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High-level telithromycin resistance in laboratory-generated mutants of *Streptococcus pneumoniae*

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Resistance to macrolides in *Streptococcus pneumoniae* is usually mediated by methylation of 23S ribosomal RNA, encoded by the *erm*(B) methylation gene, or by efflux mediated by the *mef*(A) gene. Changes in the L4 and L22 ribosomal proteins have also been associated with macrolide resistance and reduced telithromycin activity. This study generated *in vitro* mutants from three parent strains of *S. pneumoniae*: 02J1175 [*mef*(A) +], 02J1095 [*erm*(B) +] and NCTC 13593 (macrolide susceptible). The *erm*(B) and the *erm*(B) upstream region, the *mef*(A) genes and the *mef*(A) upstream and downstream regions, the 23S rRNA genes encoding domains II and V and the L4 and L22 genes of the telithromycin-resistant strains were all amplified by PCR and all, except the *mef*(A) upstream and downstream regions, were sequenced. No changes were present in any of the genes of the *mef*(A) + mutants. No changes were found in the *erm*(B) genes, the 23S rRNA genes or the L4 protein genes of the *erm*(B) + mutants. However, a Lys-94 to Gln-94 amino acid mutation did occur in a mutant derived from *erm*(B) + with a telithromycin MIC of >32 mg/L. A 210 base pair deletion in the *erm*(B) upstream region was also present in this strain. We believe this is the first incidence of a Lys-94 to Gln-94 change in L22 associated with telithromycin resistance and also the first time that such a large deletion in the *erm*(B) upstream region has been identified in *S. pneumoniae*.

Keywords: telithromycin, *Streptococcus pneumoniae*, resistance

Introduction

Macrolide resistance is currently an increasing problem in *Streptococcus pneumoniae* throughout the world. The countries with the highest levels of erythromycin A resistance between 1999 and 2001 worldwide were South Korea (86.2%), Japan (77.9%) and Hong Kong (72.9%). In Europe, France (58%) and Italy (44.6%) have the highest levels of erythromycin A resistance. The UK and USA had resistance levels of 13.2% and 30%, respectively.[1]

The two most regularly identified mechanisms of macrolide resistance in *S. pneumoniae* are target modification and efflux. Target modification causes resistance not only to macrolides but also to lincosamides and streptogramin B antibiotics and is associated with high-level macrolide resistance.[2] *erm* genes cause methylation of the binding site of the MLS_B antibiotics within the peptidyl transferase centre of the 23S rRNA. In *S. pneumoniae*, the *erm*(B) gene mediates methylation, although recently an *erm*(A) gene has also been associated with macrolide-resistant *S. pneumoniae*.[3,4] Methylation can occur as monomethylation or dimethylation. It has recently been shown that variations in mono- or dimethylation lead to different resistance phenotypes. Erm(B) and Erm(A)(TR) are both A2058 dimethyltransferases and expression of resistance due to *erm* methylation can be inducible or constitutive.[5]

The inducible expression of MLS_B resistance is putatively controlled at a post-transcriptional level by a regulatory region upstream from the *erm* gene.[6] The exact mechanism or mechanisms of constitutive resistance have not been fully elucidated in *S. pneumoniae*. Constitutive expression of MLS_B resistance in various bacteria has been associated with deletions or mutations in the regulatory region upstream from the *erm* gene.[7] Two *S. pneumoniae* clinical isolates with deletions in the leader peptides of their *erm*(B) genes have been isolated, one each from Mexico and Canada, and both were ketolide-resistant. In these strains, however, there were also other mutations; both had three amino acid mutations in the Erm protein itself, and the Mexican strain also had mutations in the L4 riboprotein.[8]

The *mef*(A) genetic element was first described in 2000 as a chromosomal element designated Tn*1207.1*, 7244 base pairs in size.[9] In 2001, Gay & Stephens described a 5.4 or 5.5 kb genetic element containing a *mef*(E) gene called MEGA.[10] In MEGA, the open reading frame (ORF) sequence 3′ of the *mef*(E) gene was designated *mel*. The *mel* ORF is also a homologue of *msrA* in staphylococci, which encodes an ATP-binding cassette to provide the energy for efflux. The *mef*(E) and *mel* are co-transcribed, which suggests that both are required for efflux.

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Macrolide resistance in clinical isolates and laboratory-derived strains of *S. pneumoniae* have been linked to alterations of specific nucleotides in the 23S rRNA of the ribosomal subunit. Mutations at adenine 2058 are the most frequently identified ribosomal mutations associated with macrolide resistance in 23S rRNA.[11] Specifically in *S. pneumoniae*, mutations have been located at nucleotides 2058, 2059, 2062 and 2611.[12–15] These ribosomal mutations were identified in clinical isolates, which did not contain the *erm*(B) or *mef*(A) genes. In addition to these mutation sites, a mutation in the hairpin 35 region of the domain II of the 23S rRNA has been associated with macrolide and ketolide resistance in a laboratory-derived mutant of *S. pneumoniae*.[16] This laboratory-derived *S. pneumoniae* strain was found to have a deletion of one adenine in the series of four located at positions 749–752 in the hairpin 35 of domain II.[16]

The ribosome consists not only of rRNA but also riboproteins, which interact with the rRNA to form the ribosomal subunits. Two such riboproteins are L4 and L22. Recently, mutations in both of these riboproteins have been associated with macrolide resistance in *S. pneumoniae*. Positively charged residues of both L4 and L22 interact with the negatively charged phosphate groups of the RNA forming nucleic acid–protein complexes.[17,18] Erythromycin resistance mutation studies of these riboproteins implied that they also have interactions with the central loop of domain V of the rRNA. Gregory & Dahlberg also showed that these proteins have multiple contacts with rRNA in domains II, III and V of the rRNA.[19]

Mutations in the L4 riboprotein and L22, to a lesser extent, have been associated with macrolide and, on occasion, telithromycin resistance, either alone or in combination with 23S rRNA mutations or the presence of an *erm*(B) gene.[4,14–16] From the studies carried out to date, it appears that the L4 amino acid region from 67 to 72 is the hotspot for mutations conferring macrolide resistance.[4,14–16]

Mutations in L22 associated with macrolide resistance have been mainly described in laboratory-derived strains of *S. pneumoniae*. In fact, only two types of L22 mutation have been identified in clinical isolates. They are a Gly-95 to Asp-95 amino acid change, and a six amino acid insertion (RTAHIT) at amino acid 109.[12,20] Three strains selected *in vitro* on telithromycin were identified recently with two types of L22 mutation; one strain had a Gln-95 to Asp-95 mutation and the other an Ala-97 to Asp-97 change. These strains were not telithromycin-resistant but the mutation did cause the telithromycin MIC to increase 32-fold for the Gln-95 to Asp-95 mutant and 8- or 16-fold for the Ala-97 to Asp-97 mutants.[21]

The generation of telithromycin-resistant *S. pneumoniae* mutants to date has mainly used macrolide-susceptible *S. pneumoniae* to try to develop telithromycin-resistant mutants. Therefore, we decided to investigate telithromycin resistance development from *S. pneumoniae*, which were already macrolide resistant. The aim of this study was to investigate the development of telithromycin resistance in *S. pneumoniae* by generating telithromycin-resistant mutants *in vitro*, from strains of varying MLS_B resistance profiles. Once the mutants were generated, the mechanisms by which these strains became telithromycin-resistant and finally the influence of erythromycin and clarithromycin on the development of telithromycin resistance were studied.

Materials and methods

Bacterial strains

The three *S. pneumoniae* strains used as the parents for the step-wise selection of telithromycin-resistant mutants were 02J1175 [*mef*(A)+], 02J1095 [*erm*(B) +] and NCTC 13593, which is a macrolide-susceptible strain and does not contain either gene.

Mutation studies

Each of the three parent strains were inoculated into Todd Hewitt broth (Oxoid) and incubated in 5% CO_2 overnight. Varying dilutions, from neat to 10^{-6}, of the cultures were spread on Columbia agar (Sigma–Aldrich) plates supplemented with 5% defibrinated horse blood containing telithromycin at concentrations equal to or 2 × MIC for the strain. Plates with each concentration of telithromycin were inoculated in triplicate and a control with no antibiotic was also included. All plates were incubated in 5% CO_2 for 48 h. The resulting mutants were purified by subculturing twice on plates with the selectant telithromycin concentration. The MICs of telithromycin against the mutants were tested at this point according to the BSAC guidelines.[22] Successive generations of mutants were derived in the same way as the first generation. This process was repeated until high-level telithromycin resistance occurred or the MICs for successive generations remained constant. Serially subculturing the mutants on antibiotic-free medium for 10 generations and then retesting their telithromycin MICs was used to determine the stability of a representative of each generation.

MIC determination

MICs were determined on Columbia agar supplemented with 5% defibrinated horse blood and doubling dilutions of antibiotic according to the BSAC guidelines.[22] All plates were incubated in 5% CO_2 for 16–18 h. The antibiotics were stored and prepared according to the manufacturer's guidelines. Telithromycin was obtained from Aventis Pharma Ltd.

PCR conditions

S. pneumoniae strains were emulsified in 200 µL of Milli-Q water and boiled for 10 min in order to extract the total DNA. The supernatant was used as the DNA template in the PCR experiments. The PCR conditions and primers for the detection of *erm*(B) and *mef*(A) genes are described in Table 1 and are based on those previously devised by Sutcliffe *et al.* and Tait-Kamradt *et al.*[23,24] The *mef*(A) gene reverse primer was designed using the primer design website Primer 3 at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi in order to amplify the entire *mef*(A) gene. The three parents 02J1095, 02J1175 and NCTC 13593 and the selected generation representative mutants, shown in Table 2, were investigated for the presence of *erm*(B) or *mef*(A) genes.

The *mel* gene and *mef*(A) upstream region of the bacterial strain 02J1175 and the mutants M I 2, M II 15, M III 3 and M IV were amplified by PCR. The primers used to amplify the *mel* genes and upstream regions were previously described by Gay & Stephens and Sutcliffe *et al.*, except for the *mef*(A) upstream forward primer (Table 1).[10,23] This was designed with the primer design website Primer 3 at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. The *mef*(A) upstream reverse primer was the same as the reverse primer used to amplify the *mef*(A) gene and the *mel* forward primer was the forward primer used to amplify the *mef*(A) gene. Their respective positions in the MEGA insertion element are *mef*(A) upstream reverse primer, nucleotides 1506–1525 and the *mel* forward primer, nucleotides 1181–1201.

The *erm*(B) upstream regions of the parent strain 02J1095 and mutants J I 1, J II 1, J II 4, J II 5, J II 6, J II 7, J II 8 and J II 9 were investigated by amplification by PCR. The forward primer was designed using the primer design website Primer 3 at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi and the reverse primer was the same as the reverse primer used to amplify the *erm*(B) gene described in Table 1.

Table 1. PCR primers

Primer name	Sequence 5′ to 3′
erm(B) forward	GAA AA(AG) GTA CTC AAC CAA ATA
erm(B) reverse	AGT AA(CT) GGT ACT TAA ATT GTT TAC
mef(A) forward	AGT ATC ATT AAT CAC TAG TGC
mef(A) reverse	TTC TTC TGG TAC TAA AAG TGG
erm(B) upstream forward	GAA GCA AAC TTA AGA GTG TGT TGA
erm(B) upstream reverse	AGT AA(CT) GGT ACT TAA ATT GTT TAC
mef(A) upstream forward	GAG CAT TCA TTA GTT ACG GTG AGG
mef(A) upstream reverse	TTC TTC TGG TAC TAA AAG TGG
mel forward	AGT ATC ATT AAT CAC TAG TGC
mel reverse	CTT CAC GGT CTA AAT GGC TCG
23S 3′ forward	CGG CGG CCG TAA CTA TAA CG
23S 3′ reverse	
DS 18	GCC AGC TGA GCT ACA CCG CC
DS 23	TAC ACA CTC ACA TAT CTC TG
DS 30	TTT TAC CAC TAA ACT ACA CC
DS 91	TAC CAA CTG AGC TAT GGC GG
Inner primers	
forward	GTT CCC TCA GAA TGG TTG GA
reverse	CAT AGC TAC CCA GCG ATG C
Domain II	
forward	GGT TAA GTT AAT AAG GGC GC
reverse	TTT CGA CTA CGG ATC TTA GC
L4 forward	AAA TCA GCA GTT AAA GCT GG
L4 reverse	GAG CTT TCA GTG ATG ACA GG
L22 forward	GCA GAC GAC AAG AAA ACA CG
L22 reverse	ATT GGA TGT ACT TTT TGA CC

The PCR mixture was the same as that of the *erm*(B) gene PCR except the $MgCl_2$ concentration was 3 mM. The cycle parameters were the same as those of the *erm*(B) PCR.

In order to detect mutations in the peptidyl transferase region of the 23S rRNA, all four contigs of the domain V section were amplified using the four downstream primers and PCR conditions as published by Tait-Kamradt *et al.* and shown in Table 1.[15] Problems were encountered in obtaining the sequence data for the nucleotide section from 2350 to 2650 of the DS 18 and DS 23 genes, as they were both 2000 nucleotides in size. As a result, primers were designed to amplify this inner portion of each of the genes. The PCR products of the downstream primers were purified and this product was then used as the DNA template for the inner PCR experiments. The primers are shown in Table 1. The inner primer PCR consisted of 94°C for 3 min, 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min and a final step of 72°C for 10 min. The domain II portion of the 23S rRNA was amplified by using the domain II primers (Table 1) and conditions of Tait-Kamradt *et al.*[15] The L4 and L22 genes were also amplified as previously published using the primers shown in Table 1.[15]

The PCR products were purified by the gel extraction method or directly using the Qiagen PCR product purification kit.

Sequencing

Sequencing of purified DNA was determined by the chain termination method.[25] The PCR primers were also used as sequencing primers. Individual PCR fragments were set up in the Ready Reaction Format for fluorescence based on dideoxy cycle sequencing (PE Applied Systems, Warrington, UK).

Induction experiments

Disc diffusion: The strains 02J1095, J I 1, J II 1, J II 8 and the control strain NCTC 13593, described in Table 2, were each inoculated into 4.5 mL of sterile distilled water and adjusted to a turbidity equivalent to that of a 0.5 McFarland standard. Using a sterile swab, each strain was spread onto separate Columbia blood agar plates. Discs containing 15 µg of each of the following agents—erythromycin, clarithromycin and telithromycin—were placed onto the plate approximately 2 cm apart. Each strain was tested in duplicate.

Induction assays: In order to investigate inducibility further, two induction assays were carried out.

1 The parent 02J1095 [*erm*(B) +] was subcultured on erythromycin, and the resulting mutants were tested for induction of telithromycin resistance. 02J1095 was inoculated into sterile saline and adjusted to a turbidity equivalent to that of a 0.5 McFarland standard. One hundred microlitres was spread on Columbia blood agar plates containing 4, 8, 16, 32, 64 and 128 mg/L each of erythromycin and a control plate with no antibiotic and incubated in 5% CO_2 overnight. Colonies were randomly chosen from each plate and subcultured on plates containing the appropriate erythromycin concentration and incubated overnight in 5% CO_2. The MIC of telithromycin against these mutants was investigated as previously described. *S. pneumoniae* NCTC 13593, *S. aureus* NCTC 6571 and *E. coli* NCTC 10418 were all used as controls in the MIC tests.
2 The MICs of telithromycin in the presence of erythromycin (4, 8, 16, 32, 64 and 128 mg/L) for strains 02J1095 and all the mutants were determined as previously described. Control strains consisted of the *S. pneumoniae* NCTC 13593 and *S. aureus* NCTC 6571.

Table 2. Selected representative mutants

Parent	NCTC 13593 (0.016 mg/L)	02J1095 *erm*(B) + (0.06 mg/L)	02J1175 *mef*(A) + (0.5 mg/L)
Generation 1	NCTC 1 (0.032 mg/L)	J I 1 (1 mg/L)	M I 2 (2 mg/L)
Generation 2	NCTC 2 (0.12 mg/L)	J II 1 (4 mg/L) J II 4 (16 mg/L) J II 5, 6 (32 mg/L) J II 7 to 9 (>32 mg/L)	M II 15 (4 mg/L)
Generation 3	NCTC 3 (0.5 mg/L)		M III 3 (8 mg/L)
Generation 4	NCTC 4 (0.5 mg/L)		M IV (8 mg/L)

Telithromycin MICs are given in parentheses.

Results

Mutation studies

Telithromycin-resistant mutants were created from the macrolide-resistant strains 02J1095 [*erm*(B) +] and 02J1175 [*mef*(A) +]. Selection of the macrolide-susceptible NCTC 13593 strain on telithromycin failed to produce telithromycin-resistant mutants (Table 2).

The MIC of 02J1095 and its mutants increased from 0.06 mg/L for the parent 02J1095 to >32 mg/L over two generations (Table 2). The first generation increase from 0.06 to 1 mg/L was a 16-fold increase; from first to second generation, the increase was greater than 32-fold. These increases were stable, such that when the mutants were serially subcultured 10 times, without the selective pressure of telithromycin, they still maintained the same telithromycin MIC.

The telithromycin MIC of the parent 02J1175 was 0.5 mg/L. The first generation mutants had telithromycin MICs between 1 and 4 mg/L (Table 2). The representative mutant, M I 2, selected to create the second generation had an MIC of 2 mg/L. This gave rise to a second generation with MICs between 2 and 4 mg/L. The third and fourth generations both had telithromycin MICs of 8 mg/L and further mutants with higher telithromycin MICs could not be obtained from them. The telithromycin MIC of the most resistant strain, fourth generation 02J1175 mutant, reverted from 8 to 1 mg/L after 10 passages in antibiotic-free medium. The MICs of M III 3 and M II 15 also decreased but only by one doubling dilution. The MIC of M I 2 decreased by two doubling dilutions. For the third generation strain, this means that its telithromycin resistance remained stable with an MIC of 4 mg/L. When the reverent strain of the fourth generation 02J1175 mutant was mutated on agar plates containing either 1 or 2 mg/L, all the resulting mutants returned to an MIC of 8 mg/L, the same as the fourth generation mutant. Therefore, in order for *S. pneumoniae* strains with a *mef*(A) gene to maintain their telithromycin MIC, selective pressure must be maintained. This has previously been noted by Davies *et al.*,[26] when the telithromycin and macrolide MICs reverted back to baseline MICs (or close to) after 10 passages on antibiotic-free media. This is not true for those containing the *erm*(B) gene. Once resistance or an elevated telithromycin MIC has been achieved, it is stable with or without the selective pressure.

The NCTC 13593 strain, which was macrolide-susceptible, had a telithromycin MIC of 0.016 mg/L. This increased sequentially to 0.032, 0.12, 0.5 and 0.5 mg/L for the four mutant generations, respectively (Table 2). The final generation mutants were telithromycin-susceptible.

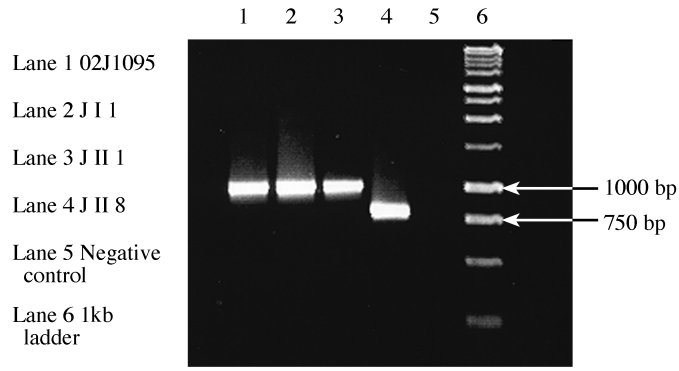

Figure 1. *erm*(B) promoter PCR gel.

PCR and sequencing of erm(B) and mef(A)

A representative of each mutant generation was chosen for further molecular analysis. The 02J1095 parent and all mutants tested contained the *erm*(B) gene, determined by PCR amplification. In order to investigate the mechanism of telithromycin resistance, the *erm*(B) gene from each representative mutant was amplified by PCR and sequenced. The same procedure was carried out on 02J1175 and its representative mutants with the *mef*(A) gene (Table 2). No mutations were found in either the *erm*(B) or *mef*(A) genes. No *erm*(B) or *mef*(A) genes were present in the NCTC 13593 parent or its mutants. The strains only contained an *erm*(B) gene or a *mef*(A) gene, and no strains were positive for both genes.

The *erm*(B) upstream region of 02J1095 and the selected mutants, described in Table 2, was amplified by PCR in order to investigate the nucleotide region from the promoter to the *erm*(B) gene. Deletions in this region have been associated with a change from inducible to constitutive erythromycin A resistance.[7,27] The corresponding bands of approximately 1000 base pairs of the *erm*(B) upstream region PCR products of 02J1095 and the selected mutants (Table 2) were visualized on the agarose gels. The J II 8 band however was just above the 750 bp band of the 1 kb ladder as shown in Figure 1.

The sequencing results showed some anomalies not only in J II 8 but also in each of the other strains, in comparison to the *erm*(B) upstream sequence of the Tn*1545* of *S. pneumoniae* in the NCBI website. The gene comparator was accession number X52632. The nucleotide changes are listed in Table 3 and their positions relative to the putative promoter (–35 and –10 sequences), the ribosome binding

Table 3. Nucleotide mutations in the *erm*(B) upstream region

Mutant strains (telithromycin MIC mg/L)	Nucleotide changes in comparison to *erm*(B) of Tn*1545*
02J1095 (0.06)	A292G, C297T, T318A, A319G
J I 1 (1)	A292G, C297T, T318A, A319G
J II 1 (4)	A292G, C297T, T318A, A319G, C324A
J II 4 (16)	A292G, C297T, T318A, A319G, C307T
J II 5 (32)	A292G, C297T, T318A, A319G
J II 6 (32)	A292G, C297T, T318A, A319G
J II 7 (>32)	A292G, C297T, T318A, A319G, G302A
J II 8 (>32)	210 bp deletion from 113 to 322
J II 9 (>32)	A292G, C297T, T318A, A319G, T322G

Figure 2. Sequence alignment of *erm*(B) upstream region.

sites (RBS) and control peptide are indicated in Figure 2. The G-292, T-297 and G-319 are all present in the *erm*(B) carrying plasmid pAM 77 of *Streptococcus sanguis* and the T318A mutation has previously been identified in an oral streptococci.[7,27] The C297T, A292G and the T318A, A319G mutations have been located in clinical isolates of *S. pneumoniae*, which had inducible expression of the *erm*(B) gene.[7,27] The mutations that were present only in J II 1, J II 4, J II 7 and J II 9 have not been previously identified. Similar deletions of large fragments of nucleotides upstream from the *erm*(B) gene have been located in *Streptococcus agalactiae* (HM1081) and *S. pneumoniae* (HM36), which had constitutive *erm*(B) expression.[7,27] The plasmid pAMβ1 of *Enterococcus faecalis* also contained the same deletion as these strains.[7,27] However, in these strains this deletion removed the first RBS and the control peptide but J II 8 had a deletion of the second RBS and the control peptide.

The *mef*(A) upstream region PCR products and the *mel* genes of the strains were amplified and the corresponding PCR bands at 614 and 1680 bp, respectively were visualized on the agarose gel. There were no changes in the sizes of the *mef*(A) upstream region, containing the promoter, or the downstream *mel* region in any of the mutants. The sequencing results of the entire *mef*(A) gene indicated no changes in either the parent 02J1175 or the representative mutants.

Induction experiment results

On both plates, with 02J1095 and J I 1 (Figure 3), the zone of inhibition around telithromycin had two straight edges, one each from erythromycin and clarithromycin. The D-shaped zone indicates that the antimicrobial agent to the left of the D induces resistance. From these photographs (Figure 3), it can be seen that erythromycin and clarithromycin both induce telithromycin resistance. The growth of J II 1 to J II 9 was either up to the discs or within 1 mm of the discs and so were recorded as resistant to the three antimicrobial agents and, as such, had constitutive expression of the *erm*(B) gene.

Induction assays

The MICs of the mutant strains derived from growth of 02J1095 on erythromycin are shown in Table 4. The telithromycin MICs of the strains tested in the presence of erythromycin are shown in Table 5.

The first set of MICs indicated that when 02J1095 was subcultured in the presence of erythromycin, the telithromycin MIC of all the mutant strains increased to 1 mg/L, regardless of the concentration of erythromycin that it was exposed to.

With regard to the second MIC test, the MICs of 02J1095 and all of the first generation mutants did increase. The telithromycin MICs of the already highly resistant second-generation strains increased or decreased regardless of the concentration of erythromycin. The telithromycin MIC of the parent 02J1095 increased from 0.06 to 1 mg/L or 2 mg/L and that of the first generation mutants increased gradually from 0.25 to 32 mg/L and that of the second generation mutants increased from 4 to >32 mg/L depending on the strain and the concentration of erythromycin. Therefore, as indicated by the disc diffusion test and the two induction tests, erythromycin does induce telithromycin resistance. However, the level of induction varies depending on the initial telithromycin MIC and does not increase beyond a telithromycin MIC of 64 mg/L.

Ribosomal mutations

Mutations in the domains II and V of the 23S rRNA have previously accounted for increases in MIC of both macrolides and telithromycin.[12,14,15,28–30] Therefore, it was plausible to speculate that changes in either or both of these regions could be responsible for the increase in telithromycin MIC. The region of 23S rRNA from nucleotide 1 to 1011, representing domain II, of the two most resistant strains J II 8 and M IV was amplified and sequenced. No heterogeneity in the nucleotide sequences of the hairpin 35 was observed. Thus no changes were present in any of the four alleles of the 23S rRNA.

All four genes of the domain V region of 23S rRNA were amplified and sequenced individually. Again the domain V sequences were identical to those of the TIGR 4 strain sequences.[31] This indicates that no changes were present in the peptidyl transferase region of the 23S rRNA

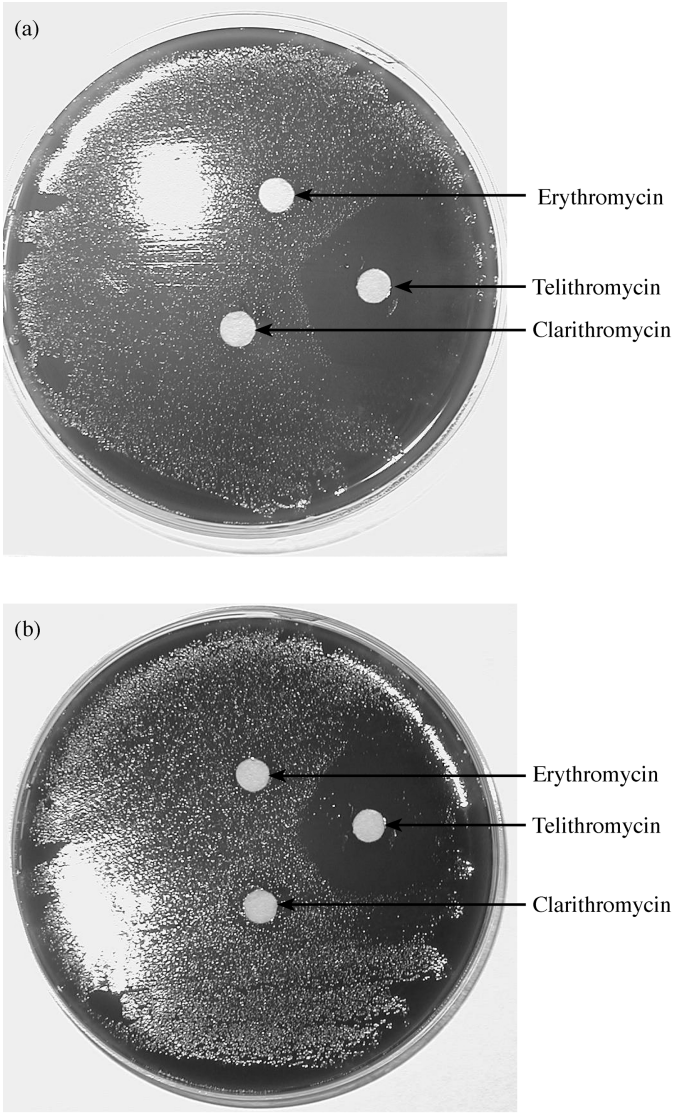

Figure 3. Induction experiment results. (a) Parent strain 02J1095 with discs of erythromycin, clarithromycin and telithromycin; (b) mutant J I 1 with discs of erythromycin, clarithromycin and telithromycin.

The lack of mutation in the 23S rRNA suggested that changes could be present in L4 and L22 ribosomal proteins associated with the peptidyl transferase region. Changes in the L4 and L22 have been implicated in decreased susceptibility of *S. pneumoniae* to telithromycin and erythromycin.[15,16,32] The L4 protein genes of 02J1095, J II 8 [*erm*(B) +], 02J1175 and M IV [*mef*(A) +] were amplified and sequenced. No changes, insertions or deletions occurred in either resistant strain in comparison to the susceptible TIGR 4 strain or the macrolide-resistant parent strains. Using primers described by Tait-Kamradt *et al.*, the genes encoding the L22 protein of 02J1095, J I 1, J II 1, J II 4, J II 5, J II 6, J II 7, J II 8, J II 9, 02J1175 and M IV were amplified and sequenced.[15] Again, there were no changes present in any of the strains tested except one strain, J II 8, in which a Lys-94 to Gln-94 change was determined (Figure 4). This change was due to an adenine to cytosine change. This is the first incidence of a Lys-94 to Gln-94 change in L22 being associated with telithromycin resistance.

Table 4. Telithromycin MICs of erythromycin generated mutants

Strain	Telithromycin MIC (mg/L)
02J1095	0.06
No antibiotic	0.06
E4 1–3	1
E8 1–3	1
E16 1–3	1
E32 1–3	1
E64 1–3	1
E128 1–3	1

E4, E8, E16, E32, E64 and E128 numbered 1 to 3 are the mutants subcultured on erythromycin at 4, 8, 16, 32, 64 and 128 mg/L, respectively.

Discussion

The mutants generated from a strain with the *erm*(B) gene were stable, unlike the mutants from a *mef*(A)-positive strain. However, the *mef*(A) revertants only required the presence of telithromycin to return immediately to the highest telithromycin MIC once more. They did not return to the telithromycin MIC of 8 mg/L by stepwise selection but merely in one step, which suggests a compensatory mutation. Strain NCTC 13593 did not have either the *erm*(B) or *mef*(A) genes present. The highest telithromycin MIC of any NCTC 13593 mutant was 0.5 mg/L, which is still clinically susceptible. It is interesting to note that in the erythromycin A-resistant strains either due to the *erm*(B) gene or *mef*(A) gene, telithromycin resistance emerged after only two generations. To date telithromycin-resistant mutants have usually been derived from macrolide-susceptible parent strains, which could explain why the results of this study vary from many of those previously published. However, Davies *et al.* passaged *S. pneumoniae* strains, which were *mef*(A)-positive, *erm*(B)-positive or macrolide-susceptible on a variety of antimicrobial agents.[26] The number of passages required for telithromycin resistance to emerge varied. The macrolide-susceptible strain required 24 passages on telithromycin for a strain with a telithromycin MIC of 8 mg/L to emerge, whereas a strain with an *erm*(B) gene required only three passages in telithromycin to result in a mutant with a telithromycin MIC of 8 mg/L.

The results of the *erm*(B) and *mef*(A) gene sequencing indicated that no changes in either gene were associated with telithromycin resistance. Therefore, whereas these genes are required for the selection of telithromycin resistance, they themselves do not change to facilitate telithromycin resistance.

Deletions in the *erm* gene attenuator region in *Streptococcus pyogenes*, *S. pneumoniae* and *S. agalactiae* have all been associated with constitutive expression of their Erm methylases. In *S. pyogenes*, deletions of 163 base pairs or six base pairs and a duplication of 101 base pairs in the *erm*(TR) upstream region resulted in an increase in clindamycin MIC from 1 to 128 mg/L when transformed into *E. coli*.[33] The *S. pyogenes* strains with mutated attenuators were mutants, which had been selected on clindamycin, and the parent strain was inducibly intermediate to erythromycin and fully susceptible to clindamycin. Tait-Kamradt *et al.* described two *S. pneumoniae* clinical isolates with truncated *erm*(B) leader peptides of 15 and 19 amino acids.[8]

Table 5. Telithromycin MICs of *erm*(B) + mutants in the presence of increasing concentrations of erythromycin

Strain	TEL MIC (no ERY)	TEL MIC (ERY 4 mg/L)	TEL MIC (ERY 8 mg/L)	TEL MIC (ERY 16 mg/L)	TEL MIC (ERY 32 mg/L)	TEL MIC (ERY 64 mg/L)	TEL MIC (ERY 128 mg/L)
02J1095	0.06	1	1	1	1	2	2
J I 1	1	8	4	8	8	16	16
J I 2	2	8	8	8	4	32	16
J I 3	0.25	4	4	4	4	8	16
J I 4	0.5	8	8	16	8	16	32
J I 5	0.5	4	4	4	4	8	8
J I 6	0.5	8	8	8	8	32	32
J I 7	2	8	8	8	8	32	32
J II 1	4	16	16	16	16	32	32
J II 2	32	64	64	32	64	64	64
J II 3	32	16	32	16	16	32	32
J II 4	16	4	4	4	4	4	8
J II 5	32	32	32	32	32	64	32
J II 6	32	32	32	32	32	32	32
J II 7	>32	32	32	32	32	32	32
J II 8	>32	64	32	64	32	32	64
J II 9	>32	32	32	32	32	64	32

TEL, telithromycin; ERY, erythromycin.

These two strains had telithromycin MICs of 1 and 256 mg/L, respectively and both also had changes in the gene itself, leading to three amino acid changes. The strain with the 256 mg/L telithromycin MIC also contained L4 riboprotein amino acid mutations of glycine, threonine and glycine at amino acids 69 to 71 to threonine, proline and serine, respectively.

The results of the PCR experiments of the *erm*(B) upstream region carried out with the parent 02J1095 and the mutants derived from it showed that the J II 8 strain had a large deletion of 210 bp in this section. This result is very similar to that found with the clindamycin-resistant *S. pyogenes*. Therefore, it is possible that a mechanism of resistance exists that alters the *erm*(B) attenuator of strains inducibly resistant to erythromycin in order to confer resistance to other antimicrobial agents within the same group, such as clindamycin and telithromycin. The results of Tait-Kamradt *et al.* are similar to the findings of this study in that the telithromycin-resistant strains both had truncated regions upstream from the *erm*(B) gene.[8] The other mutants investigated in this study, which were also highly telithromycin-resistant, did not however have such a deletion. Therefore, whereas the mutation in the *erm*(B) attenuator may be at least partly responsible for telithromycin resistance in J II 8, this is not the case for the other five highly telithromycin-resistant strains.

The mutated nucleotides of the strains investigated at positions 292, 297 and 319 are the same nucleotides as those of plasmid pAM 77 from *Streptococcus sanguis* and the nucleotide change at position 318 was found previously in an oral streptococcus.[7,27] These mutations were in the parent 02J1095 and all the mutants, except J II 8, and as such do not appear to be involved in the development of telithromycin resistance. It is, however, interesting to note the nucleotide changes at 318 and 319 are just at the end of the deletion in J II 8. Two previously reported *S. pneumoniae* isolates with the same 318 and 319 mutations were both inducibly erythromycin-resistant but susceptible to the ketolide HMR 3004.[27] The other single mutations in J II 1, J II 4, J II 7 and J II 9 have not previously been associated with changes in resistance patterns. But as each strain has a different mutation, it is unlikely that these mutations individually lead to telithromycin resistance.

The disc diffusion experiments indicated, from the D-shaped zones of inhibition around the telithromycin disc, that erythromycin and clarithromycin are both inducers of telithromycin resistance in the strains 02J1095 and J I 1. This finding was verified by two further induction studies with erythromycin at concentrations from 4 to 128 mg/L. There were variations in the increase in telithromycin MIC but the increase in telithromycin MICs in all strains caused a change from telithromycin-susceptible to non-susceptible or resistant. This is the first report of erythromycin induction of telithromycin resistance in *S. pneumoniae*.

The high-level telithromycin MICs for the mutants are not all caused by the same mutation in the *erm*(B) attenuator. Although the large deletion in J II 8 is such that it is probably part of the mechanism used by this strain to overcome telithromycin, the sizes of the upstream and downstream regions from the *mef*(A) genes suggest that no large nucleotide deletions have occurred. The presence of the *mel* genes in these strains confirms previous studies of the *mef*(A) operon, which suggested the presence of the *mel* gene downstream from the *mef*(A) gene. From this study, it can be seen that deletions of large numbers of nucleotides from either the upstream or downstream nucleotide regions of the *mef*(A) gene are not responsible for the increase in telithromycin MIC and resistance.

The main regions of interest to date for macrolide resistance in *S. pneumoniae* in the 23S rRNA are the domains II and V. The area of consequence in domain II is the hairpin 35. A deletion in one adenine in the series of four located at positions 749 to 752 resulted in a 500-fold increase in the telithromycin MIC for a *S. pneumoniae* strain. In this case, it became resistant to telithromycin (4 mg/L).[16] Previously, a single point mutation (U754A) in a laboratory strain of

```
atggcagaaattacttcagctaaagcaatggctcgtacagtacgt
 M  A  E  I  T  S  A  K  A  M  A  R  T  V  R
gtttcacctcgtaaatcacgtcttgttcttgataacatccgtggt
 V  S  P  R  K  S  R  L  V  L  D  N  I  R  G
aaaagcgtagccgatgcaatcgcaatcttgacattcactccaaac
 K  S  V  A  D  A  I  A  I  L  T  F  T  P  N
aaagctgctgaaatcatcttgaaagttttgaactcagctgtagct
 K  A  A  E  I  I  L  K  V  L  N  S  A  V  A
aacgctgaaaacaactttggtttggataaagctaacttggtagta
 N  A  E  N  N  F  G  L  D  K  A  N  L  V  V
tctgaagcattcgcaaacgaaggaccaactatgaaacgtttccgt
 S  E  A  F  A  N  E  G  P  T  M  K  R  F  R
ccacgtgcgcaaggttcagcttcaccaatcaacaaacgtacagct
 P  R  A  Q  G  S  A  S  P  I  N  K  R  T  A
cacatcactgtagctgttgcagaaaaa
 H  I  T  V  A  V  A  E  K
```

Figure 4. L22 gene sequence and amino acid translation of J II 8.

E. coli resulted in the cells being resistant to telithromycin.[30] For domain V there is less specificity with regard to one macrolide-resistant region. Nucleotide changes have been located at 2058, 2059 and 2611. There is also variation in the nucleotide changes for these sites.[13,15,16,32] These two regions were the starting points of the search for answers to the increase in telithromycin MIC or resistance. However, as no nucleotide changes or deletions were identified in either region, the possibility of domain II or V providing the answers was ruled out.

The L4 protein has been associated with large increases in telithromycin MIC in *S. pneumoniae*. Insertion of six amino acids into a highly conserved area of ribosomal L4 protein (^{63}KPWRQKGTGRAR^{74}) has been associated with a 500-fold increase in the MIC of telithromycin.[32] Once again, there were no changes in the nucleotide sequences and so the amino acid sequence of either J II 8 or M IV. Changes within the L22 protein amino acid sequence have also been reported as a cause of increased telithromycin MIC from 0.008 to 0.25 mg/L. This was due to three simultaneous amino acid mutations: alanine-93 to glutamic acid-93, proline-91 to serine-91 and glycine-83 to glutamic acid-83.[16] The L22 protein binds primarily to the 23S rRNA. Mutations in this protein could change the conformation of 23S rRNA and thus affect ketolide binding.[19] In this study, the only mutation that was found was in the L22 of J II 8, a highly telithromycin-resistant strain. The mutation did not occur in mutants of the same parent with MICs of 4 or 1 mg/L. The change at amino acid 94 from lysine to glutamine was between two previously documented changes: glycine-95 to aspartic acid-95 and alanine-93 to glutamic acid-93. The glycine-95 to aspartic acid-95 mutation was associated with increases in erythromycin MIC from 0.03 to 1 mg/L and 0.015 to 0.25 mg/L. The two strains were selected with erythromycin and roxithromycin, respectively.[16] The alanine-93 to glutamic acid-93 mutation was in combination with two other changes as previously mentioned. The change from ionic lysine to the neutral glutamine near the 3′ end of the protein could result in a conformational change of the protein and thus prevent telithromycin binding. The previously documented changes at amino acids 93 and 95 may also cause a conformational change in the L22 protein and thus prevent erythromycin binding.

No telithromycin resistance could be created from a macrolide-susceptible strain. Therefore although telithromycin has high activity against macrolide-resistant strains, if the strain is exposed to telithromycin *in vitro*, the activity of telithromycin will fall such that the strain becomes telithromycin-resistant within a few generations. The highly telithromycin-resistant strain J II 8 had two mutations, one in the *erm*(B) attenuator and the other in the L22 riboprotein. Whereas this strain had two mutations, none of the other strains had any mutations. Therefore, whereas in this strain these mutations are the most probable cause of telithromycin resistance, this is not the case with the other telithromycin-resistant strains.

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