and their corresponding complemented strains. The MIC for the wild-type and the complemented strains was 8 mg/L, compared with <0.25 mg/L

Table 2. MICs of tetracyclines for *S. australis* FRStet12 and the tetAB(46) mutant strains

Strain	Oxytetracycline	Doxycycline	Chlortetracycline	Tigecycline
FRStet12	8	0.5	4	1
FRStet12 Δ ABC1	1	<0.25	0.5	<0.25
FRStet12 Δ ABC2	2	<0.25	0.5	<0.25

for the Δ tetA(46) and Δ tetB(46) mutants. MIC determination of the other tetracyclines (Table 2) showed that the mutants were 2- to 8-fold more sensitive to oxytetracycline, doxycycline, chlortetracycline and tigecycline, indicating that TetAB(46) is also able to export these molecules. As MDR-type transporters often are capable of extrusion of other toxic compounds,^{21,22,24} we determined the MIC of acriflavine and ethidium bromide but found no difference between the wild-type and mutant strains. In addition, we found no difference in the zone size in disc diffusion assays with ciprofloxacin, metronidazole, azithromycin, ampicillin, methicillin, oxacillin, penicillin or gentamicin, indicating that TetAB(46) is specific for the transport of tetracyclines.

To determine whether similar genes were present in other bacteria, BLAST searches were performed. This analysis revealed orthologues of TetAB(46) in the one draft genome of *S. australis* present in the database (NCTC 13166) and in all six of the *Streptococcus parasanguinis* draft genomes present in the database (ATCC 903, ATCC 15912, F0405, F0449, FW213 and SK236). The predicted proteins within these streptococcal genomes share \geq 95% amino acid identity with TetA(46) and TetB(46). Since both *S. australis* NCTC 13166 and *S. parasanguinis* NCTC 55898 (ATCC 15912) are resistant to tetracycline (2 mg/L), it is possible that these tetAB(46) orthologues confer this phenotype.

Discussion

Most tetracycline resistance genes reported in oral bacteria encode RPPs,^{6,27,28} whereas efflux genes are rarely detected. Here, we report the discovery of a predicted tetracycline efflux determinant, tetAB(46) from *S. australis*. This species, first isolated from the oral cavities of children in Australia,²⁹ is an opportunist pathogen reported in sputum samples of adult cystic fibrosis patients³⁰ and in a case of invasive infection, a community-acquired meningitis in an elderly patient.³¹

Most tetracycline efflux proteins belong to the MFS family of transporters, which are membrane located and exchange a proton for a tetracycline-cation complex against a concentration gradient. These have been described in both Gram-positive and Gram-negative bacteria.⁷ ABC transporters conferring resistance to tetracyclines have also been reported. There is one example currently in the tet nomenclature database: OtrC from *S. rimosus*,⁴ which consists of an NBD and an MSD encoded by separate genes. While other ABC transporters capable of exporting tetracycline have been reported, e.g. SmdAB in *S. marcescens*,²² these are capable of exporting a number of other compounds

and have not therefore been given a tetracycline resistance gene designation.

Analysis of *S. australis* FRStet12 revealed two orfs responsible for tetracycline resistance, which are most closely related to YheI and YheH of *B. subtilis*. YheI and YheH are non-identical ABC transporter subunits, each containing an NBD and an MSD, which were shown to interact to form a heterodimeric multidrug ABC transporter capable of transporting several structurally dissimilar drugs, such as fluorescently labelled ethidium bromide and daunomycin.²¹ Another related MDR transporter is LmrCD of *L. lactis*, also shown to be capable of extrusion of a range of structurally unrelated drugs.²⁴ Expression of *E. faecalis* EfrA and EfrB together in *E. coli* confers resistance to a range of drugs, including acriflavine, norfloxacin and doxycycline. Further, energy-dependent efflux of acriflavine in *E. coli* harbouring *efrAB* was also demonstrated.²⁵ MDR-type ABC transporters from *S. marcescens* and *E. coli* are also related to TetAB(46): although there are no functional data for the *E. coli* system,²³ SmdAB of *S. marcescens* has been shown to confer multidrug, including tetracycline, resistance on *E. coli* and to be inhibited by ATPase inhibitors.²²

Of the tetracycline (*tet*) and oxytetracycline (*otr*) resistance genes currently listed in the tetracycline gene nomenclature database (<http://faculty.washington.edu/marilynr/>), 28 code for active efflux, 12 for ribosomal protection, 3 for enzymatic drug inactivation and 1 has an unknown mechanism. The two genes required for tetracycline resistance in *S. australis* FRStet12 were designated *tetA*(46) and *tetB*(46) under the current naming standards.²⁰

In-frame deletions in either *tetA*(46) or *tetB*(46) demonstrated that both were required for tetracycline resistance in *S. australis*. The fact that the genes encode non-identical proteins, each containing a predicted MSD and NBD, suggests that they may function as a heterodimeric ABC transporter, although confirmation of this requires demonstration of a physical interaction between the two proteins, experiments that are beyond the scope of the present study.

During the course of this work, we made two observations on the molecular biology of *S. australis* that are worthy of discussion. Firstly, we report the maintenance of pVA838 after multiple passages in the absence of selection, which is unusual in our experience with this plasmid in other streptococci. The stability of 'empty' pVA838 in the mutant strains may have contributed to the second unexpected result obtained in this work: in experiments designed to introduce the wild-type alleles *in trans*, transformation of both mutants with the wild-type alleles cloned in pVA838 resulted in chromosome repair, i.e. double crossover recombination occurred, resulting in replacement of the mutant allele with the wild-type allele in the chromosome, and this restored the tetracycline resistance phenotype. However, instead of detecting the mutant allele in the plasmid as expected following double crossover, we detected only pVA838 without an insert in these strains and did not detect the presence of the mutant alleles. This result was reproduced in triplicate for both mutants. One explanation is that following allelic exchange, the plasmid carrying the mutant allele was lost from the population because of the fitness disadvantage associated with its replication, compared with replication of the plasmid without the insert.

Despite homology to proven multidrug transporters, TetA(46) and TetB(46) were found to confer resistance only to tetracyclines.

The MFS efflux systems transport only the naturally occurring tetracyclines, with the exception of TetA(B), which also transports the semi-synthetic analogue, minocycline.⁷ The reduced MICs in the *tetA*(46) and *tetB*(46) mutants indicate that TetAB(46) is able to transport both tetracycline and its semi-synthetic derivatives. We have previously shown that *S. australis* FRStet12 is sensitive to minocycline (MIC of 0.25 mg/L).³² Since MDR-type ABC transporters have been shown to extrude toxic molecules other than antibiotics,^{24,25} we determined the MICs of acriflavine and ethidium bromide, but found no difference between the wild-type and mutant strains. Thus, our data suggest that TetAB(46) is specific for tetracyclines.

In conclusion, we have identified a novel tetracycline resistance determinant, *tetAB*(46), in an oral viridans *Streptococcus* species. TetAB(46) is related to known MDR-type ABC transporters in both Gram-positive and Gram-negative bacteria, but confers resistance only to tetracyclines. Genes highly related to *tetAB*(46) are present in the genomes of other tetracycline-resistant oral streptococci, including several strains of *S. parasanguinis* and another strain of *S. australis*.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figure S1 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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