High-level tetracycline resistance mediated by efflux pumps Tet(A) and Tet(A)-1 with two start codons

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INTRODUCTION

Tetracyclines are broad-spectrum antibiotics that exhibit activities against a wide range of Gram-positive and Gram-negative bacteria, including Chlamydia, Mycoplasma and Rickettsiae (Soares et al., 2012; Shlaes, 2006). However, the extensive use of tetracyclines in clinical practice and feed stock has imposed a continuing selective pressure for resistant organisms (Thaker et al., 2010). In clinical settings, resistance to tetracyclines is common among Enterobacteriaceae, and the resistance levels and rates have been very high (Chopra & Roberts, 2001). Efflux is presumed to be the most common and most clinically significant mechanism of tetracycline resistance. The efflux resistance genes are often associated with mobile elements, among which the class A tetracycline resistance (tet) determinant was the first identified of the RP1/Tn1721 system (Levy, 1992; Waters et al., 1983; Tuckman et al., 2000).

The GenBank/EMBL/DDBJ accession number for the tet(A)-1-tetP(A) sequence of pH528 is KC495078.

Traditionally, it was suggested that a single efflux pump often provided low-level antibiotic resistance; however, tetracycline efflux pumps may confer high-level tetracycline resistance. For instance, a single Tn10-encoded TetB (A) protein was sufficient to confer tetracycline resistance with a MIC of 128 µg ml⁻¹ (Nguyen et al., 1983; Sapunaric & Levy 2005).

Although limited research has been done on the mechanisms of tetracycline resistance in recent decades, an allele of tet(A) was found in a Shigella sonnei strain in 2003 and was designated tet(A)-1 (Hartman et al., 2003). tet(A)-1 had mutations in the last 28 nt of tet(A), corresponding to the C-terminal 9 aa of the protein and including the stop codon. It was reported that translation of the 1275 bp tet(A)-1 gene started at ATG (Hartman et al., 2003), while translation of tet(A) was presumed to start at GTG, located 75 bp downstream of ATG (Waters et al., 1983; Allmeier et al., 1992). This finding raised the question of whether one or both of these two start codons are the actual start codon for these tet determinants.

Efflux is the most common mechanism of tetracycline resistance. Class A tetracycline efflux pumps, which often have high prevalence in Enterobacteriaceae, are encoded by tet(A) and tet(A)-1 genes. These genes have two potential start codons, GTG and ATG, located upstream of the genes. The purpose of this study was to determine the start codon(s) of the class A tetracycline resistance (tet) determinants tet(A) and tet(A)-1, and the tetracycline resistance level they mediated. Conjugation, transformation and cloning experiments were performed and the genetic environment of tet(A)-1 was analysed. The start codons in class A tet determinants were investigated by site-directed mutagenesis of ATG and GTG, the putative translation initiation codons. High-level tetracycline resistance was transferred from the clinical strain of Klebsiella pneumoniae 10-148 containing tet(A)-1 plasmid pH27 to Escherichia coli J53 by conjugation. The transformants harbouring recombinant plasmids that carried tet(A) or tet(A)-1 exhibited tetracycline MICs of 256–512 µg ml⁻¹, with or without tetR(A). Once the ATG was mutated to a non-start codon, the tetracycline MICs were not changed, while the tetracycline MICs decreased from 512 to 64 µg ml⁻¹ following GTG mutation, and to ≤ 4 µg ml⁻¹ following mutation of both GTG and ATG. It was presumed that class A tet determinants had two start codons, which are the primary start codon GTG and secondary start codon ATG. Accordingly, two putative promoters were predicted. In conclusion, class A tet determinants can confer high-level tetracycline resistance and have two start codons.

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In this study, we found that the class A tet determinants tet(A) and tet(A)-1 could confer high-level tetracycline resistance, and that they had two start codons, GTG and ATG.

METHODS

Bacterial strains. Klebsiella pneumoniae 10-148 carrying tet(A)-1 and K. pneumoniae 10-1750 harbouring tet(A) were isolated from sputum and urine, respectively, from patients of the Neurosurgery Department in 2010 at Huashan Hospital, a teaching hospital of Fudan University in Shanghai. Both strains had a tetracycline MIC of 512 μg ml⁻¹ and were resistant to most of the antimicrobials tested (Table 1).

Antimicrobial susceptibility testing. MICs were determined by the Clinical and Laboratory Standards Institute agar dilution methodology (CLSI, 2009) and interpreted according to (CLSI, 2012) guidelines. A broth dilution method was used to detect changes in tetracycline susceptibilities for E. coli strains containing recombinant plasmids. Escherichia coli ATCC 25922 was used as quality control strain in susceptibility testing experiments.

Conjugation and cloning of tet determinants. Conjugation and subcloning experiments were carried out to identify the determinant mediating high-level tetracycline resistance and the DNA sequences flanking tet(A)-1 in K. pneumoniae 10-148. Conjugation was performed as described previously (Wang et al., 2003), with E. coli J53 AzIR as the recipient strain. Plasmid DNA was isolated from the transconjugant using a Plasmid Midi kit (Qiagen), and digested with EcoRI and PstI. The EcoRI–PstI fragment was ligated into pUC18 AmpR (resistance to ampicillin). The recombinant plasmid was transformed into E. coli TOP10 (Invitrogen) and sequenced, with the templates. PCR fragments were then ligated into the pMD 18-T vector (Takara). Transformants containing recombinant plasmids were selected on MHA plates containing tetracycline (20 μg ml⁻¹) and ampicillin (100 μg ml⁻¹).

Determination of the start codons of tet(A)-1 and tet(A). The putative translation initiation codons ATG and GTG of tet(A)-1 and tet(A) (Fig. 1a) were mutated using a QuikChange Lightning Site-Directed Mutagenesis kit (Stratagene) to determine the start codons of class A tet genes. Mutagenesis was carried out on the plasmids pHS29 [tet(A)-1-tetR(A)] and pH531 [tet(A)-tetR(A)] following the manufacturer’s protocol. The primers were designed to alter the DNA sequence from 5’ ATG to 5’ ACG, from 5’ ATG to 5’ GTG, from 5’ GTG to 5’ GTC, and from 5’ GTG to 5’ ATG (Table 2). After confirmation by sequencing, the resultant plasmid constructs were renamed (Table 3).

RESULTS

Conjugation

High-level tetracycline resistance could be transferred from K. pneumoniae 10-148 to E. coli J53 AzIR by conjugation. A plasmid of about 90 kb was isolated from the transconjugant and termed pHS27. Plasmid pHS27 was shown to mediate high-level tetracycline resistance with an MIC of 128 μg ml⁻¹ (Table 1).

Genetic environment of tet(A)-1

A recombinant plasmid with an approximately 5.3 kb EcoRI-PstI insert was designated pHS28. E. coli TOP10 containing pHS28 had a tetracycline MIC of 256 μg ml⁻¹ (Table 1). DNA sequencing showed that the 5330 bp EcoRI-PstI DNA insert contained tet(A)-1-tetR(A) with a nucleotide sequence identical to that of the originally reported sequence from S. sonnei (GenBank accession no. AF502943), except for a single nucleotide change in tetR(A) (Ser53→Gly), which did not alter the level of tetracycline resistance compared with the wild-type (data not shown).

Table 1. Susceptibilities of clinical strains (K. pneumoniae 10-1750 and K. pneumoniae 10-148), E. coli transconjugant and transformant to three tetracyclines and other antimicrobials

<table>
<thead>
<tr>
<th>Strains</th>
<th>TET (μg ml⁻¹)</th>
<th>DOX</th>
<th>MH</th>
<th>TGC</th>
<th>PIP</th>
<th>CRO</th>
<th>IMP</th>
<th>CIP</th>
<th>AMK</th>
<th>CHL</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. pneumoniae 10-1750</td>
<td>512</td>
<td>64</td>
<td>32</td>
<td>1</td>
<td>512</td>
<td>256</td>
<td>0.125</td>
<td>1</td>
<td>2</td>
<td>256</td>
</tr>
<tr>
<td>K. pneumoniae 10-148</td>
<td>512</td>
<td>64</td>
<td>32</td>
<td>0.5</td>
<td>512</td>
<td>128</td>
<td>0.125</td>
<td>256</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>E. coli J53/pHS27*</td>
<td>128</td>
<td>16</td>
<td>6</td>
<td>0.25</td>
<td>64</td>
<td>32</td>
<td>0.125</td>
<td>0.125</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>E. coli TOP10/pHS28†</td>
<td>256</td>
<td>16</td>
<td>2</td>
<td>0.125</td>
<td>512</td>
<td>0.06</td>
<td>0.125</td>
<td>0.06</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>E. coli J53</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0.25</td>
<td>0.125</td>
<td>0.06</td>
<td>0.125</td>
<td>0.06</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td>E. coli TOP10</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.06</td>
<td>0.125</td>
<td>0.06</td>
<td>0.125</td>
<td>0.06</td>
<td>0.5</td>
<td>2</td>
</tr>
</tbody>
</table>

TET, tetracycline; DOX, doxycycline; MH, minocycline; TGC, tigecycline; PIP, piperacillin; CRO, ceftriaxone; IMP, imipenem; CIP, ciprofloxacin; AMK, amikacin; CHL, chloramphenicol.

*The E. coli J53 transconjugant containing plasmid pHS27 carrying tet(A)-1 from K. pneumoniae 10-148.

†E. coli TOP10 transformant carrying recombinant plasmid pHS28 with EcoRI-PstI insert containing tet(A)-1 from pHS27.
strA, strB and tetR(A) genes were located upstream of tet(A)-1, and tnp, orf1 and orf2 were located downstream (Fig. 1a).

Cloning of tet(A)-1 and tet(A)

After confirmation by sequencing, the recombinant plasmids containing PCR fragments of tet(A)-1-tetR(A), tet(A)-1, tet(A)-tetR(A) and tet(A) were designated pHS29 [tet(A)-1-tetR(A)], pHS30 [tet(A)-1], pHS31 [tet(A)-tetR(A)] and pHS32 [tet(A)], respectively (Table 3). The nucleotide sequences of tet(A)-1-tetR(A) in pHS29 and tet(A)-tetR(A) in pHS31 were identical to those in pSSTAV (GenBank accession no. AF502943) and Tn1721 (GenBank accession no. X61367), except for a mutation leading to an amino acid substitution (Leu380→Pro) in tet(A). Using the site-directed mutagenesis method, we found that this point mutation did not alter the tetracycline MICs (data not shown).

Remarkably, all four clones containing the recombinant plasmids pHS29, pHS30, pHS31 and pHS32 conferred a high level of tetracycline resistance, with MICs of 256–512 μg ml⁻¹ (Table 3).

Determination of the translational start codons of class A tet determinants

In both pHS29 [tet(A)-1-tetR(A)] and pHS31 [tet(A)-tetR(A)], when ATG was changed to ACG (a non-initiation codon, amino acid change Met→Thr), the tetracycline MICs of E. coli TOP10 harbouring pHS29-ATGtoACG and pHS31-ATGtoACG were as high as those with pHS29 and pHS31 (512 μg ml⁻¹) (Table 3). When GTG was mutated to GTC (a non-initiation codon, no amino acid change), the tetracycline MICs for E. coli TOP10 harbouring pHS29-GTGTtoGTC and pHS31-GTGTtoGTC decreased from 512 to 64 μg ml⁻¹ (Table 3), still higher than the CLSI tetracycline resistance breakpoint of ≥16 μg ml⁻¹. When both GTG and ATG were replaced with the non-start codons GTC and ACG, the tetracycline MICs for E. coli TOP10 carrying the mutant plasmids decreased to a level similar to that for E. coli TOP10 (≤4 μg ml⁻¹) (Table 3). Furthermore, a predicted Shine–Dalgarno sequence (GGAGG) was found to be located 6 nt upstream of the GTG initiation codon, an optimum distance for initiation of translation, whereas only two conserved nucleotides (GA) were found 10 nt upstream of the ATG initiation (Fig. 1b). Therefore, GTG appeared to be the primary start codon, while ATG seemed to be the secondary translational initial codon of both tet(A)-1 and tet(A).

Using the SoftBerry BPROM program (http://linux1.softberry.com/berry.phtml), two potential promoter sequences and transcriptional start sites were predicted (Fig. 1b). The transcript predicted to start translation at GTG was designated tetA-P1, and a second, longer transcript that may arise from translation beginning at ATG was designated tetA-P2.

DISCUSSION

A study in 1980 showed that WT tet(A) alone conferred resistance to tetracycline with a MIC of 125 μM (equivalent to about 64 μg ml⁻¹) (McMurry et al., 1980), as determined by pre-induced cells grown in medium A with glucose (McMurry & Levy, 1978). However, the tetracycline resistance caused by the tetA or tet(A)-1 gene alone has not yet been well clarified. This study found that both the transconjugant and transformant carrying recombinant plasmids containing tet(A)-1 showed high-level tetracycline resistance, with MICs of 128–256 μg ml⁻¹. Both tet(A) and

<table>
<thead>
<tr>
<th>Target region</th>
<th>Primer name</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA cloning</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tet(A)-1-tetR(A)</td>
<td>loc tetA-1-F</td>
<td>TGCTGTAAGTCTGAAGTGGT</td>
</tr>
<tr>
<td></td>
<td>loc tetA-1-R</td>
<td>AGACGAGGAAAGGAAT</td>
</tr>
<tr>
<td>tet(A)-1</td>
<td>tetA-1-F</td>
<td>AGTTTGGGTGTCGAGA</td>
</tr>
<tr>
<td></td>
<td>tetA-1-R</td>
<td>CAGAGCAGGAAAGGAAT</td>
</tr>
<tr>
<td>tet(A)-tetR(A)</td>
<td>loc tetA-F</td>
<td>CTTCTGCAAGTGTCAGG</td>
</tr>
<tr>
<td></td>
<td>loc tetA-R</td>
<td>CTAGGATGTTGTCTTTGGA</td>
</tr>
<tr>
<td>tet(A)</td>
<td>tetA-F</td>
<td>GCTTCTAATCGGAGGAG</td>
</tr>
<tr>
<td></td>
<td>tetA-R</td>
<td>GTTCACACCGACTCCT</td>
</tr>
<tr>
<td>Site-directed mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATG</td>
<td>tetA-ATGtoFG</td>
<td>CACTGATAAACATACTGCTGACAAACTTATC</td>
</tr>
<tr>
<td></td>
<td>tetA-ATGtoACG-R</td>
<td>GATAAGTTGTTGCGAGTGTTATGTTATCAGT</td>
</tr>
<tr>
<td>ATG</td>
<td>tetA-ATGtoGTG-F</td>
<td>CACTGATAAACATACTGCTGACAAACTTATC</td>
</tr>
<tr>
<td></td>
<td>tetA-ATGtoGTG-R</td>
<td>GATAAGTTGTTGCGAGTGTTATGTTATCAGT</td>
</tr>
<tr>
<td>GTG</td>
<td>tetA-GTGTtoGT-C</td>
<td>CGGAGGCGAGGAGGAGGAGGAGGAGGAGGAG</td>
</tr>
<tr>
<td></td>
<td>tetA-GTGTtoGT-C-R</td>
<td>GTCTGTGTGTGGTGGAGGAGGAGGAGGAGGAG</td>
</tr>
<tr>
<td>GTG</td>
<td>tetA-GTGTtoATG-F</td>
<td>CGGAGGCGAGGAGGAGGAGGAGGAGGAGGAG</td>
</tr>
<tr>
<td></td>
<td>tetA-GTGTtoATG-R</td>
<td>GTCTGTGTGTGGTGGAGGAGGAGGAGGAGGAG</td>
</tr>
</tbody>
</table>

Table 2. Primers used in this study
tet(A)-1 could mediate high-level tetracycline resistance (MICs 256–512 \( \mu \)g ml\(^{-1}\)) with or without tetR(A). As a negative regulator of tetA, TetR blocks the transcription of the TetA efflux pump in the absence of tetracycline (Orth et al., 2000; Berens & Hillen, 2003). However, in the presence of tetracycline, the drug binds to TetR, which dissociates from the tet operator to induce expression of the efflux pump (Saenger et al., 2000; Nguyen et al., 2014). A tetR(A) mutant strain would thus exhibit a high level of constitutive tetracycline resistance (Coleman & Foster, 1981). Although the C-terminal 9 aa of tet(A)-1 and tet(A) were different from each other, the \( E.\ coli \) TOP10 containing pHS29 [tet(A)-1-tetR(A)] or pHS31 [tet(A)-tetR(A)] had the same tetracycline MICs, indicating that the mutations in the last 28 nt had little to no influence on the function of tet(A)-1 and tet(A).

In prokaryotic mRNAs, the leader region preceding the initiator AUG codon is thought to be important in directing mRNA binding to the ribosome at the translation initiation site (Laursen et al., 2005). In class A tet determinants, however, the primary promoter we predicted, which was the same as the traditional one, was shown to overlap with the initiator codon AUG (Waters et al., 1983), implying that it contains no leader sequence for ribosome binding. There is possibly a secondary promoter in the upstream region of ATG, similar to the Prm and P\( \beta \) promoters in phage \( \lambda \) cl gene transcription, where the Prm mRNA starts at AUG (Walz et al., 1976). Likewise, two promoters are also involved in the transcription of tetR(A) (Waters et al., 1983), resulting in the primary transcript tetR1 and a secondary transcript tetR2 (Fig. 1b). tetR1 may act as a autoregulator of tetR(A) gene, as does Prm for the cl gene.

With regard to the two start codons (ATG and GTG) in class A tet determinants, the predicted Shine–Dalgarno sequence for GTG start codon is GGAGG, which closely matches the consensus AGGAGG. No typical Shine–Dalgarno sequence is found for the ATG start codon, with the possible exception of GA upstream of the ATG initiation. The absence of a Shine–Dalgarno sequence for tetracycline resistance mediated by efflux pumps

Fig. 1. Genetic environment and start codons of class A tet determinants. (a) Genetic environment of the tet(A)-1-tetR(A) gene in the 5330 bp EcoRI–PstI fragment of plasmid pHS28 (upper). \( E.\ coli \) TOP10 cells containing pHS28 had a tetracycline MIC of 256 \( \mu \)g ml\(^{-1}\). tet(A)-1 (GenBank accession no. AF502943) is an allele of tet(A) (GenBank accession no. X61367) (below), with mutations in the last 28 nt (middle), while tetR(A) is identical. (b) Portion of the intercistronic region containing regulatory and transcription signals of the divergently oriented tetA and tetR genes. Primary and secondary promoters and the initiation of the tetA (tetA-P1, tetA-P2) and tetR (tetR1, tetR2) transcripts are marked above and below the double strand respectively. For tetA, GUG and AUG are supposed to be the translational initial codons (underlined) for transcripts tetA-P1 and tetA-P2. Notably, the primary transcript tetR1 begins with AUG (italic) the presumptive tetR translational start codon (Waters et al., 1983).
the ATG start codon would dramatically influence the ribosome recruitment onto the mRNA strand. Therefore, the Tet(A) protein translated at GUG may confer high-level tetracycline resistance, compared with lower-level tetracycline resistance when translation starts at AUG. This was in agreement with our results of site-directed mutagenesis on initiator codons. Thus, GTG appeared to be the primary start codon of both tet(A)-1 and tet(A), and ATG was presumed to be the secondary translational initial codon for class A tet genes when GTG did not work. With regard to the two putative promoters (tetA-P1 and tetA-P2), we propose that the primary transcript tetA-P1 may start translation at GUG, the supposed start codon of tet(A), and that the secondary transcript tetA-P2 begins translation at AUG. The tetA-P1 transcript may be responsible for both the positive and negative autoregulation of class A tet determinants, including tet(A) and tet(A)-1.

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