Molecular characterization of a novel mosaic \textit{tet}(S/M) gene encoding tetracycline resistance in foodborne strains of \textit{Streptococcus bovis}

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The presence of antibiotic-resistance (AR) genes in foodborne bacteria of enteric origin represents a relevant threat to human health in the case of opportunistic pathogens, which can reach the human gut through the food chain. \textit{Streptococcus bovis} is a human opportunistic pathogen often associated with infections in immune-compromised or cancer patients, and it can also be detected in the environment, including fermented foods. We have focused on the molecular characterization of a tetracycline (Tet)-resistance gene present in 39 foodborne isolates of \textit{S. bovis} phenotypically resistant to this drug. The gene was identified as a novel \textit{tet}(S/M) fusion, encoding a mosaic protein composed of the N-terminal 33 amino acids of \textit{tet}(S), in-frame with the \textit{tet}(M) coding sequence. Heterologous expression of the mosaic gene was found to confer Tet resistance upon \textit{Escherichia coli} recipients. Moreover, the \textit{tet}(S/M) gene was found to be transcriptionally inducible by Tet under the endogenous \textit{tet}(S) promoter in both \textit{S. bovis} and \textit{E. coli}. Nucleotide sequencing of the surrounding genomic region of 16.2 kb revealed large blocks of homology with the genomes of \textit{Streptococcus infantarius} and \textit{Lactococcus lactis}. A subregion of about 4 kb containing mosaic \textit{tet}(S/M) was flanked by two copies of the IS\textit{1216} mobile element. PCR amplification with primers directed outwards from the \textit{tet}(S/M) gene identified the presence of a 4.3 kb circular form corresponding to the intervening chromosomal region between the two IS\textit{1216} elements, but lacking a replication origin. The circular element shared extensive overall homology with a region of the multidrug-resistance plasmid pK214 from \textit{L. lactis}, containing \textit{tet}(S), as well as the IS\textit{1216} transposase-containing element and intervening non-coding sequences. Linear reconstruction of the insertion events likely to have occurred within this genomic region, inferred from sequence homology, provides further evidence of the chromosomal rearrangements that drive genomic evolution in complex bacterial communities such as the gut and food microbiota.

**INTRODUCTION**

\textit{Streptococcus bovis} is an indigenous resident of the gastrointestinal (GI) tract in humans and animals. It is classified as a member of the group D streptococci, which includes the highly related species \textit{Streptococcus equinus}, \textit{Streptococcus caprinus}, \textit{Streptococcus galalyticus}, \textit{Streptococcus infantarius}, \textit{Streptococcus macedonius} and \textit{Streptococcus waius} (Herrera et al., 2009). Several \textit{Streptococcus} species represent serious invasive pathogens, often associated with wound infections, sepsis, abscesses and dental caries in immunocompromised or cancer patients (Moet et al., 2007; Fernández-Ruiz et al., 2010; Al-Jashamy et al., 2010). The association of \textit{S. bovis} with endocarditis (Gupta et al., 2010), colon cancer (Boleij et al., 2009) and colon adenoma (Kahveci et al., 2010) has also recently been reported. This species is therefore considered a potential pathogen.

The widespread use of antibiotics has applied strong selective pressure in the environment, favouring the survival and spread of antibiotic-resistant (AR) bacterial species. Such environmental selection is reflected in the increasing presence of AR commensal bacteria in the gut microbiota of livestock, which in turn leads to an increased frequency of AR species within the microbiota of fermented foods of animal origin, such as dairy and meat products. AR commensal bacteria do not by themselves represent a threat to human health, but their presence in the gut microbiota of humans and animals is increasingly viewed as a reservoir with the potential of being transmitted to pathogens through genomic exchange (Ammon...
et al., 2007). This is especially relevant for opportunistic pathogens, which are also capable of acquiring virulence genes and are reported to be important agents of AR nosocomial infection. The key role played by mobile elements in AR gene transfer was further emphasized by the publication of the first sequenced genome of a vancomycin-resistant clinical isolate of Enterococcus faecalis, revealing the presence of up to 38 insertion sequences (ISs) (Paulsen et al., 2003). Transposon-mediated inter-species transfer of AR genes has now been further characterized as one of the main mechanisms contributing to AR spread in bacteria (Wozniak & Waldor, 2010).

In the present work we report the molecular characterization of a novel functional mosaic tetracycline (Tet)-resistance gene that was identified in foodborne isolates of S. bovis. We also studied the genomic context surrounding the mosaic gene, whose sequence shares long stretches of homology with the genomes of other streptococcal species.

**METHODS**

**Bacterial strains and growth conditions.** S. bovis strains were cultured in de Man, Rogosa and Sharpe (MRS) medium. Growth conditions and antibiotic concentrations were as previously described (Devirgiliis et al., 2010).

**Cloning procedures.** A 2603 bp DNA fragment containing full-length mosaic tet(S/M) and the 5’ and 3’ untranslated regions was amplified using the TetS/M-F-Sall and TetS/M-R-PstI primers (Table 1). The purified PCR product was digested with SalI and PstI and cloned into pBluescript KS-I and subsequently treated with DNase I (RNase-free). One microgram of genomic DNA was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) in a final volume of 20 µl, using random hexamers as primers. Two microlitres of the reaction mix was then used as template in PCRs with primers amplifying either the 16S rDNA (control) or the tet(S/M) mosaic gene (primers TetS-1-FW and Rev-Ext; Table 1).

**DNA extraction and molecular analysis.** Genomic DNA was extracted using the MagPrep Bacterial Genomic DNA kit (Merck), according to manufacturer’s instructions. PCR amplifications were performed as previously described (Devirgiliis et al., 2008), with the primers listed in Table 1 (Pirmm, Italy). PCR products were purified with the Nucleospin Extract II purification kit (Macherey-Nagel), and sequenced by the M-Medical sequencing service (Italy). Southern hybridizations were carried out using standard protocols, with probes labelled with digoxigenin-11-dUTP (Roche Diagnostics). Restriction endonucleases were purchased from Promega.

**RT-PCR.** Bacterial RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions and subsequently treated with DNase I (RNase-free). One microgram of total RNA was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) in a final volume of 20 µl, using random hexamers as primers. Two microlitres of the reaction mix was then used as template in PCRs with primers amplifying either the 16S rRNA (control) or the tet(S/M) mosaic gene (primers TetS-1-FW and Rev-Ext; Table 1).

**Gene walking.** The two-step gene walking method consisted of a walking-PCR (step 1) followed by direct sequencing of the PCR product (step 2), as described in (Pilhofer et al., 2007). Walking-PCRs were performed in a final volume of 50 µl containing 0.5 µM of the specific primer (Table 1), 2.5 U AccuTaq (Sigma), 1× AccuTaq buffer, 2.5 mM of each dNTP and 50 ng template DNA. The cycling program was the same as that reported in (Pilhofer et al., 2007), except for the annealing temperature of specific primers (see Table 1) and the AccuTaq extension temperature (68 °C). Using this method, overlapping DNA fragments ranging from 3 to 4 kb were obtained with walking primers directed outwards, upstream of tet(S/M) (Rev-Ext, TetS-2-REV, KUP-FW, IS1068-FW, STRINF2-REV in Table 1). The sequence of the region downstream of tet(S/M) was obtained following three rounds of walking with primers Tet-int-FW, Orf6-FW and STRINF1-REV (Table 1). Walking-PCR products were purified using a Nucleospin Extract II kit according to the manufacturer’s instructions and were fully sequenced. Computer-assisted joining of

Table 1. Primers used in PCR amplifications

Nucleotides corresponding to restriction site sequences are in italic type. Primers marked with ‘×’ in the Walking primer column were also used for gene walking.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Target region</th>
<th>Annealing temperature (°C)</th>
<th>Walking primer</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TetS/M-F-Sall</td>
<td>gggcccGTGACTAGCCATTCTGAAGGTTATCTTC</td>
<td>−379ATG tet(S)</td>
<td>54</td>
<td>TetS-1-FW</td>
<td>This work</td>
</tr>
<tr>
<td>TetS/M-R-PstI</td>
<td>gggcccCTGCACAGAATTATCGGCTCTGCGTCTTTGC</td>
<td>Orf6 stop codon</td>
<td>68</td>
<td>TetS-2-REV</td>
<td>This work</td>
</tr>
<tr>
<td>STRINF2-REV</td>
<td>GGGATTCTTCCAATATGAGGACCG</td>
<td>STRINF_00828</td>
<td>68</td>
<td>STRINF2-REV</td>
<td>This work</td>
</tr>
<tr>
<td>IS1068-FW</td>
<td>CCTTGATCTCCTAAASAAAACAAGTG</td>
<td>llkf,p0022</td>
<td>68</td>
<td>IS1068-FW</td>
<td>This work</td>
</tr>
<tr>
<td>KUP-FW</td>
<td>CTTTAATAGGGCCAGATGAGCAG</td>
<td>llkf,p0020</td>
<td>68</td>
<td>KUP-FW</td>
<td>This work</td>
</tr>
<tr>
<td>TetS-1-FW</td>
<td>GCCATACATGCTGTAGTAGGATGATACGC</td>
<td>tet(S) promoter</td>
<td>68</td>
<td>TetS-1-FW</td>
<td>This work</td>
</tr>
<tr>
<td>TetS-2-REV</td>
<td>CCTGTACTACATACACTGATAGGCCACAGATG</td>
<td>tet(S) promoter</td>
<td>68</td>
<td>TetS-2-REV</td>
<td>This work</td>
</tr>
<tr>
<td>Rev-Ext</td>
<td>GCTTTGCTTCTGGCAGGTCCAATGC</td>
<td>tet(M) promoter</td>
<td>68</td>
<td>Rev-Ext</td>
<td>This work</td>
</tr>
<tr>
<td>For-Ext</td>
<td>CATTCAGATGAGCGGCGCAAATCC</td>
<td>tet(M)</td>
<td>68</td>
<td>For-Ext</td>
<td>Devirgiliis et al. (2009)</td>
</tr>
<tr>
<td>Tet-int-FW</td>
<td>CCGATAGATAAAGTGACGATA</td>
<td>tet(M)</td>
<td>54</td>
<td>Tet-int-FW</td>
<td>Devirgiliis et al. (2009)</td>
</tr>
<tr>
<td>Orf6-FW</td>
<td>GTGGATATTGCTGCGATGATGG</td>
<td>Orf6</td>
<td>58</td>
<td>Orf6-FW</td>
<td>This work</td>
</tr>
<tr>
<td>STRINF1-REV</td>
<td>GGAGTTTTCGCTCTCATGATTGG</td>
<td>STRINF_00831</td>
<td>62</td>
<td>STRINF1-REV</td>
<td>This work</td>
</tr>
</tbody>
</table>
the sequenced fragments to obtain the complete sequence was performed using the web resource CLC Sequence Viewer 6.0.2 (www.clcbio.com).

RESULTS

Identification of a novel tet(S/M) mosaic gene

The Tet-resistance determinant in phenotypically resistant foodborne strains of S. bovis isolated in our laboratory was previously reported as tet(M) on the basis of PCR amplification with tet(M)-specific primers (Devirgiliis et al., 2010). To further characterize the resistance gene, three representative isolates belonging to different repetitive extragenic palindromic (rep) groups were analysed at the molecular level. Since tet(M) is most commonly found within mobile elements in Gram-positive bacteria (Flórez et al., 2008), the PCR strategy chosen used primers directed outwards from the tet(M) gene (Table 1, For-Ext and Rev-Ext). These primers could yield an amplicon only in the case of a circular template. This approach yielded a 3.7 kb amplicon (Fig. 1a), which was sequenced fully, revealing a novel Tet-resistance gene represented by a tet(S)–tet(M) fusion. The DNA sequence of the relevant portion of the mosaic tet gene is shown in Fig. 1(b) with the corresponding primary sequence of the protein, consisting of the amino-terminal 33 amino acids of Tet(S), fused to Tet(M) residues 28–639. To ensure that the mosaic gene was indeed responsible for conferring phenotypic resistance to Tet upon the S. bovis isolates, we first tested for the possible presence of other Tet-resistance determinants by PCR and/or by Southern blotting. With these approaches, we could exclude the presence of the most commonly occurring tet resistance genes in Gram-positive bacteria: tet(O), tet(S), tet(L), tet(K) and tet(W) (data not shown). We then tested the ability of the mosaic tet(S/M) gene to confer Tet resistance by heterologous expression in E. coli. A 2.6 kb fragment containing the entire tet(S/M) ORF, with long stretches of 5' and 3' untranslated sequences, was cloned into the plasmid vector pBluescript KS+, and the resulting construct was transformed into the Tet-sensitive E. coli DH5α strain. Three independent recombinant colonies were tested for their ability to grow in the presence of Tet in liquid culture, using the MIC for E. coli cells in Tet as a reference concentration (5 mg l⁻¹). As shown in Fig. 2, E. coli transformants expressing S. bovis tet(S/M) were able to survive at the maximum Tet concentration tested (12 mg l⁻¹), with growth curves displaying the same slope as the Tet-resistant E. coli strain XL1Blue, which harbours the Tn10 transposon and is therefore intrinsically resistant to Tet.

Genomic localization of tet(S/M)

To begin analysing the genomic context of the newly identified resistance gene, we extracted genomic DNA from the three representative S. bovis strains, and subjected it to Southern blot analysis. Results in Fig. 3 show that tet(S/M) is present in single copy within a genomic EcoRI fragment larger than 10 kb and a HindIII fragment of 3 kb, specifically...
hybridizing with the tet(M) probe (Fig. 3a). A tnpA probe was used as an internal control, because sequencing of the 3.7 kb amplicon had shown the presence of an IS belonging to the IS1216 family (Mahillon & Chandler, 1998). This probe hybridized with the same genomic EcoRI and HindIII fragments recognized by the tet(M) probe, but also with other fragments, indicating the presence of additional ISs in the genome of S. bovis (Fig. 3b).

Since the S. bovis genome has not been fully sequenced, we sought to extend sequence information in the chromosomal region containing tet(S/M) using the two-step gene-walking method of Pilhofer et al. (2007) (see Methods). Oligonucleotide primers corresponding to both termini of tet(S/M) were first synthesized, and then used in PCRs to extend into the surrounding sequence in both directions. As more DNA sequence was determined, the same steps were employed to ‘walk’ further into the genome on both sides. The resulting sequence of a chromosomal region of 16,244 nt is schematically represented in Fig. 4(a). Major observations are as follows: a chromosomal subregion containing the tet(S/M) gene is flanked by IS1216 sites, suggesting that excision events could lead to a 4.3 kb circular form. The results of our previous Southern hybridizations were confirmed at the sequence level, as the position of HindIII restriction sites identified the following digestion products: (1) the 3 kb HindIII fragment detected by both tet(S/M) and tnpA probes (Fig. 3, lanes 4–5, 9–10); and (2) the 5 kb fragment detected by the tnpA probe alone (Fig. 3b, lanes 9–10). The remaining sequence showed the presence of three additional ISs, two of them sharing homology with IS6 family members (IS1216), and one with IS3 family members (IS1068) (Mahillon & Chandler, 1998) (Fig. 4b). All IS transposase-encoding ORFs appeared to be truncated by premature stop codons, with the exception of ORF B within IS1068 (Fig. 4b). Overall, BLAST searches for sequence similarity showed that this region contains stretches of homology to the pkF147A plasmid of Lactococcus lactis subsp. lactis, strain KF147.

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**Fig. 2.** Heterologous expression of Tet(S/M) in E. coli confers Tet resistance. Growth curves of three independent recombinant colonies of E. coli strain DH5α expressing S. bovis tet(S/M) in the absence (a) or presence of Tet at 6 mg l⁻¹ (b) and 12 mg l⁻¹ (c). The three transformants are labelled with □, △ and ◇. Negative controls: E. coli strain DH5α (●), strain DH5α with empty vector (○); positive controls: E. coli strain XL1-Blue with empty vector (○, dashed line) or with S. bovis tet(S/M) (●, dashed line).

**Fig. 3.** Genomic localization of the tet(S/M) gene. Southern blot analysis of EcoRI- and HindIII-digested genomic DNA from independent isolates of S. bovis probed with tet(M) (a) and tnpA (b) gene fragments. Lanes 1–3 and 6–8, EcoRI-digested DNA from isolates 1315, 1357 and 1400; lanes 4–5 and 9–10, HindIII-digested DNA from isolates 1315 and 1357.
Novel mosaic tetracycline-resistance gene

Tet induction of the mosaic **tet(S/M)** gene

Tet is well known to induce transcription of the corresponding resistance genes in Gram-negative bacteria (Berens & Hillen, 2003). Tet-dependent regulation of tet gene expression in Gram-positive organisms has been shown to be associated with increased transposition frequency of Tn916 in *Ent. faecalis* and *Bacillus subtilis*, by a mechanism involving transcriptional attenuation (Su et al., 1992; Celli & Trieu-Cuot, 1998; Roberts & Mullany, 2009). We have also reported transcriptional induction of tet(M) expression in foodborne strains of *Lactobacillus paracasei* (Comunian et al., 2010), and we therefore sought to analyse expression of the **tet(S/M)** mosaic gene in *S. bovis* strains, where transcription is driven by the **tet(S)** promoter. To this aim, total RNA was extracted from the Tet-resistant *S. bovis* strains, grown in the presence or absence of the antibiotic, and subjected to RT-PCR with specific primers mapping on the **tet(S)** and **tet(M)** sequences (TetS-1-FW and Rev-Ext in Table 1). As shown in Fig. 6(a), **tet(S/M)** expression was very low in the absence of Tet in all three strains examined, while it was strongly induced following antibiotic addition to the growth medium at a final concentration of 8 mg l⁻¹. Previously described *E. coli* transformants expressing an *S. bovis* tet(S/M) construct (Fig. 2) were also tested for induction by growth in the presence or absence of 12 mg Tet⁻¹. The results of RT-PCR assays in Fig. 3(b) show that...
Table 2. List of known *S. infantarius* and *Lc. lactis* genes homologous to the ORFs detected in the sequenced region of the *S. bovis* genome depicted in Fig. 4

<table>
<thead>
<tr>
<th>ORF no.</th>
<th>Gene name or locus tag</th>
<th>Protein</th>
<th>Putative function</th>
<th>DNA homology (%)</th>
<th>Amino acid identity (%)</th>
<th>Accession no.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>STRINF_00827</td>
<td>Hypothetical protein</td>
<td>ATPase component (ABC-type uncharacterized transport system)</td>
<td>99</td>
<td>99</td>
<td>ABJK02000017</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>STRINF_00828</td>
<td>Hypothetical protein</td>
<td>Permease component (ABC-type uncharacterized transport system)</td>
<td>99</td>
<td>99</td>
<td>ABJK02000017</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>tnpA</em></td>
<td>IS1216 transposase A portion (77–152 aa)</td>
<td>Transposase</td>
<td>90</td>
<td>96</td>
<td>X92946</td>
<td>Perreten et al. (1997)</td>
</tr>
<tr>
<td>4</td>
<td>LLKF_p0016</td>
<td>Resolvase</td>
<td>Resolvase</td>
<td>92</td>
<td>97</td>
<td>CP001835</td>
<td>Siezen et al. (2010)</td>
</tr>
<tr>
<td>5</td>
<td>LLKF_p0022</td>
<td>IS1068 transposase (C terminus)</td>
<td>Transposase</td>
<td>99</td>
<td>98</td>
<td>CP001835</td>
<td>Siezen et al. (2010)</td>
</tr>
<tr>
<td>6</td>
<td>LLKF_p0021</td>
<td>IS1068 transposase (N terminus)</td>
<td>Transposase</td>
<td>96</td>
<td>88</td>
<td>CP001835</td>
<td>Siezen et al. (2010)</td>
</tr>
<tr>
<td>7</td>
<td>LLKF_p0020</td>
<td>Potassium transport system protein kup 1</td>
<td>Potassium transporter</td>
<td>99</td>
<td>99</td>
<td>CP001835</td>
<td>Siezen et al. (2010)</td>
</tr>
<tr>
<td>8</td>
<td><em>tnpA</em></td>
<td>Truncated IS1216 transposase A (1–105 aa)</td>
<td>Transposase</td>
<td>99</td>
<td>100</td>
<td>X92946</td>
<td>Perreten et al. (1997)</td>
</tr>
<tr>
<td>9</td>
<td><em>tet</em> (S/M)</td>
<td>Tet(S/M)</td>
<td>Tet resistance</td>
<td>79</td>
<td>77</td>
<td>X92946</td>
<td>Perreten et al. (1997)</td>
</tr>
<tr>
<td>10</td>
<td><em>orf6</em></td>
<td>Hypothetical protein</td>
<td>Unknown function</td>
<td>87</td>
<td>81</td>
<td>X92946</td>
<td>Perreten et al. (1997)</td>
</tr>
<tr>
<td>12</td>
<td>STRINF_00831</td>
<td>Hypothetical protein</td>
<td>Catalytic activity (polysaccharide deacetylase domain)</td>
<td>99</td>
<td>98</td>
<td>ABJK02000017</td>
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<td>14</td>
<td>STRINF_00836</td>
<td>Hypothetical protein</td>
<td>Regulator of disulfide bond formation</td>
<td>99</td>
<td>99</td>
<td>ABJK02000017</td>
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the presence of a circular template. Furthermore, sequence tet
mosaic tet
As described above, amplification of the region containing
Identification of an intermediate circular form
Tet-dependent induction of the mosaic gene, driven by the S.
Disccussion
Foodborne bacteria that survive the harsh conditions of the
upper GI tract and reach the colon become part of the
crowded gut microbiota, thus contributing to its composi-
tion, which in turn can affect the health/disease balance of
the host (Garrett et al., 2010). Although fermented food
products mostly contain probiotic species, they can also
contribute a small fraction of bacteria which are not
favourable for health, such as opportunistic pathogens
capable of acquiring virulence traits in immune-compromised
individuals. Among them, enterococci are the most
abundant genus found in the human GI tract (Ogier &
Serror, 2008), but streptococci are also represented (Herrera
et al., 2009). In the latter case, some species have been
identified as responsible for common infections (Fernández-
Ruiz et al., 2010; Srivastava et al., 2010). Horizontal transfer
of AR determinants to pathogens raises growing concern,
especially in light of the potential inter-species transferabil-
ity of AR genes, suggested by their association with mobile
elements (Freitas et al., 2011). For this reason it is import-
ant not only to identify the occurrence of AR genes in
foodborne bacteria but also to characterize their genomic
context, with special focus on the regions involved in inter-
species and intra-species transfer. Conjugative plasmids
containing transposon Tn1546 carrying the van(A) operon
seem to have played a relevant role in the recent increase of
vancomycin-resistant enterococci (VRE) isolated from
poultry and swine sources. Tn1546 variants are associated
with IS1216 ISs, which are capable of autonomous trans-
position and greatly contribute to mobilization of flanking
analysis of the genomic context of the newly identified
resistance gene revealed that it localizes within a 4.3 kb
chromosomal subregion delimited by ISs, suggestive of
potential excision and circularization events. However,
no sequence resembling that of a replication origin was
identified within the entire 4.3 kb region, suggesting that
an excised circle containing tet(S/M) would be unable to
sustain autonomous replication. The results of Southern
blot hybridization in Fig. 3 seem to confirm the absence of
detectable levels of the circular form, as the 4.3 kb HindIII
band predicted from the DNA sequence as a linearization
product of the circular form could not be detected with
either tet(M) or tnpA probes. However, since we were indeed
able to amplify a tet(S/M)-containing circle by PCR (Fig.
1a), this result suggests the low abundance or transient
nature of the circle. BLAST sequence similarity searches with
the genomic region containing the mosaic tet(S/M) gene
revealed homology with a region of Lc. lactis plasmid pK214
GenBank accession no. X92946, nucleotides 25513–29826)
carrying the Tet-resistance gene tet(S) and the IS1216 mobile
element (Perreten et al., 1997) (Fig. 5). However, the DNA
sequence of the transposase-encoding tnpA gene within the
IS1216 element of S. bovis contained a nonsense mutation,
leading to a translational STOP codon (UGA) at amino acid
position 105.

**Fig. 5.** Molecular structure of the 4341 bp circular form containing
mosaic tet(S/M). Light grey identifies tet(S) ORF sequences in the
mosaic gene, dark grey highlights tet(M) sequences. Sequence
comparison between the tet(S/M)-containing circle and nucleo-
tides 25513–29826 of Lc. lactis plasmid pK214 (accession no.
X92946) is shown below the circle. Percentage homology is
shown for each coding and non-coding subregion.

[Tet-dependent induction of the mosaic gene, driven by the S.
bovis tet(S) promoter, could also be retained in the E. coli
heterologous expression system, suggesting that proteins
involved in Tet-dependent regulation of gene expression are
well conserved in Gram-negative and Gram-positive bacteria.

**Identification of an intermediate circular form**
As described above, amplification of the region containing
mosaic tet(S/M) was achieved using diverging primers on
the tet(M) sequence that could yield an amplicon only in
the presence of a circular template. Furthermore, sequence

**Fig. 6.** Tet-dependent induction of tet(S/M) expression. RT-PCR
analysis of mosaic tet(S/M) gene expression in: (a) three in-
dependent S. bovis isolates grown in the presence (lanes 2, 4 and
6) or absence (lanes 1, 3 and 5) of Tet (8 mg l⁻¹); (b) three
independent E. coli DH5α transformants expressing S. bovis
Tet(S/M), grown in the presence (lanes 2, 4 and 6) or absence
(lanes 1, 3 and 5) of Tet (12 mg l⁻¹). Reverse-transcribed 16S
rRNA was used as an internal control.
in only a subset of cells and mobilized only within the same cell. Circular elements often represent transient replication intermediates, typical of transposable elements such as Tn916 (Clewel et al., 1995). Replication of such elements is promoted by transposases and generates circular molecules, which can either integrate at different chromosomal locations or be horizontally transferred to other cells (Churchward, 2002). The two IS1216-like repeats flanking the S. bovis tet (S/M) gene might be involved in circularization via homologous recombination. Such non-replicative circular elements harbouring Tet-resistance genes were first described in a B. fibrisolvens transconjugant, where two direct repeats flanking tet(W) in the donor strain were postulated to give rise to a circular intermediate via homologous recombination (Kazimi, 2006). IS1216-like modules have been reported in association with AR determinants in streptococcal strains. The tet(S) gene in Tet-resistant clinical isolates of S. dysgalactiae, as well as the erm(T) gene in erythromycin-resistant clinical isolates of S. galolyticus, are both flanked by two copies of IS1216 in the same orientation (Tsai et al., 2005). In both cases, the structural organization reported for the sequences flanking the AR genes is similar to that of the tet(S)-flanking region in pK214.

Sequencing of a longer, 16 kb genomic region surrounding the mosaic AR gene shows the presence of several ISs, suggestive of a mobilizable region. Although most of the ORFs encoding transposases appear to be truncated, we cannot exclude that some of them might be functional, as evidenced by the presence of the amplifiable tet(S/M)-containing circular form. Notably, it has been reported that C-terminal-truncated transposases play a role in the reduction of transposition activity (Gueguen et al., 2006). Transposase inactivation appears to be a common feature of IS elements, and is recognized as a regulatory mechanism (Mahillon & Chandler, 1998).

The S. bovis genome has not yet been fully sequenced, and very little information is available in databases. The closest species whose entire genome sequence is available is S. infantarius (accession no. ABJK02000000). The taxonomic status of S. bovis strains has been evolving in the past few decades and has progressively changed in parallel with the characterization of new species originally described as S. bovis (Facklam, 2002). Our Tet-resistant foodborne isolates belong to the S. bovis–equinus complex, which includes different species and subspecies isolated from infected humans or animals (Farrow & Collins, 1984; Schlegel et al., 2003). The S. bovis–equinus complex is divided into four DNA clusters, one of which consists of S. infantarius subsp. coli and subsp. infantarius. The latter subspecies has been isolated from foodstuffs and from infected humans, where it was found to be associated with systemic disease in infants (Bouvet et al., 1997; Schlegel et al., 2000, 2004). Due to their association with several human and animal diseases, as well as their occurrence in fermented foods, we believe that it is extremely important to determine the genomic sequences of the most widespread species.
belonging to the S. bovis–equinus complex, which will serve as a basis to identify the genetic determinants in infectious strains as well as their potential association with AR determinants.

We take these results as strongly indicative of extensive genomic exchanges, possibly through conjugative events, between species that are natural components of the human microbiota, and which should be more thoroughly investigated in light of their potential role as disease-causing agents.

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