RESEARCH LETTER

# Minocycline resistance in an oral *Streptococcus infantis* isolate is encoded by *tet*(S) on a novel small, low copy number plasmid

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## Abstract

We have determined the genetic basis of minocycline resistance in a strain of *Streptococcus infantis* isolated from a healthy human oral cavity. We demonstrate that *tet*(S), identical to *tet*(S) found on the enterococcal conjugative transposon Tn*6000*, is responsible for the observed resistance. The gene is located on a small, low copy number plasmid and is flanked by IS*1216* elements. The *tet*(S) gene is capable of excising from the plasmid together with one of the IS*1216* elements. The plasmid contains a putative toxin/antitoxin system related to *relBE*. Deletion of the toxin, *relE*, did not result in plasmid instability but did increase the fitness of the mutant compared to the wild-type strain.

## Introduction

Antibiotic resistance is one of the biggest challenges currently facing modern health care. Excessive and inappropriate use of antibiotics has resulted in a high selective pressure for bacteria to either evolve or acquire resistance genes (Andersson, 2003).

The oral cavity of healthy humans is a reservoir for antibiotic resistance genes (Seville *et al.*, 2009; Roberts & Mullany, 2011), and resistance to antimicrobials such as tetracyclines (Lancaster *et al.*, 2003; Villedieu *et al.*, 2003), macrolides (Villedieu *et al.*, 2004; Seville *et al.*, 2009) and amoxicillin (Ready *et al.*, 2004) has been found.

Tetracycline resistance is particularly common in the oral cavity microbial communities (Villedieu *et al.*, 2003; Seville *et al.*, 2009; Ciric *et al.*, 2012), and the genes primarily responsible are those encoding ribosomal protection proteins (RPPs), which also confer resistance to minocycline (Seville *et al.*, 2009). Of these RPP-encoding genes, *tet*(M) is the most common and is usually found within conjugative transposons of the Tn*916* family (Seville *et al.*, 2009; Roberts & Mullany, 2011; Ciric *et al.*, 2012). Another RPP gene, *tet*(S), which is found much less often in the human oral cavity (Seville *et al.*, 2009), shows 79% amino acid identity with *tet*(M) (Charpentier *et al.*, 1993). The *tet*(S) gene was originally found in *Listeria monocytogenes* (Charpentier *et al.*, 1993) and was recently discovered within a conjugative transposon of the Tn*916* family (designated Tn*6000*) from *Enterococcus casseliflavus* (Roberts *et al.*, 2006; Brouwer *et al.*, 2010). Recently, *tet*(S) has been reported in a variety of *Enterococcus* spp., often on a derivative of Tn*6000* and often associated with the insertion sequence IS*1216* (Novais *et al.*, 2012).

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In this work, we have determined the molecular basis of tetracycline resistance in a *Streptococcus infantis* isolate from the oral cavity of a healthy human. We show *tet*(S) is present on a small, low copy number plasmid and is capable of excising from the plasmid with one of the two IS*1216* elements which flank it and likely mediate its excision.

## Materials and methods

### Sampling

Saliva was obtained from 10 healthy volunteers who had not received antibiotics in the previous 3 months as previously described (Ciric *et al.*, 2011). Ethical approval for the project was obtained from the University College London (UCL) Ethics Committee (Project No. 1364/001).

### Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table 1. *Streptococcus infantis* C.MI.8 was cultured in 5% $CO_2$ on Columbia blood agar (Oxoid) containing 4 mg $L^{-1}$ minocycline at 37 °C. *Streptococcus australis* FRStet12 was cultured under the same conditions without antibiotics. *Escherichia coli* was grown on or in LB agar or broth containing 100 mg $L^{-1}$ ampicillin when required.

**Table 1.** Bacterial strains and plasmids used in this study

| Bacterial strain name | Comments | Reference or source |
|---|---|---|
| *Streptococcus infantis* C.MI.8 | Original isolate containing pSI01. $Min^R$ | This study |
| *Streptococcus australis* FRStet12 | Recipient strain. $Tet^R$, $Min^S$ | Seville (2007) |
| *Streptococcus australis* FRStet12::pSI01 | Recipient strain transformed with pSI01. $Min^R$, $Tet^R$ | This study |
| *Streptococcus australis* FRStet12::pSI01Δ*relE* | Recipient strain transformed with pSI01Δ*relE*. $Min^R$, $Tet^R$ | This study |
| *Escherichia coli* Alpha-Select Bronze | Used for general cloning | Bioline |
| Plasmids | | |
| pGEM T-easy | Vector for TA cloning. $Amp^R$ | Promega |
| pSI01 | *S. infantis* C.MI.8 plasmid. $Min^R$ | This work |
| pSI01Δ*relE* | *S. infantis* C.MI.8 plasmid *relE* knockout. $Min^R$ | This work |

Min, minocycline; Tet, tetracycline; Rif, rifampicin; Fus, fusidic acid; Strep, streptomycin; R, resistant; S, susceptible; TA, toxin–antitoxin.

### DNA extraction

Total genomic DNA was extracted using the ArchivePure DNA Yeast and Gram Positive Bacteria Kit (5Prime) according to the manufacturer's instructions. Mutanolysin at a final concentration of 15 μg $mL^{-1}$ and lysozyme at a final concentration of 3 μg $mL^{-1}$ were added at the cell lysis step of the protocol. DNA was stored at −20 °C. Plasmid DNA extractions were performed using the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions with the following modification; mutanolysin and lysozyme were added to buffer P1 at the above concentrations and the cells were incubated for 1 h at 37 °C before proceeding with the remainder of the protocol. Plasmid DNA was eluted in sterile water heated to 70 °C.

### PCR and sequencing

The sequences of all primers used in the study are shown in Table 2. Standard PCR was carried out using BioMix™ Red (Bioline) and long PCR was carried out with BIO-X-ACT™ Long Mix (Bioline) according to the manufacturer's instructions. RPP PCR using degenerate primers (RPPF and RPPR; Warburton *et al.*, 2009) was used to identify RPP genes within *S. infantis* C.MI.8. The *S. infantis* C.MI.8 isolate was identified by sequencing of the superoxide dismutase gene, *sod*(A), as described by Poyart *et al.* (1998). Single-specific primer (ssp) PCR was performed in order to ascertain the sequence of the DNA flanking the *S. infantis* C.MI.8 RPP gene. All DNA sequencing was carried out at the UCL Wolfson Institute DNA sequencing facility. Quantitative PCR was used to estimate the copy number of the plasmid as described previously (Chen *et al.*, 2011). Reactions were run using the Sensimix SYBR No-ROX kit (Bioline) on the Rotor-Gene 6500 system (QIAGEN). Primers were designed to amplify regions of *c.* 100 bp of the pSI01 *rep* gene and the *S. infantis* C.MI.8 *sod*(A) gene. The copy number was calculated as the mean threshold cycle ($C_T$) of the amplicons of the *sod*(A) gene (single-copy chromosomal gene) compared to the amplicon of the plasmid *rep* gene. The formula $2^{\Delta C_T}$, $\Delta C_T$ being the difference between the threshold cycle number of the reference *sod*(A) gene and that of *rep*.

### Southern blot hybridisation

Southern blot hybridisation was performed on HindIII-digested genomic DNA of *S. infantis* C.MI.8, *S. australis* FRStet12 and *S. australis* FRStet12::pSI01 using a probe specific to the *tet*(S) gene (JX275965, 7148–8174 bp;


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**Table 2.** Primers used in this study

| Name | Sequence (5′–3′) | Gene target | References |
|---|---|---|---|
| RPP-F | CCIGGVCAYATGGAYTTYHTDGC | RPP genes | Warburton *et al.* (2009) |
| RPP-R | CKRAARTCIGMIGGIGTRCTIACHGG | RPP genes | Warburton *et al.* (2009) |
| d1 | CCITAYICITAYGAYGCIYTIGARCC | sod(A) | Poyart *et al.* (1998) |
| d2 | ARRTARTAIGCRTGYTCCCAIACRTC | sod(A) | Poyart *et al.* (1998) |
| Single-specific primer PCR and sequencing primers | | | |
| tet(S)-IS-5R-1 | CGTGAGTGCTCCAATCAGCTGG | | This study |
| tet(S)-IS-5R-2 | GGATTGTAGTAGTCCGGCACG | | This study |
| tet(S)-IS-3R-1 | GCCCAGGTGCGCCTTGTACG | | This study |
| tet(S)-IS-3R-2 | AGCTACTCGTTGTGATAACC | | This study |
| tet(S)-1F | GGAACGCTACATTTGCGAGACTC | | This study |
| tet(S)-2F | GTCTGCTTTCCAACCACGTCGTCC | | This study |
| tet(S)-3F | CCCTTACGGTATCTATGATTTAA | | This study |
| tet(S)-IS-1F | GGTCGTTATACACGGGGCAT | | This study |
| tet(S)-IS-2F | CCAAGCTTCTATTGTGAAGGC | | This study |
| tet(S)-IS-3F | GAACACAAGTTTCCAAGGGC | | This study |
| tet(S)-3R | AGGGCACAGTATCCACCTAGT | | This study |
| tet(S)-1R | CGAGTTTGTGACTGTACTCCATC | | This study |
| tet(S)-2R | CCTGCATCAACATGTGCTAAGATACCG | | This study |
| tet(S)-IS-1R | CAGGTCAATAGGACAACTGTCC | | This study |
| tet(S)-IS-2R | TTACGCACTGCCTCACCCACG | | This study |
| tet(S)-IS-3R | GGGTTTTGCATCAACATATTTAG | | This study |
| tet(S)-IS-4R | GAACGAGCGAGACGTAACCG | | This study |
| tet(S)-IS-5R | GGGATAAAGACACTTCTGACC | | This study |
| tet(S)-IS-6R | ACTTCTCTTACCAATAGCAATGAC | | This study |
| pTETS-1 | CACCAGTATGTATTAAACCATAG | | This study |
| PCR1-1F | GATACCTGAAGCACAAGATG | | This study |
| PCR1-2F | GAGACGAGCGTTCTGACGC | | This study |
| pTETS-2 | CAATGATCTGACGACCAATTTCG | | This study |
| PCR2-1F | GGAAGTGTAGATAGCGGTAC | | This study |
| pTETS-3 | GATAAGGCAGAGCCTGGTGAG | | This study |
| pTETS-4 | GTAGAAGGGGATCACTATCGG | | This study |
| pTETS-5 | TTCCCTTACCGAGTCAGCTTG | | This study |
| pTETS-6 | GATGCTGAGATAGTCATTCTG | | This study |
| IS*1216* element excision | | | |
| tet(S)IS-1 | GCGCAGCTGAAATTATCAGC | | This study |
| tet(S)IS-2 | TCAGGTCAATAGGACAACTG | | This study |
| tet(S)IS-3 | GATGAAGTACAGAGTTACTCG | | This study |
| tet(S)IS-4 | AAGCCTCACTTTCAACACG | | This study |
| Probe amplification for Southern blot | | | |
| ISF | GATAGCAGGAGGAATGACGATG | | Ciric *et al.* (2011) |
| ISR | CCCTTACGGCATCTATGA | | Ciric *et al.* (2011) |
| PCR2-1F | As above | | |
| pTETS-4 | As above | | |
| qPCR primers for copy number | | | |
| rep-F | CTGGATCATACGTGCCAACGAC | | This study |
| rep-R | CGATAGGGCGGTCATCACTCCG | | This study |
| C. MI.8 sodA-F | GCTTGCTGATGTAGAATCTATCC | | This study |
| C. MI.8 sodA-R | GTTGAAGTCACTTCAAGCTTACC | | This study |
| relE-knockout primers | | | |
| relE check F | GATCAGAATGACTATCTCAGCATCA | | This study |
| relE check R | GAGCGTTCAAATCAGCGTTA | | This study |
| mut relE F | AACTGGCTATTGCTTGTTTCAATTATGT | | This study |
| mut relE R | AACTCAATCCCAACCATCCTCTACATTT | | This study |

RPP, ribosomal protection protein.

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primers PCR2-1F and pTETS-4 in Table 2) and the IS*1216* sequence (JX275965, 5719–6437 and 9535–10 253 bp; primers ISF and ISR in Table 2) from pSI01. Probes were labelled and the blots detected with the DIG-High Prime DNA Labelling and Detection Kit (Roche, UK).

### Transformation studies

*Streptococcus infantis* C.MI.8 pSI01 plasmid DNA (20 ng) was added to minocycline-susceptible *S. australis* FRStet12 (Table 1) while competent during the exponential phase of growth (Warburton *et al.*, 2013). Transformants were selected on media containing 4 mg $L^{-1}$ minocycline.

### Minimal inhibitory concentrations

Minimum inhibitory concentrations (MICs) were determined by agar dilution according to the British Society for Antimicrobial Chemotherapy guidelines (http://www.bsac.org.uk/) using minocycline. The concentrations tested ranged from 0.125 to 64 mg $L^{-1}$. These were carried out using *S. infantis* C.MI.8, *S. australis* FRStet12 (used as a control strain as the MIC to minocycline had been previously determined; Ciric *et al.*, 2011) and *S. australis* FRStet12::pSI01. Iso-sensitest agar containing 5% blood was used and the plates were incubated at 37 °C for 48 h.

### Plasmid stability and mutation of *relBE*

To assess the contribution of the putative RelBE toxin–antitoxin (TA) system to the stability of pSI01, a deletion was made in the *relE* toxin component using the Phusion Site-Directed Mutagenesis Kit (Thermo Scientific). The mutant plasmid pSI01Δ*relE* was transformed into *S. australis* FRStet12. PCR across the deletion was performed (primer pair: relEcheck F + R), and the products of appropriate length were sequenced to confirm the deletion of *relE*.

To assess the stability of pSI01, *S. infantis* C.MI.8, *S. australis* FRStet12::pSI01 and FRStet12::pSI01Δ*relE* were cultured without selection for minocycline resistance in BHI broth for 24 h. Dilutions of the cultures were spread onto BHI and 5% defibrinated horse blood without minocycline. After 24 h, 100 single colonies were picked from nonselective plates onto plates containing minocycline (4 mg $L^{-1}$). The minocycline plates were cultured for 48 h, and the proportion of resistant colonies was determined. Colony PCR was performed to establish whether both *tet*(S) and the plasmid backbone were present.

In subsequent experiments, FRStet12::pSI01 and FRStet12::pSI01Δ*relE* were serially cultured in antibiotic-free BHI broth for 5 and 10 days. A 1 : 10 dilution of the culture was made with fresh broth every 24 h. Stability of the plasmids in these experiments was determined as above.

### Competitive fitness experiments

Overnight cultures of *S. australis* FRStet12::pSI01 and FRStet12::pSI01Δ*relE* were diluted to $OD_{600}$ of 0.1, mixed and grown in competition for 5 h. Dilutions of the cultures were then spread onto BHI and 5% defibrinated horse blood without minocycline, and after 24 h, 100 colonies were picked from nonselective plates onto plates containing minocycline (4 mg $L^{-1}$). After 24 h, the ratio of wild-type and RelE mutant colonies was determined by PCR across the *relE* region of the plasmid. The size difference in the PCR product identified whether either the wild-type or mutant *relE* containing plasmid was present.

## Results

### Isolation and genotypic characterisation of pSI01

A minocycline-resistant (MIC $\geq$ 32 mg $L^{-1}$) *Streptococcus* strain designated C.MI.8 was isolated from pooled saliva collected from healthy volunteers. The strain was identified as *S. infantis* by the sequence of the superoxide dismutase gene, *sod*(A) (ID 98.39%, JN181310).

To determine whether an RPP gene was responsible for minocycline resistance, PCR using degenerate primers designed to detect RPP genes was performed. The resulting amplicon was sequenced and shown to be identical to *tet*(S) from the conjugative transposon Tn*6000* (FN555436; Roberts *et al.*, 2006; Brouwer *et al.*, 2010).

The DNA sequence flanking *tet*(S) was determined and this demonstrated that the gene was contained on a plasmid which we designated pSI01 (Fig. 1 and Table 3. Accession number JX275965). The plasmid had an overall GC content of 34.73%, which was consistent throughout the ORFs; it possesses a predicted TA addiction system with homology to the *relBE* module found in the *Streptococcus parasanguinis* plasmid pFW213 (EU685104, 99.27%). The antitoxin *relB* differs from that of pFW213 by two SNPs and the toxin *relE* by one SNP. The SNPs result in one amino acid sequence change in RelB (S52F) and no amino acid changes in RelE. *Orf3* is predicted to encode a hypothetical protein that has 91.41% amino acid similarity to a hypothetical protein found in *S. infantis* ATCC 700779 (ZP_08062518). *rep* encodes a predicted replication initiation protein belonging to the Rep3


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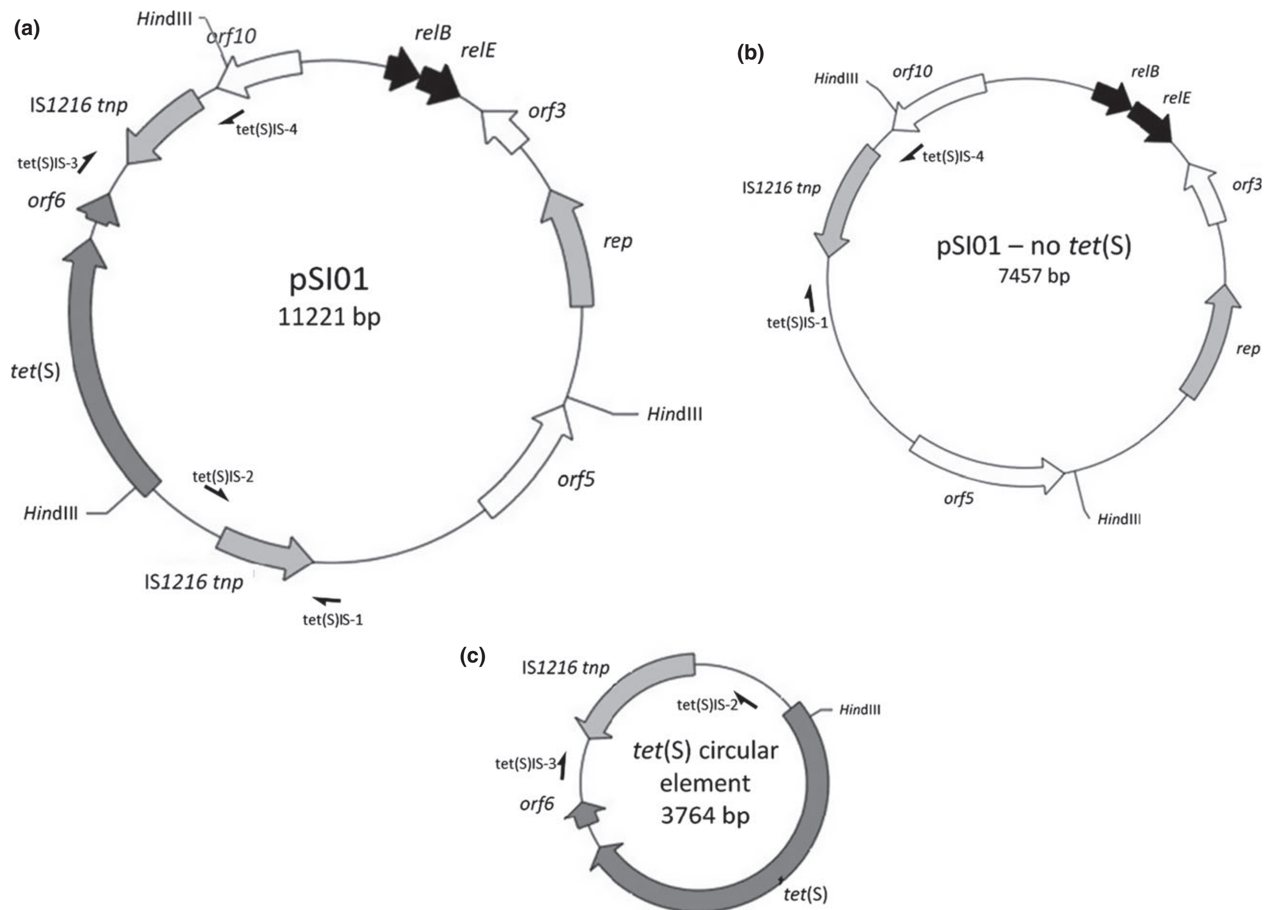


**Fig. 1.** (a) Schematic of pSI01. (b, c) The recombinant forms of the plasmid detected by PCR.

**Table 3.** Details of predicted ORFs on pSI01

| Coding region | Position on pSI01 (bp) | Size (bp) | Strand | Closest homologue (BlastN) | Closest homologue (BlastX) | Percentage identity and coverage (%) | Accession numbers |
|---|---|---|---|---|---|---|---|
| *relB* | 432–680 | 249 | + | RelB – addiction module antitoxin | – | 98.80, 100 | EU685104 |
| *relE* | 671–976 | 306 | + | RelE – addiction module toxin | – | 99.67, 100 | EU685104 |
| *orf3* | 1155–1550 | 396 | – | – | Hypothetical protein | 91.41, 96 | ZP_08062518 |
| *rep* | 1914–2795 | 882 | – | – | Replication initiation protein – Rep3 superfamily | 89.04, 98 | ZP_08064587 |
| *orf5* | 3501–4433 | 933 | – | – | Hypothetical protein | 48.28, 92 | EHC01600 |
| IS*1216 tnp* | 5738–6424 | 687 | – | IS*1216* transposase | – | 99.42, 100 | CP002208 |
| *tet*(S) | 7034–8974 | 1941 | + | Tet(S) – tetracycline resistance protein | – | 100, 100 | FN555436 |
| *orf6* | 9098–9229 | 132 | + | Tn*916*-like Orf6 – conjugative transposon protein | – | 100, 100 | FN555436 |
| IS*1216 tnp* | 9554–10 240 | 687 | – | IS*1216* transposase | – | 99.71, 100 | JN172912 |
| *orf10* | 10 410–11 027 | 618 | – | – | Hypothetical protein | 77.56, 99 | ZP_10793128 |

superfamily, which is related (89.04% aa sequence identity) to one found in *S. parasanguinis* ATCC 903 (ZP_08064587) and to (68% aa sequence identity) a proven Rep of *Streptococcus bovis* plasmid pSB01 (BAA35196; Nakamura *et al.*, 2001). *Orf5* encodes another hypothetical protein with identity (48.28% aa sequence) to a hypothetical protein found in *Streptococcus suis* R61 (EHC01600). The plasmid has two IS*1216* sequences that

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flank the *tet*(S) region. The two insertion sequences are not identical (ID 99.63%), differing by three SNPs. The closest homologue of the IS*1216* element which follows *orf5* is found in *Enterococcus faecalis* strain EF-01 plasmid pEF-01 (ID 99.42%, CP002208). The IS*1216* element closest to *orf10* is highly similar to one found in the *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* plasmid pVF22 (ID 99.71%, JN17912). The region encoding *tet*(S) flanked by the two IS*1216* elements is almost identical to the *E. casseliflavus* conjugative transposon Tn*6000* (ID 99.93%, FN555436). This region includes the *tet*(S) gene and a Tn*916*-like *orf6*. Finally, *orf10* is predicted to encode a hypothetical protein similar to one found in *Streptococcus ratti* FA-1 (aa ID 77.56%, ZP_10793128).

qPCR was performed in order to estimate the copy number of pSI01 in *S. infantis* C.MI.8. As the efficiency of both plasmid and chromosomal assays was near identical (*rep*: $M = -3.434$, $R^2 = 0.999$; *sod*(A): $M = -3.401$, $R^2 = 0.997$), the copy number was estimated at two to three copies per cell using the $2^{\Delta C_T}$ method (Pfaffl, 2001).

As there are two near-identical IS*1216* elements flanking *tet*(S), we hypothesised that this could lead to genetic instability. In order to test this, PCRs were carried out to determine whether any genetic rearrangement occurred (see Fig. 1b and c for position of primers and Table 2 for primer sequences). Four PCRs were performed using the following primer combinations: tet(S)IS-1 and tet(S)IS2; tet(S)IS-3 and tet(S)IS-4; tet(S)IS-1 and tet(S)IS-4; and tet(S)IS-2 and tet(S)IS-3. The first two assays yielded the expected products which included the IS*1216* elements and the flanking regions. However, the PCR which was expected to amplify the entire region including the two IS*1216* elements and the *tet*(S) region (using primers tet (S)IS-1 and tet(S)IS-4) resulted in an amplicon of *c.* 1 kb. Subsequent sequencing showed that *tet*(S), *orf6* and one IS*1216* element had excised, leaving the IS*1216* element closest to *orf10* within the replicon (see Fig. 1b). Furthermore, the assay designed to amplify a circular molecule using the primers tet(S)IS-2 and tet(S)IS-3 also resulted in an amplicon of *c.* 1 kb. Sequencing of the amplicon showed that the IS*1216* element closest to *orf5* had excised from pSI01 along with the *tet*(S) region (see Fig. 1c).

Southern blot hybridisation was performed using *S. infantis* C.MI.8 genomic DNA digested with HindIII. Both the *tet*(S) and IS*1216 tnp* genes were used as probes to determine whether copies of either of the genes were found anywhere other than pSI01. One copy of the *tet* (S) gene was found on a 3.4-kb fragment predicted by *in silico* digestion of pSI01 (Fig. 2a). Similarly, two copies of the IS*1216 tnp* gene were found on two fragments 3.7 and 3.4 kb in size, as predicted by *in silico* digestion (Fig. 2b). Therefore, the recombinant forms of the plasmid that were detected by PCR (shown in Fig. 1b and c) were not detectable in the genomic DNA extracted from the original *S. infantis* C.MI.8 isolate, suggesting that these molecules are rare.

## pSI01 can transfer by transformation

pSI01 was transferred by transformation using plasmid DNA and the minocycline-susceptible recipient *S. australis* FRStet12. The transformation rate was *c.* $8.9 \times 10^{-5}$ transformants per microgram of plasmid DNA. Four minocycline-resistant transformants were picked and screened for the presence of pSI01 by PCR amplification of *tet*(S). The *sod*(A) sequences of the four transformants were found to be identical to that of the recipient and different from the donor.

*Streptococcus infantis* C.MI.8 had an MIC of 32 mg $L^{-1}$. The transfer of pSI01 into *S. australis* FRStet12 increased its minocycline MIC from 0.25 to 32 mg $L^{-1}$, a 128-fold increase.

Southern blot hybridisation of *S. australis* FRStet12::pSI01 genomic DNA digested with HindIII using both the *tet*(S) and IS*1216 tnp* genes as probes showed one 3.4-kb fragment hybridising to *tet*(S) probe and two hybridising fragments of 3.7 and 3.4 kb to IS*1216 tnp* probe.

## pSI01 stability is not affected by *relE* deletion but the mutant has a growth advantage

The stability of pSI01 was tested in the original host *S. infantis* C.MI.8. After 24-h growth in antibiotic-free medium, all 108 colonies tested were resistant to minocycline, showing that the plasmid was 100% stable after 24-h growth (with a detection limit of 0.93%).

Using PCR-based mutagenesis, we constructed pSI01Δ*relE* (which lacks most of the sequence of the toxin component *relE*; bases 683–1029 of pSI01) to study the effect of the toxin on pSI01 stability. pSI01Δ*relE* was transformed into *S. australis* FRStet12 and PCR across the joint of the deleted *relE* and subsequent sequencing of the product confirmed the deletion.

The stability of pSI01 and pSI01Δ*relE* in *S. australis* FRStet12 was tested. After 24-h growth in antibiotic-free medium, cells were plated out onto antibiotic-free agar plates. The following day, 100 colonies were subcultured onto agar containing minocycline. 100% of the colonies containing pSI01 grew up on minocycline plates compared to 99% of colonies containing pSI01Δ*relE* (average of four experiments, detection limit is 1%). The experiment was repeated with cells that were grown in antibiotic-free medium for 5 and 10 days. Again, 100 colonies were subcultured from antibiotic-free agar plates onto


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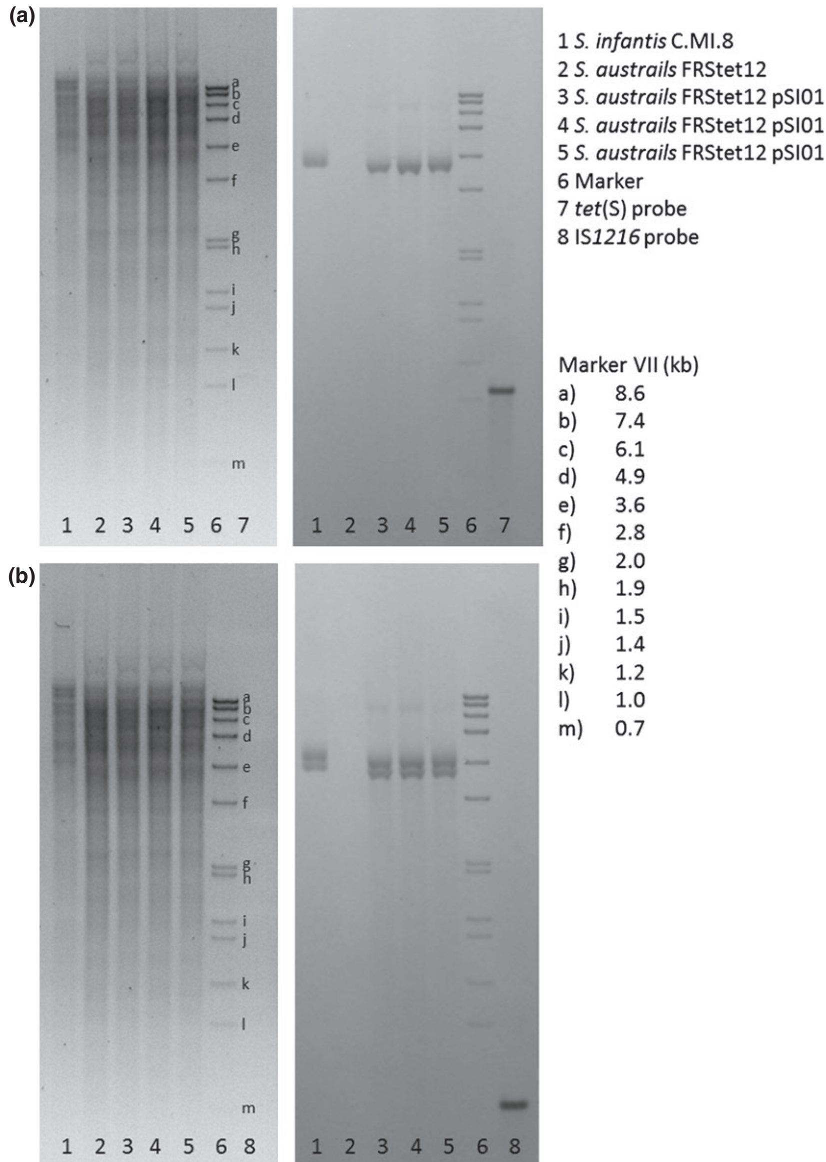


**Fig. 2.** Southern blot analysis of the genomic DNA of *Streptococcus infantis* and *Streptococcus australis* transformants. We did not detect the recombinant forms shown in Fig. 1b and c in the genomic DNA of the original *S. infantis* isolate and the *S. australis* transformants. Hybridisation with (a) the *tet*(S) gene-derived probe and (b) the IS*1216*-derived probe.

agar containing minocycline. When the cells were grown in antibiotic-free medium for 5 days, 100% of the colonies, containing either the wild-type or mutant plasmid, grew on minocycline (experiment performed once, detection limit 1%). When the cells were grown in antibiotic-free medium for 10 days, an average of 98% of colonies grew on minocycline regardless of which plasmid was present (average of three experiments, detection limit 1%).

To test whether all colonies that grew on minocycline plates actually contained the plasmid, PCR was conducted using primers to amplify *relE* or the point where *relE* was deleted, respectively, for pSI01 and pSI01Δ*relE* (primer pair relE check F + R) and to amplify *tet*(S) (RPP-F + R). Both for the cells containing pSI01 and pSI01Δ*relE*, the PCR for *tet*(S) was positive in all colonies. The PCR for *relE* in the cells containing pSI01 was positive in all colonies; however, the PCR across the joint where *relE* was deleted in pSI01Δ*relE* was negative in *c.* 1% of the colonies that contained pSI01Δ*relE*.

To test whether all colonies that grew on minocycline plates actually contained the plasmid, PCR was conducted using primers to amplify part of the plasmid backbone (primer pair relE check F + R). A total of 500 colonies were tested containing either the wild-type or mutant plasmid. These colonies were acquired from the 24-h, 5-day and 10-day experiments in antibiotic-free medium. Three colonies that were minocycline resistant and were

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derived from cells containing the mutant plasmid were negative for the PCR of the plasmid backbone. PCR for *tet*(S) (primers RPP-F + R) was positive on each of these, confirming that minocycline resistance did not occur through spontaneous mutation.

As no clear difference in stability of pSI01 was found, a competition assay was set up using FRStet12 pSI01 and FRStet12 pSI01Δ*relE*. Overnight cultures of both strains were mixed and the mixed culture was incubated for 5 h after which the cells were spread onto antibiotic-free plates. One hundred colonies were subcultured onto plates containing minocycline and colony PCR was performed for *relE* in pSI01 or the junction from where *relE* was deleted in the recombinant plasmid (primer pair relEcheck F + R). The size of the PCR products, *c.* 0.9 kb for pSI01Δ*relE* and 1.2 kb for pSI01, was used to determine which of the plasmids was present, see Table 4. On average, 80% of the colonies contained pSI01Δ*relE* and 20% contained pSI01, based on three independent experiments.

## Discussion

We have shown that *tet*(S) is carried on a novel, small, low copy number plasmid in *S. infantis* C.MI.8 isolated from the human oral cavity. The *rep* of the plasmid is related to the Rep3 superfamily and the *tet*(S) itself is flanked by directly repeated IS*1216* copies. The plasmid is predicted to have a copy number of 2–3 per cell and confers high-level minocycline resistance upon its host.

In the original host strain *S. infantis* C.MI.8, an infrequent recombination event can occur that allows the region of the plasmid containing *tet*(S), *orf6* and one copy of IS*1216* to excise from the plasmid and form a small circular molecule, whereas the remainder of pSI01 circularises but no longer contains a copy of *tet*(S) (Fig. 1b and c). This has important implications for the dissemination of *tet*(S); many different genetic platforms containing *tet*(S) have been found, for example in *Enterococcus* sp. (Novais *et al.*, 2012), and it is likely that *tet*(S) has transferred from the conjugative transposon Tn*6000*, or a derivative, to other DNA molecules catalysed by recombination reactions involving IS*1216* which are very often found associated with it. Additionally, the observation of circular molecules containing resistance genes and single copies of IS elements (or other repeat sequences which flank resistance genes) has been made previously. The tetracycline resistance gene *tet*(W), for example, has been detected on circular molecules in both *Butyrivibrio fibrisolvens* (Kazimierczak *et al.*, 2006) and *Rothia* sp. (Villedieu *et al.*, 2007). Various IS elements, including IS*1216*, have been shown to be responsible for the formation of circular molecules containing resistance genes, for example the multidrug resistance gene *cfr* in *Enterococcus* spp. (Liu *et al.*, 2012, 2013) and *S. suis* (Wang *et al.*, 2013) and the antiseptic resistance gene *qrg* in *Streptococcus oralis* (Ciric *et al.*, 2011). Our observation of the circular molecule containing *tet*(S) and IS*1216* extends the number of resistance genes shown to exist as nonreplicative circular forms and supports the view that these 'unconventional circularisable structures' (Palmieri *et al.*, 2013) may be an evolutionarily important mechanism for the dissemination of resistance genes between different replicons.

pSI01 encodes a TA addiction module with high similarity to the *relBE* module found in the plasmid pFW213 of *S. parasanguinis*. TA addiction modules function to prevent the loss of the element on which they are encoded. After constructing a mutant pSI01 that does not contain the *relE* toxin component, stability was tested in *S. australis* FRStet12. We could not find any difference in the stability of pSI01Δ*relE* compared to pSI01 over a 10-day period of continuous subculturing.

During the stability assays of pSI01 and pSI01Δ*relE* in *S. australis* FRStet12, it was observed occasionally that PCRs on colonies were positive for *tet*(S), whereas they were negative for the plasmid backbone. It is hypothesised that after recombination the circular element containing *tet*(S) has integrated into the chromosome in these colonies. These pSI01 backbone negative colonies were only found in the cultures that originally contained pSI01Δ*relE*, which may indicate that plasmid stability is mildly affected or the recombination events take place somewhat more often for the mutant plasmid.

A competition assay was set up between *S. australis* FRStet12 pSI01 and FRStet12 pSI01Δ*relE*. The starter culture for the assay contained equal numbers of both cell types but after 5 h 80% of the mixed cell culture consisted of FRStet12 pSI01Δ*relE*. These results indicate that the toxin mutant strain had a growth advantage over the strain containing the wild-type pSI01, which indicates that the production of the toxin itself may have a considerable cost to the cell or that the effects of the toxin, despite the presence of the antitoxin, slow down the growth rate of the host cells.

**Table 4.** Results of competition assays between pSI01 and pSI01Δ*relE* in *Streptococcus australis* FRStet12. PCR was performed on 100 minocycline-resistant colonies in three independent experiments

| | Experiment 1 | Experiment 2 | Experiment 3 | Average (%) |
|---|---|---|---|---|
| pSI01 | 21 | 19 | 20 | 20 |
| pSI01Δ*relE* | 79 | 81 | 80 | 80 |


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In conclusion, we have determined the genetic basis of minocycline resistance in a human-derived oral *S. infantis* isolate. We have shown that it is due to *tet*(S) present on a novel low copy number plasmid flanked by IS*1216* elements. It is capable of excising, together with *orf6* and one of the IS*1216* into a free, presumably nonreplicative circular form. We suspect that this type of recombination between IS*1216* elements associated with *tet*(S) is aiding the dissemination of *tet*(S) amongst different DNA molecules.

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