



RESEARCH LETTER

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

Lena Ciric, Michael S. M. Brouwer, Peter Mullany & Adam P. Roberts

Department of Microbial Diseases, UCL Eastman Dental Institute, University College London, London, UK

Correspondence: Adam P. Roberts,
Department of Microbial Diseases, University
College London, UCL Eastman Dental
Institute, 256 Gray's Inn Road, London WC1X
8LD, UK. Tel.: +00 44 020 3456 1044;
fax: +00 44 020 3456 1127;
e-mail: adam.roberts@ucl.ac.uk

Present addresses: Lena Ciric, Department
of Civil, Environmental and Geomatic
Engineering, University College London,
Gower Street, London WC1E 6BT, UK
Michael S. M. Brouwer, Central Veterinary
Institute of Wageningen UR, PO Box 65,
8200 AB, Lelystad, The Netherlands

Received 3 February 2014; revised 25
February 2014; accepted 25 February 2014.
Final version published online 19 March 2014.

DOI: 10.1111/1574-6968.12410

Editor: Stefan Schwarz

Keywords

IS1216; Tn6000; toxin–antitoxin; *relBE*.

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

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 Tn6000, is responsible for the observed resistance. The gene is located on a small, low copy number plasmid and is flanked by IS1216 elements. The *tet(S)* gene is capable of excising from the plasmid together with one of the IS1216 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.

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 Tn916 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 Tn916 family (designated Tn6000) 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 Tn6000 and often associated with the insertion sequence IS1216 (Novais *et al.*, 2012).

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 *IS1216* 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% CO₂ 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
<i>Streptococcus infantis</i> C.MI.8	Original isolate containing pSI01. Min ^R	This study
<i>Streptococcus australis</i> FRStet12	Recipient strain. Tet ^R , Min ^S	Seville (2007)
<i>Streptococcus australis</i> FRStet12::pSI01	Recipient strain transformed with pSI01. Min ^R , Tet ^R	This study
<i>Streptococcus australis</i> FRStet12::pSI01Δ <i>relE</i>	Recipient strain transformed with pSI01Δ <i>relE</i> . Min ^R , Tet ^R	This study
<i>Escherichia coli</i> Alpha-Select Bronze	Used for general cloning	Bioline
Plasmids		
pGEM T-easy	Vector for TA cloning. Amp ^R	Promega
pSI01	<i>S. infantis</i> C.MI.8 plasmid. Min ^R	This work
pSI01Δ <i>relE</i>	<i>S. infantis</i> C.MI.8 plasmid <i>relE</i> 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 BioMixTM Red (Bioline) and long PCR was carried out with BIO-X-ACTTM 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 $2^{\Delta C_T}$, ΔC_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;

Table 2. Primers used in this study

Name	Sequence (5'–3')	Gene target	References
RPP-F	CCIGGVCAAYATGGAYTTYHTDGC	RPP genes	Warburton <i>et al.</i> (2009)
RPP-R	CKRAARTCIGMIGGIGTRCTIACHGG	RPP genes	Warburton <i>et al.</i> (2009)
d1	CCITAYICITAYGAYGCIYTIGARCC	sod(A)	Poyart <i>et al.</i> (1998)
d2	ARRTARTAIGCRTGYTCCCAIACRTC	sod(A)	Poyart <i>et al.</i> (1998)
Single-specific primer PCR and sequencing primers			
tet(S)-IS-5R-1	CGTGAGTGCTCCAATCAGCTGG		This study
tet(S)-IS-5R-2	GGATTGTAGTAGTCCGGCAGC		This study
tet(S)-IS-3R-1	GCCCAGGTGCGCCTTGATCC		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	GGTCGTATACACGGGGCAT		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	CAGGTCAATAGGACAACGTGCC		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	ACTTCTTACCAATAGCAATGAC		This study
pTETS-1	CACCAGTATGTATTAACCATAG		This study
PCR1-1F	GATACCTGAAGCACAAGATG		This study
PCR1-2F	GAGACGAGCGTTCTGACGC		This study
pTETS-2	CAATGATCTGACGACCAATTTCC		This study
PCR2-1F	GGAAGTGTAGATAGCGGTAC		This study
pTETS-3	GATAAGGCAGAGCCTGGTGAG		This study
pTETS-4	GTAGAAGGGGATCACTATCGG		This study
pTETS-5	TTCCCTTACCGAGTCAGCTTG		This study
pTETS-6	GATGCTGAGATATCATTCTG		This study
IS1216 element excision			
tet(S)IS-1	GCGCAGCTGAAATTATCAGC		This study
tet(S)IS-2	TCAGGTCAATAGGACAACCTG		This study
tet(S)IS-3	GATGAAGTACAGAGTTACTCG		This study
tet(S)IS-4	AAGCCTCACTTCAACACG		This study
Probe amplification for Southern blot			
ISF	GATAGCAGGAGGAATGACGATG		Ciric <i>et al.</i> (2011)
ISR	CCCTTACGGCATCTATGA		Ciric <i>et al.</i> (2011)
PCR2-1F	As above		
pTETS-4	As above		
qPCR primers for copy number			
rep-F	CTGGATCATACTGCCAACGAC		This study
rep-R	CGATAGGGCGGTCACTACCCG		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	AACTGGCTATTGCTTGTTCGAATTATGT		This study
mut relE R	AACTCAATCCCAACCATCCTCTACATTT		This study

RPP, ribosomal protection protein.

primers PCR2-1F and pTETS-4 in Table 2) and the IS1216 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 OD₆₀₀ 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 ≥ 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 Tn6000 (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

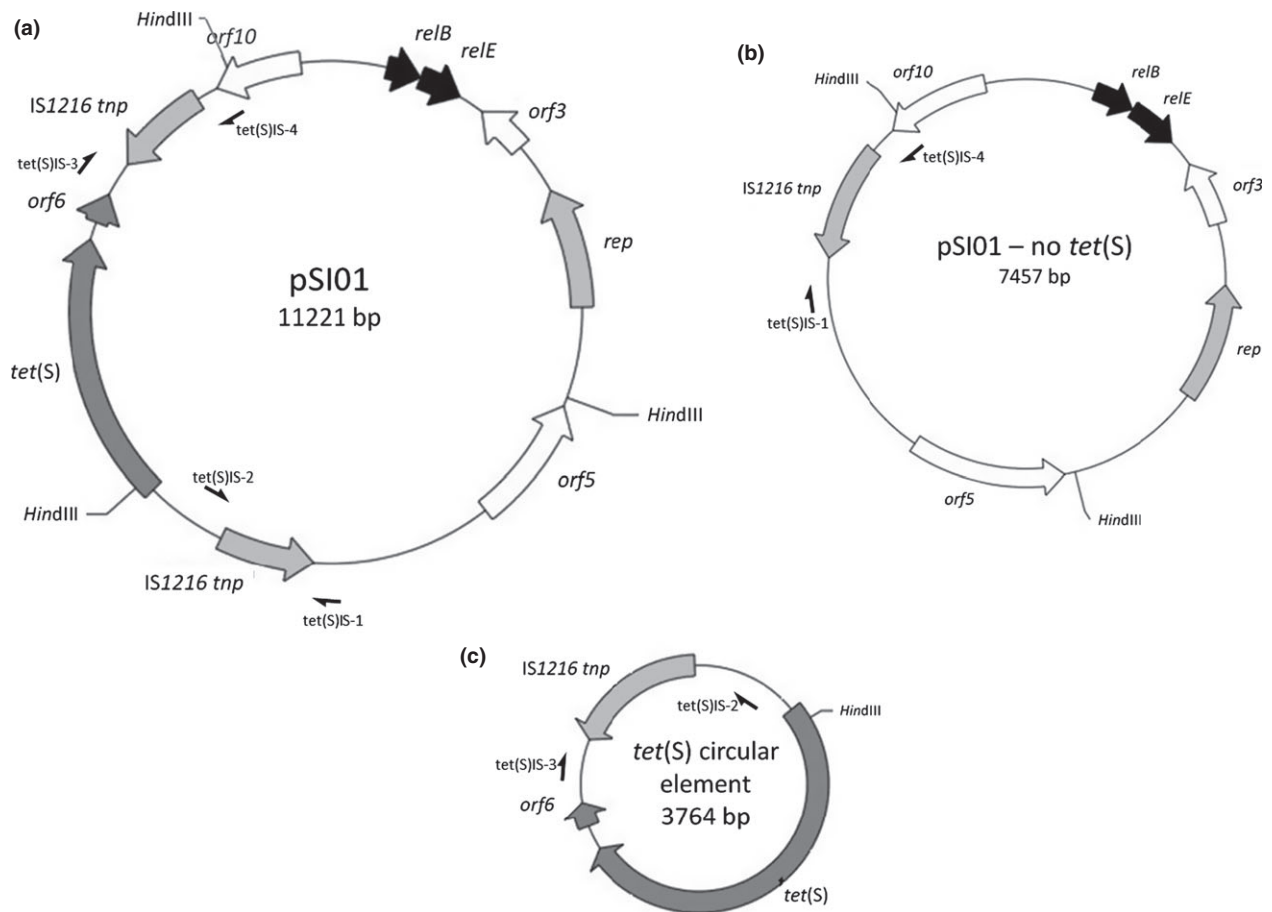


Fig. 1. (a) Schematic of pSI01. (b, c) The recombinant forms of the plasmid detected by PCR.

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
<i>relB</i>	432–680	249	+	RelB – addiction module antitoxin	–	98.80, 100	EU685104
<i>relE</i>	671–976	306	+	RelE – addiction module toxin	–	99.67, 100	EU685104
<i>orf3</i>	1155–1550	396	–	–	Hypothetical protein	91.41, 96	ZP_08062518
<i>rep</i>	1914–2795	882	–	–	Replication initiation protein – Rep3 superfamily	89.04, 98	ZP_08064587
<i>orf5</i>	3501–4433	933	–	–	Hypothetical protein	48.28, 92	EHC01600
<i>IS1216 tnp</i>	5738–6424	687	–	<i>IS1216</i> transposase	–	99.42, 100	CP002208
<i>tet(S)</i>	7034–8974	1941	+	Tet(S) – tetracycline resistance protein	–	100, 100	FN555436
<i>orf6</i>	9098–9229	132	+	Tn916-like Orf6 – conjugative transposon protein	–	100, 100	FN555436
<i>IS1216 tnp</i>	9554–10 240	687	–	<i>IS1216</i> transposase	–	99.71, 100	JN172912
<i>orf10</i>	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 *IS1216* sequences that

flank the *tet(S)* region. The two insertion sequences are not identical (ID 99.63%), differing by three SNPs. The closest homologue of the IS1216 element which follows *orf5* is found in *Enterococcus faecalis* strain EF-01 plasmid pEF-01 (ID 99.42%, CP002208). The IS1216 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 IS1216 elements is almost identical to the *E. casseliflavus* conjugative transposon Tn6000 (ID 99.93%, FN555436). This region includes the *tet(S)* gene and a Tn916-like *orf6*. Finally, *orf10* is predicted to encode a hypothetical protein similar to one found in *Streptococcus rattii* 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 $2^{\Delta Ct}$ method (Pfaffl, 2001).

As there are two near-identical IS1216 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 IS1216 elements and the flanking regions. However, the PCR which was expected to amplify the entire region including the two IS1216 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 IS1216 element had excised, leaving the IS1216 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 IS1216 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 IS1216 *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 IS1216 *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.* 8.9×10^{-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 IS1216 *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 IS1216 *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 pSI01 Δ *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. pSI01 Δ *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 pSI01 Δ *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 pSI01 Δ *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

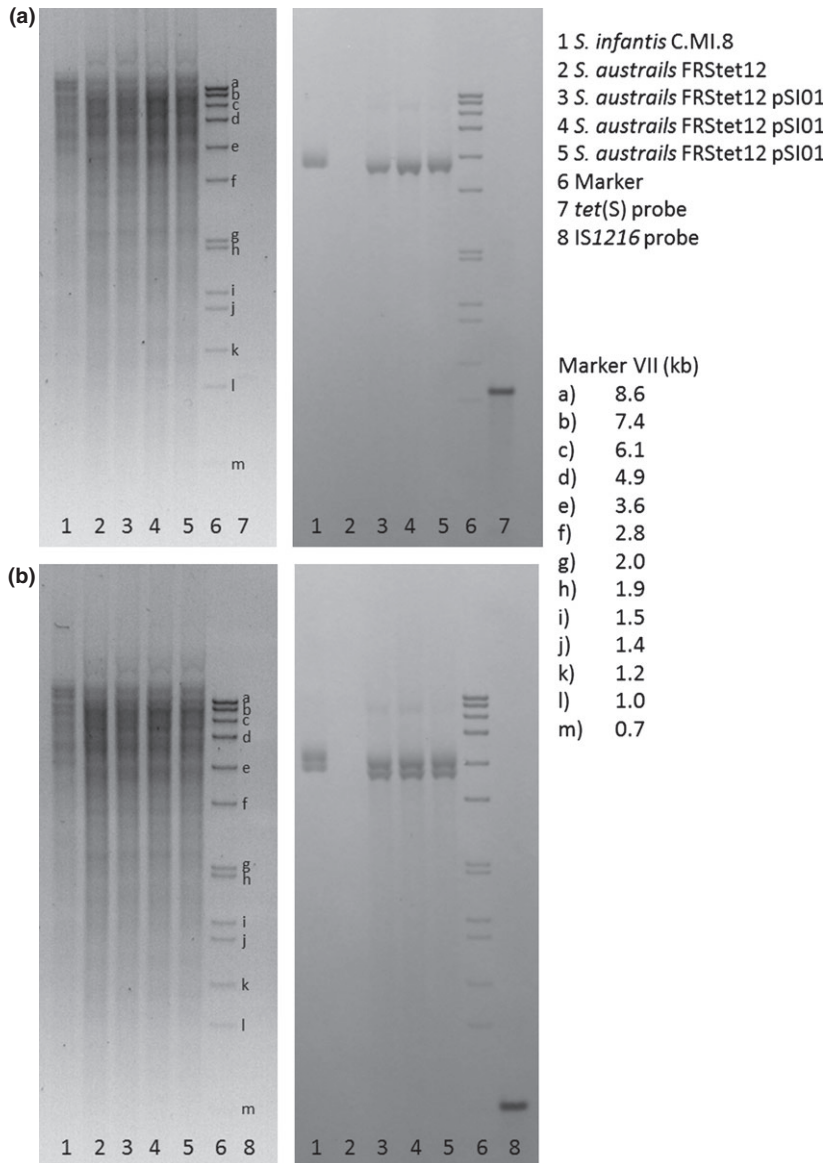


Fig. 2. Southern blot analysis of the genomic DNA of *Streptococcus infantis* and *Streptococcus australis* transformants. We did not detect the recombinant forms shown in Fig. 1b and c in the genomic DNA of the original *S. infantis* isolate and the *S. australis* transformants. Hybridisation with (a) the *tet(S)* gene-derived probe and (b) the IS1216-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 pSI01 Δ *relE* (primer pair *relE* check F + R) and to amplify *tet(S)*

(RPP-F + R). Both for the cells containing pSI01 and pSI01 Δ *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 pSI01 Δ *relE* was negative in *c*. 1% of the colonies that contained pSI01 Δ *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

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 IS1216 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 IS1216 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 Tn6000, or a derivative, to other DNA molecules catalysed by

recombination reactions involving IS1216 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 IS1216, 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 IS1216 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 <i>relE</i>	79	81	80	80

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 IS1216 elements. It is capable of excising, together with *orf6* and one of the IS1216 into a free, presumably nonreplicative circular form. We suspect that this type of recombination between IS1216 elements associated with *tet(S)* is aiding the dissemination of *tet(S)* amongst different DNA molecules.

Acknowledgements

This research was funded by the Commission of the European Communities, specifically the Infectious Diseases research domain of the Health theme of the 7th Framework Programme, contract 241446, 'The effects of antibiotic administration on the emergence and persistence of antibiotic-resistant bacteria in humans and on the composition of the indigenous microbiotas at various body sites'. The authors have no conflict of interest to declare.

References

- Andersson DI (2003) Persistence of antibiotic resistant bacteria. *Curr Opin Microbiol* **6**: 452–456.
- Brouwer MS, Mullany P & Roberts AP (2010) Characterization of the conjugative transposon Tn6000 from *Enterococcus casseliflavus* 664.1H1 (formerly *Enterococcus faecium* 664.1H1). *FEMS Microbiol Lett* **309**: 71–76.
- Charpentier E, Gerbaud G & Courvalin P (1993) Characterization of a new class of tetracycline-resistance gene *tet(S)* in *Listeria monocytogenes* BM4210. *Gene* **131**: 27–34.
- Chen YYM, Shieh HR, Lin CT & Liang SY (2011) Properties and construction of plasmid pFW213, a shuttle vector with the oral *Streptococcus* origin of replication. *Appl Environ Microbiol* **77**: 3967–3974.
- Ciric L, Mullany P & Roberts AP (2011) Antibiotic and antiseptic resistance genes are linked on a novel mobile genetic element: Tn6087. *J Antimicrob Chemother* **66**: 2235–2239.
- Ciric L, Ellatif M, Sharma P, Patel R, Song X, Mullany P & Roberts AP (2012) Tn916-like elements from human, oral, commensal streptococci possess a variety of antibiotic and antiseptic resistance genes. *Int J Antimicrob Agents* **39**: 360–361.
- Kazimierczak KA, Flint HJ & Scott KP (2006) Comparative analysis of sequences flanking *tet(W)* resistance genes in multiple species of gut bacteria. *Antimicrob Agents Chemother* **50**: 2632–2639.
- Lancaster H, Ready D, Mullany P, Spratt D, Bedi R & Wilson M (2003) Prevalence and identification of tetracycline-resistant oral bacteria in children not receiving antibiotic therapy. *FEMS Microbiol Lett* **228**: 99–104.
- Liu Y, Wang Y, Wu C, Shen Z, Schwarz S, Du XD, Dai L, Zhang W, Zhang Q & Shen J (2012a) First report of the multidrug resistance gene *cfr* in *Enterococcus faecalis* of animal origin. *Antimicrob Agents Chemother* **56**: 1650–1654.
- Liu Y, Wang Y, Schwarz S, Li Y, Shen Z, Zhang Q, Wu C & Shen J (2012b) Transferable multiresistance plasmids carrying *cfr* in *Enterococcus* spp. From swine and farm environment. *Antimicrob Agents Chemother* **57**: 42–48.
- Nakamura M, Ogata K, Nagamine T, Tajima K, Matsui H & Benno Y (2001) The replicon of the cryptic Plasmid pSBO1 isolated from *Streptococcus bovis* JB1. *Curr Microbiol* **43**: 11–16.
- Novais C, Freitas AR, Silveira E, Baquero F, Peixe L, Roberts AP & Coque TM (2012) Different genetic supports for the *tet(S)* gene in enterococci. *Antimicrob Agents Chemother* **56**: 6014–6018.
- Palmieri C, Mingoia M & Varaldo PE (2013) Unconventional circularizable bacterial genetic structures carrying antibiotic resistance determinants. *Antimicrob Agents Chemother* **57**: 2440–2441.
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.
- Poyart C, Quesne G, Coulon S, Berche P & Trieu-Cuot P (1998) Identification of streptococci to species level by sequencing the gene encoding the manganese-dependent superoxide dismutase. *J Clin Microbiol* **36**: 41–47.
- Ready D, Lancaster H, Qureshi F, Bedi R, Mullany P & Wilson M (2004) Effect of amoxicillin use on oral microbiota in young children. *Antimicrob Agents Chemother* **48**: 2883–2887.
- Roberts AP & Mullany P (2011) Tn916-like genetic elements: a diverse group of modular mobile elements conferring antibiotic resistance. *FEMS Microbiol Rev* **35**: 856–871.
- Roberts AP, Davis IJ, Seville L, Villedieu A & Mullany P (2006) Characterization of the ends and target site of a novel tetracycline resistance-encoding conjugative transposon from *Enterococcus faecium* 664.1H1. *J Bacteriol* **188**: 4356–4361.
- Seville LA (2007) Searching for novel antibiotic resistance genes and their genetic supports in the oral and faecal microbiota of six European countries. PhD Thesis, University College London, London, UK.
- Seville LA, Patterson AJ, Scott KP, Mullany P, Quail MA, Parkhill J, Ready D, Wilson M, Spratt D & Roberts AP (2009) Distribution of tetracycline and erythromycin resistance genes among human oral and fecal metagenomic DNA. *Microb Drug Resist* **15**: 159–166.
- Villedieu A, Diaz-Torres ML, Hunt N, McNab R, Spratt DA, Wilson M & Mullany P (2003) Prevalence of tetracycline resistance genes in oral bacteria. *Antimicrob Agents Chemother* **47**: 878–882.
- Villedieu A, Diaz-Torres ML, Roberts AP, Hunt N, McNab R, Spratt DA, Wilson M & Mullany P (2004) Genetic basis of

- erythromycin resistance in oral bacteria. *Antimicrob Agents Chemother* **48**: 2298–2301.
- Villedieu A, Roberts AP, Allen E, Hussain H, McNab R, Spratt DA, Wilson M & Mullany P (2007) Determination of the genetic support for *tet(W)* in oral bacteria. *Antimicrob Agents Chemother* **51**: 2195–2197.
- Wang Y, Li D, Song L, Liu Y, He T, Liu H, Wu C, Schwarz S & Shen J (2013) First report of the multiresistance gene *cfr* in *Streptococcus suis*. *Antimicrob Agents Chemother* **57**: 4061–4063.
- Warburton P, Ciric L, Lerner A, Seville LA, Roberts AP, Mullany P & Allan E (2013) TetAB46, a predicted heterodimeric ABC transporter conferring tetracycline resistance in *Streptococcus australis* isolated from the oral cavity. *J Antimicrob Chemother* **68**: 17–22.
- Warburton P, Roberts AP, Allan E, Seville L, Lancaster H & Mullany P (2009) Characterization of *tet(32)* genes from the oral metagenome. *Antimicrob Agents Chemother* **53**: 273–276.