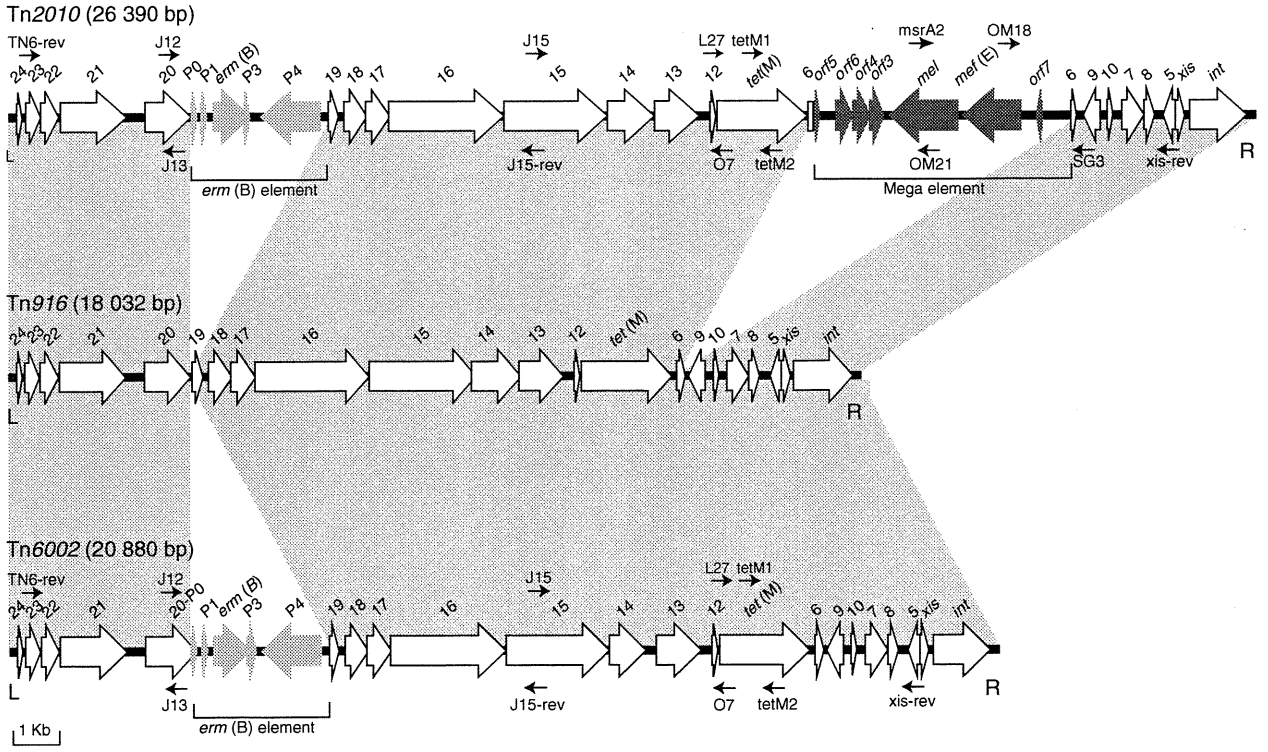

Figure 1 Comparative representation of the ORF maps of Tn*916* and Tn*916* family transposons with Tn*2010*, carrying *erm*(B) and mega element and Tn*6002*, carrying only the *erm*(B) element. The black bar represents Tn*916*, and the white arrows indicate the locations of the relevant ORFs. The light-grey and dark-grey arrows correspond to *erm*(B) and mega elements, respectively. The black arrowheads above and below ORFs show the positions and directions of the primers used to map the transposons.

Tn*6002* (Cochetti *et al.* 2007, 2008; Del Grosso *et al.* 2007). According to the previous reports, the noncoding region between the left end of the *erm*(B) element and the ATG start codon of P0 of the open reading frame (ORF) upstream of the *erm*(B) element span a region of 49 bp in case of Tn*2010* and 48 bp in the case of Tn*6002*, respectively (Fig. 1). The ATG start codon of P0 of the upstream ORF of the *erm*(B) element is located between nucleotides 50 and 52 nucleotide from left end of *erm*(B) element in the case of Tn*2010* and from the corresponding nucleotides 49–51 in the case of Tn*6002*. In Tn*6002*, nucleotide 'A' at the position 43 in the left end of the *erm*(B) element is deleted from Tn*2010*. The insertion of the *erm*(B) element into Tn*916* has resulted in the ORF20 coding sequence of Tn*916* being extended by 19 amino acids in Tn*2010*, and 71 amino acids in Tn*6002*, beyond original C-terminal amino acid of ORF20. The translation stop codon of the extended ORF20 of Tn*6002* is shared with that of P0 of the *erm*(B) element. The complete nucleotide sequence of Tn*6002* is found within these two transposons (Warburton *et al.* 2007). Thus, we compared the sequence of the Tn*2010*-like and Tn*6002*-like elements with that of Tn*6002*, with the exception of the *mega* element region.

Thirteen strains carrying the Tn*2010* element were divided into three groups (A to C) according to the genetic structure of the Tn*2010* element (Table 3, Fig. 2). Eleven type A strains, which were isolated from four different hospitals, belonged to serotype 19F and sequence type ST271. With the exception of one strain designated 05D058, which showed serotype 19A and ST320, this data implied that these strains were clonally related by PFGE analysis, and carried Tn*2010* elements that are identical to the prototype Tn*2010*. Like the *erm*(B) element of Tn*6002*, the Tn*2010* element of another two strains (i.e., 05O035 classed as type B, and 05P006 classed as type C) of the 13 strains had an adenine deletion at the noncoding region seven nucleotides upstream from the P0 open reading frame of the *erm*(B) element of Tn*2010* (Del Grosso *et al.* 2007; Warburton *et al.* 2007). The five strains of Tn*6002* except 05O071 strain of type D and the two strains of Tn*2010*, which had an adenine deletion at the noncoding region seven nucleotides upstream from the P0 open reading frame, showed the low-level resistance to macrolides. These implied that an adenine deletion associated to the low-level resistance to erythromycin. On the other hand, the data that 05P071-carrying Tn*6002* element (type D) had the deletion and still

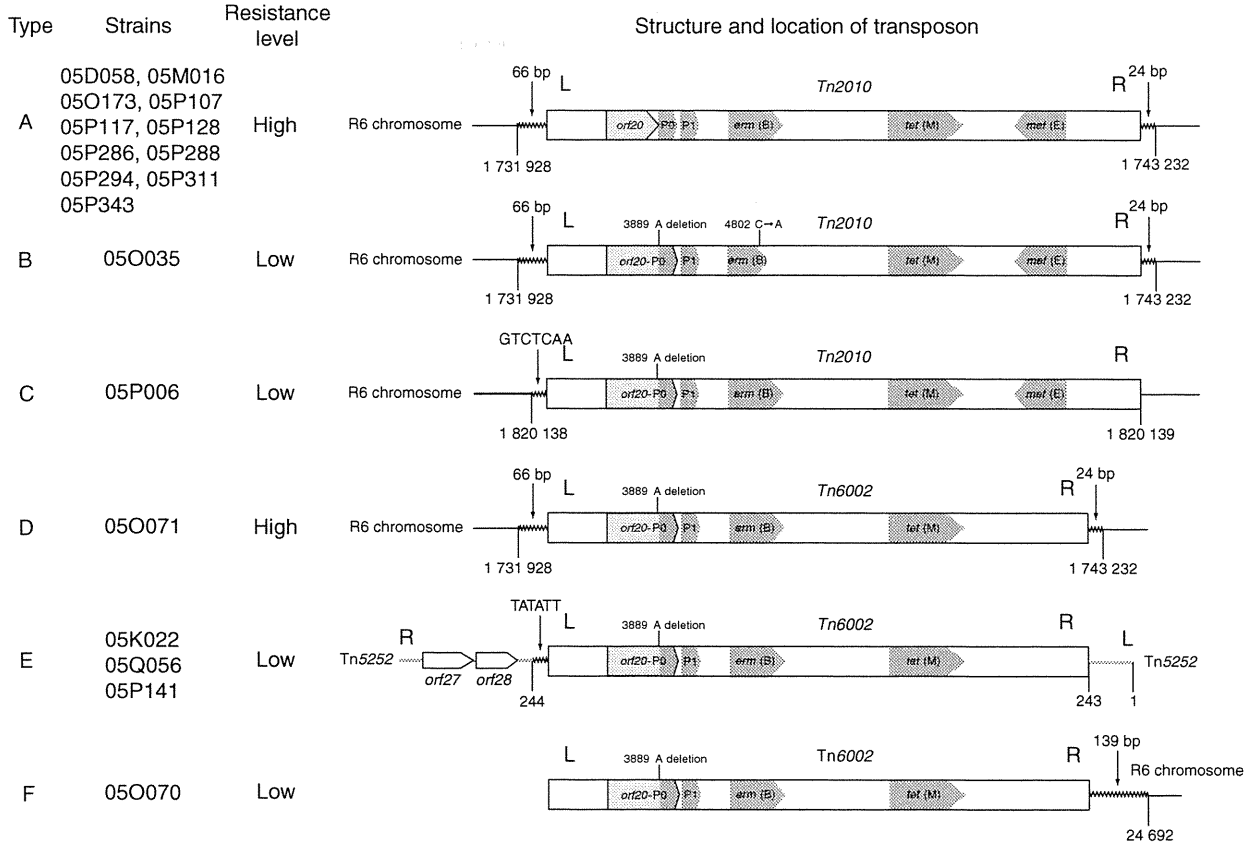

Figure 2 Junctions of Tn*2010* and Tn*6002* with the chromosome or other transposon in the 18 strains tested. 'Type' designated A to F refers to combination types based on the strains' phenotype and genotype of mobile genetic elements (Table 1). Resistance level indicates the erythromycin resistance level, high means MIC values ≥ 128 mg l^{-1} and low means MIC values between 1 to 8 mg l^{-1}. Boxes represent transposons identified in the study and the dark-grey arrows indicate the main resistance genes carrying by the transposons. Light-grey arrows indicate *orf20* of the transposons. One nucleotide 'A' deletion at 3889 position caused the gene fusion between *orf20* and P0, and resultant ORF was designated as *orf20*-P0. The black bar represents the *S. pneumoniae* R6 chromosome, and the light-grey bar represents the transposon Tn*5252*. Thin white arrows indicate the ORFs encoded on Tn*5252*. The numbers under the chromosome and transposons are the positions of the first nucleotides upstream and downstream of the inserts. The zigzag lines indicate exogenous sequences showing no homology with other bacterial sequences. In type F, only the right end of Tn*6002* could be identified, it was not possible to identify the left end.

showed the high-level resistance, also suggested that one adenine nucleotide deletion in *erm*(B) element did not simply relate to the phenotype of low-level resistance to erythromycin. The erythromycin resistance level would be affected by the gene expression, the host factor(s) (i.e. ribosomal RNAs), structure of *erm*(B) or the presence of another resistance determinant. The DNA sequence analysis of each *erm*(B) element of the isolates showed that the type B strain 05O035 had one nucleotide substitution in the *erm*(B) coding sequence (TGC converted to TGA), which resulted in a premature stop codon at amino acid number 161 in the 246 amino acid chain of the wild-type *erm*(B) (Fig. 2) (Tomich *et al.* 1980). These data implied that the low-level resistance to macrolide of strain 05O035 might be because of the nucleotide substitution. To elucidate the relations between the phenotypes and the genotypes in the other isolates, further molecular analyses are needed.

Analysis of the regions flanking from the transposons revealed that all Tn*2010* elements of type A and type B were inserted between 1 731 928 and 1 743 232 bp within the same insertion site as that of the prototype Tn*2010*, where the *orf24* end of Tn*2010* was inserted at the 3′ end of *spr1764* of the *S. pneumoniae* R6 genome and the *int* end of Tn*2010* was inserted between *spr1774* and *spr1775* of the *S. pneumoniae* strain R6, resulting in a deletion of approximately 11 300 bp (Fig. 2) (Hoskins *et al.* 2001; Del Grosso *et al.* 2007). The twelve strains with type A and type B Tn*2010* insertions, which were identical to one of the internationally spread multidrug-resistant pneumococcal clone Taiwan^{19F}-14 (clonal complex CC271). The Tn*2010* element of Taiwan^{19F}-14 strain

inserts within the same insertion site as that of prototype Tn*2010*. The Tn*2010* element of strain 05P006 named as type C was found to be inserted at a novel site at nucleotide position 1 820 138 in the middle of *spr1843* of the *S. pneumoniae* R6 strain and it had no deletions.

The genetic structures of the Tn*6002* elements of five of the strains were identical to that of the prototype Tn*6002*. A variety of Tn*6002* element insertion sites were identified, and the five strains were grouped into three types (D to F) according to their insertion site (Fig. 2). The Tn*6002* element of the type D strain was located between 1731 928 and 1743 232 bp at the same insertion site as that of the prototype Tn*6002* or Tn*2010* on the *S. pneumoniae* R6 genome.

The Tn*6002* element of strain 05K022, 05P141 and 05Q056 (type E) was located downstream of *orf28* in Tn*5252*, where the right end of the Tn*6002* element was located 243 bp upstream of the left end of Tn*5252*. Tn*5252* (47·5 kbp) is a conjugative transposon carrying the chloramphenicol-resistant determinant (Alarcon-Chaidez *et al.* 1997). Tn*5252* was originally identified in transposon Tn*5253* (65·5 kb) (Ayoubi *et al.* 1991). The composite Tn*5253* transposon is composed of a Tn*5251* insertion (18 kb, Tc^r), which is similar to the conjugative transposon Tn*916*, into Tn*5252* (Flannagan *et al.* 1994). These data implied that the Tn*6002* element in strains 05K022, 05P141 and 05Q056 might be a part of a composite transposon, which was the result of the insertion of a Tn*6002* element into a Tn*5252*-like element. The location of the large composite transposon of 05K022 and 05P141 might differ from that of 05Q056 on the chromosome because those showed different sequence types and serotypes (Table 1).

In strain 050070 classed as type F, only the right end (R end) insertion site of the Tn*6002* transposon could be identified. The left end (L end) insertion site was not identified in the reported R6 genome even by performing serial PCR analyses from the R end to a point 15 kbp upstream.

Complete nucleotide sequence

The complete nucleotide sequence analysis of the Tn*2010* element of the representative strain 05P294 showed that it was 26 390 bp in length and shared significant structural and sequence homology with Tn*6002* (accession number: AY898750), with the exception of an insertion of the 5511-bp mega element within *orf6* of the Tn*6002* element (Fig. 1). The mega element of the Tn*2010* element of 05P294 was identical to that of the prototype Tn*2010* (Warburton *et al.* 2007). With the exception of the mega element, a total of 70 nucleotide mutations were found in the Tn*2010* element (accession number: AB426620) when it was compared with Tn*6002*. These mutations are located in the upstream region of *orf20*, in the middle section of *orf16* and within *tet*(M). The *tet*(M) gene in the Tn*2010* element was completely identical to the *S. pneumoniae tet*(M) gene (accession number: X90939) (Provvedi *et al.* 1996). The nucleotide mutations found in the region corresponding to Tn*916* element of Tn*2010* implied that the origins of Tn*2010* of Chinese isolates belonging to clonal complex CC271 could be different from that of the sequenced prototype Tn*6002* found in Europe, and these Tn*2010* elements were not resultants by single event of insertion of mega element into the prototype Tn*6002*.

Concluding remarks

Our data in this study indicated that the internationally spread multidrug-resistant pneumococcal clone belonged to Taiwan^{19F}-14 type strain of CC271 was also spread in mainland China. The strains were resistant to penicillin, erythromycin and tetracycline, and carried Tn*2010* element encoded *erm(B)*, *mef(E)* and *tet(M)*. The Tn*2010* elements of Chinese strains inserted within the same insertion site of as that of prototype Tn*2010*. Beside the clonal complex CC271 strains, we found new ST-type strains carrying Tn*2010* or Tn*6002*, which were designated as ST3398, ST3397, ST3263 and ST3262, respectively. ST3398 isolate was carrying Tn*2010* at different location on the chromosome and was resistant to erythromycin and tetracycline and sensitive to penicillin. ST3397, ST3263 and ST3262 strains were carrying Tn*6002* element. These results indicated that in addition to CC271 strain, various types of *S. pneumoniae* strains carrying Tn*2010* or Tn*6002* element would be spread in China, and the strains shown in this report would become the reference *S. pneumoniae* strains in China.

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References

Alarcon-Chaidez, F., Sampath, J., Srinivas, P. and Vijayakumar, M.N. (1997) Tn*5252*: a model for complex streptococcal conjugative transposons. *Adv Exp Med Biol* **418**, 1029–1032.

Amezaga, M.R., Carter, P.E., Cash, P. and Mckenzie, H. (2002) Molecular epidemiology of erythromycin resistance in *Streptococcus pneumoniae* isolates from blood and noninvasive sites. *J Clin Microbiol* **40**, 3313–3318.

Ayoubi, P., Kilic, A. and Vijayakumar, M.N. (1991) Tn*5253*, the pneumococcal Ω(*cat tet*) BM6001 element, is a composite structure of two conjugative transposons, Tn*5251* and Tn*5252*. *J Bacteriol* **173**, 1617–1622.

Brenciani, A., Bacciaglia, A., Vecchi, M., Vitali, L.A., Varaldo, P.E. and Giovanetti, E. (2007) Genetic elements carrying *erm*(B) in *Streptococcus pyogenes* and association with *tet*(M) tetracycline resistance gene. *Antimicrob Agents Chemother* **51**, 1209–1216.

Brito, D.A., Ramirez, M. and Lencastre, H. (2003) Serotyping *Streptococcus pneumoniae* by multiplex PCR. *J Clin Microbiol* **41**, 2378–2384.

Caillaud, F., Trieu-Cuot, P., Carlier, C. and Courvalin, P. (1987) Nucleotide sequence of the kanamycin resistance determinant of the pneumococcal transposon Tn*1545*: evolutionary relationships and transcriptional analysis of aphA-3 genes. *Mol Gen Genet* **207**, 509–513.

Clinical and Laboratory Standards Institute (2006) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Document M7-A7*. Wayne, PA: Clinical and Laboratory Standards Institute.

Cochetti, I., Tili, E., Vecchi, M., Manzin, A., Mingoia, M., Varaldo, P.E. and Montanari, M.P. (2007) New Tn*916*-related elements causing *erm*(B)-mediated erythromycin resistance in tetracycline-susceptible pneumococci. *J Antimicrob Chemother* **60**, 127–131.

Cochetti, I., Tili, E., Mingoia, M., Varaldo, P.E. and Montanari, M.P. (2008) *erm*(B)-carrying elements in tetracycline-resistant pneumococci and correspondence between Tn*1545* and Tn*6003*. *Antimicrob Agents Chemother* **52**, 1285–1290.

Daneman, N., McGeer, A., Green, K. and Low, D.E. (2006) Macrolide resistance in Bacteremic pneumococcal disease: implications for patient management. *Clin Infect Dis* **43**, 432–438.

Del Grosso, M.D., d'Abusco, A.S., Iannelli, F., Pozzi, G. and Pantosti, A. (2004) Tn*2009*, a Tn*916*-like element containing *mef*(E) in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* **48**, 2037–2042.

Del Grosso, M.D., Camilli, R., Iannelli, F., Pozzi, G. and Pantosti, A. (2006) The *mef*(E)-carrying genetic element (mega) of *Streptococcus pneumoniae*: insertion sites and association with other genetic elements. *Antimicrob Agents Chemother* **50**, 3361–3366.

Del Grosso, M.D., Northwood, J.G.E., Farrell, D.J. and Pantosti, A. (2007) The macrolide resistance genes *erm*(B) and *mef*(E) are carried by Tn*2010* in dual-gene *Streptococcus pneumoniae* isolates belonging to clonal complex CC271. *Antimicrob Agents Chemother* **51**, 4184–4186.

Enright, M.C. and Spratt, B.G. (1998) A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. *Microbiology* **144**, 3049–3060.

Farrell, D.J., Jenkins, S.G., Brown, S.D., Patel, M., Lavin, B.S. and Klugman, K.P. (2005) Emergence and spread of *Streptococcus pneumoniae* with *erm*(B) and *mef*(A) resistance. *Emerg Infect Dis* **11**, 851–858.

Farrell, D.J., Couturier, C. and Hryniewicz, W. (2008) Distribution and antibacterial susceptibility of macrolide resistance genotypes in *Streptococcus pneumoniae*: PROTEKT year 5 (2003-2004). *Int J Antimicrob Agents* **31**, 245–249.

Felmingham, D., Canton, R. and Jenkins, S.G. (2007) Regional trends in beta-lactam, macrolide, fluoroquinolone and telithromycin resistance among *Streptococcus pneumoniae* isolates 2001-2004. *J Infect* **55**, 111–118.

Flannagan, S.E., Zitzow, L.A., Su, Y.A. and Clewell, D.B. (1994) Nucleotide sequence of the 18-kb conjugative transposon Tn*916* from *Enterococcus faecalis*. *Plasmid* **32**, 350–354.

Fuller, J.D., McGeer, A. and Low, D.E. (2005) Drug-resistant pneumococcal pneumonia: clinical relevance and approach to management. *Eur J Clin Microbiol Infect Dis* **24**, 780–788.

Hoskins, J., Alborn, W.E.Jr., Arnold, J. Blaszczak, L.C., Burgett, S., Detloff, B.S., Estrem, S.T., Fritz, L. *et al.* (2001) Genome of the bacterium *Streptococcus pneumoniae* strain R6. *J Bacteriol* **183**, 5709–5717.

Ko, K.S. and Song, J.H. (2004) Evolution of erythromycin-resistant *Streptococcus pneumoniae* from Asian countries that contains *erm*(B) and *mef*(A) genes. *J Infect Dis* **190**, 739–747.

Lawrence, E.R., Griffiths, D.B., Martin, S.A., George, R.C. and Hall, L.M. (2003) Evaluation of Semiautomated multiplex PCR assay for determination of *Streptococcus pneumoniae* serotypes and serogroups. *J Clin Microbiol* **41**, 601–607.

Lefevre, J.C., Faucon, G., Sicard, A.M. and Gasc, A.M. (1993) DNA Fingerprinting of *Streptococcus pneumoniae* strains by pulsed-field gel electrophoresis. *J Clin Microbiol* **31**, 2724–2728.

Li, J.T., Weinstein, A.J. and Yang, M. On behalf of China Bacterial Resistance Surveillance Study Group. (2001) Surveillance of bacterial resistance in China. *Natl Med J China* **81**, 8–16.

Mcgee, L., Klugman, K.P., Wasas, A., Capper, T., Brink, A. and the Antibiotics Surveillance Forum of South Africa. (2001) Serotype 19F multiresistant *pneumococcal* clone harboring two erythromycin resistance determinants [*erm*(B) and *mef*(A)] in South Africa. *Antimicrob Agents Chemother* **45**, 1595–1598.

Provvedi, R., Manganelli, R. and Pozzi, G. (1996) Characterization of conjugative transposon Tn*5251* of *Streptococcus pneumoniae*. *FEMS Microbiol Lett* **135**, 231–236.

Singh, A., Goering, R.V., Simjee, S., Foley, S.L. and Zervos, M.J. (2006) Application molecular techniques to the study of hospital infection. *Clin Microbiol Rev* **19**, 512–530.

Sutcliffe, J., Grebe, T., Tait-Kamradt, A. and Wondrack, L. (1996) Detection of erythromycin-resistant determinants by PCR. *Antimicrob Agents Chemother* **40**, 2562–2566.

Tenover, F.C., Arbeit, R., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H. and Swaminathan, B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233–2239.

Tomich, P.K., An, F.Y. and Clewell, D.B. (1980) Properties of erythromycin-inducible transposon Tn*917* in *Streptococcus faecalis*. *J Bacteriol* **141**, 1366–1374.

Trallero, E.P., Montes, M., Orden, B., Tamayo, E., Arenzana, J.M.G. and Marimon, J.M. (2007) Phenotypic and genotypic characterization of *Streptococcus pyogenes* isolates displaying the MLS_B phenotype of macrolide resistance in Spain, 1999 to 2005. *Antimicrob Agents Chemother* **51**, 1228–1233.

Warburton, P.J., Palmer, R.M., Munson, M.A. and Wade, W.G. (2007) Demonstration of *in vivo* transfer of doxycycline resistance mediated by a novel transposon. *J Antimicrob Chemother* **60**, 973–980.

Wu, W.J., Zhang, H., Jiang, Y.Q. and Ni, Y.X. (2006) Mechanism and dissemination of macrolide resistance in *Streptococcus pneumoniae* isolates from Shanghai. *Chin J Infect Chemother* **6**, 85–88.

Yang, H., Yang, Y.H., Nielsen, E., Yu, S.J., Yuan, L., Wang, Y.H. and Shen, X.Z. (2005) Distribution of resistance genes *erm*B, *mef*A, *tet*M and the integrase gene *intTn* of Tn*1545* in *Streptococcus pneumoniae*. *Chin J Microbiol Immunol* **25**, 677–682.

Zhao, T.M., Xiangqun, F. and Liu, Y.N. (2004) Resistance phenotypes and genotypes of erythromycin-resistant *Streptococcus pneumoniae* isolates in Beijing and Shenyang, China. *Antimicrob Agents Chemother* **48**, 4040–4041.

RESEARCH LETTER

Mutational analysis of reduced telithromycin susceptibility of *Streptococcus pneumoniae* isolated clinically in Japan

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Abstract

A total of 132 *Streptococcus pneumoniae* isolates collected between 2005 and 2006 in Japan were examined for susceptibility to telithromycin (TEL) and macrolide. The overall resistance to macrolide was 80%. Among the isolates, 128 strains had low-level TEL susceptibility (minimal inhibitory concentrations [MICs] 0.03–1 $\mu g\,mL^{-1}$), suggesting that pneumococci with reduced susceptibility to TEL have appeared without prior exposure to the drug, although none of the isolates were assigned as TEL-resistant (breakpoint, $\geq 4\,\mu g\,mL^{-1}$). Eight of these isolates (MIC 0.5–1 $\mu g\,mL^{-1}$) were analyzed for macrolide resistance determinants and genetic relatedness. They all carried *mefE-mel*, which encodes the macrolide efflux genetic assembly, and three also harbored *ermB*, which encodes rRNA methylase. Allele replacement mutagenesis of the corresponding genes in the clinical isolates revealed that reduced TEL susceptibility (MIC 1 $\mu g\,mL^{-1}$) in *S. pneumoniae* may be caused by acquisition of the *mefE-mel* element only and additionally conferred by the *ermB* determinant.

Introduction

Telithromycin (TEL) is a semi-synthetic derivative of the 14-membered macrolide erythromycin (EM), and the first ketolide approved for clinical use. It has demonstrated high efficacy against *Streptococcus pneumoniae* isolates that cause community-acquired respiratory tract disease (Bozdogan *et al.*, 2003; Fogarty *et al.*, 2003). TEL and EM bind close to the peptidyl transferase region of the 50S ribosomal subunit and inhibit bacterial protein synthesis by blocking the elongation of the peptide chain through the ribosomal tunnel (Zuckerman, 2004). The primary contact site of EM and TEL is at nucleotide A2058 of 23S rRNA gene domain V, and TEL establishes additional contacts with A752 in domain II of 23S rRNA gene (Hansen *et al.*, 1999; Douthwaite *et al.*, 2000). As a result, TEL has a stronger affinity for the ribosome and can therefore overcome common macrolide resistance mechanisms including target modification directed by the methylase encoded by *ermB*, which methylates A2058, and mutations in the 23S rRNA gene and ribosomal proteins that interrupt macrolide binding (Maglio *et al.*, 2003; Farrell & Felmingham, 2004).

High-level TEL resistance in *S. pneumoniae* was experimentally generated by mutations in domain II or V of 23S rRNA gene and ribosomal proteins L4 and L22 (Leclercq & Courvalin, 2002), and is easily created from a macrolide-resistant strain by the deletion or mutation of the region upstream of *ermB* (Walsh *et al.*, 2003). In contrast, clinical TEL resistance in *S. pneumoniae* remains rare. Farrell and Felmingham initially reported that among the worldwide collection of 13 874 *S. pneumoniae* isolates isolated between 1999 and 2003, only 10 were TEL resistant (Farrell & Felmingham, 2004). The strains isolated in France, Italy, Spain, Hungary and Japan had minimal inhibitory concentrations (MICs) of 4–8 $\mu g\,mL^{-1}$. To our knowledge, the P3084055 strain (MIC 4 $\mu g\,mL^{-1}$) is currently the only TEL-resistant *S. pneumoniae* isolate in Japan (Hirakata *et al.*, 2007). Recently, the emergence of clinical isolates of *S. pneumoniae* with a very high-level TEL resistance (MIC 256 $\mu g\,mL^{-1}$) was reported (Faccone *et al.*, 2005; Wolter

et al., 2007). Sequence analysis of the strain isolated in Argentina in 2005 identified an A2058T mutation in domain V of 23S rRNA gene, a deletion located at the C-terminal portion of L22 and an S20N mutation in L4 (Faccone *et al.*, 2005). It was negative for *ermB*, *ermA* and *ermTR*, which encode rRNA methylase. Therefore, a combination of mutational changes in 23S rRNA gene and ribosomal proteins was assumed to be responsible for the high-level TEL resistance. In contrast, it was demonstrated that *ermB* with a truncated leader peptide in combination with a $_{69}GTG_{71}$-to-TPS mutation in ribosomal protein L4 was responsible for the high-level TEL resistance in the strain isolated in Canada in 2007 (Wolter *et al.*, 2007).

The objective of this study was to investigate the occurrence of TEL resistance in 132 *S. pneumoniae* isolates collected in Japan between 2005 and 2006. The results suggest that reduced-TEL-susceptibility pneumococci have certainly appeared, although none of the isolates were TEL resistant. Further analysis using isogenic *S. pneumoniae* strains demonstrated that reduced TEL susceptibility may be caused by acquisition of only the *mefE-mel* element, which encodes the macrolide efflux pump.

Materials and methods

Bacterial strains, plasmids and media

Streptococcus pneumoniae isolates collected between 2005 and 2006 in Japan and ATCC 49619 as a drug-susceptible strain were used in this study. *Escherichia coli* strain DH5α was used as a recipient in the transformation for DNA cloning. The plasmids used are shown in Table 1. Pneumococci were routinely cultured at 37 °C and 5% CO_2 in brain–heart infusion plus 0.5% yeast extract.

Antimicrobial susceptibility testing

Susceptibility to antibiotics was determined by the serial twofold dilution method using Mueller–Hinton agar plates supplemented with 5% lysed horse blood. The susceptibility or resistance of pneumococci to TEL and EM was assessed in accordance with the recommendation of the National Committee for Clinical Laboratory Standards (2007).

Detection of macrolide-resistant genes

Bacterial cells in 1 mL of overnight pneumococcal cultures were collected, suspended in 200 μL distilled water and boiled for 10 min. A portion of the lysate supernatant was subjected to PCR. Primers for *ermA*, *ermC*, *mphA*, *mphB*, *ereA* and *ereB* were described previously (Sutcliffe *et al.*, 1996). *ermB* was identified using the forward primer ermB-F (5′-TGAAAAGGTACTCAACCAAATA-3′) and the reverse primer ermB-R (5′-AGTAACGGTACTTAAATTGTTTAC-3′). *mefA/E* was detected using the primer pair mef-F1 (5′-AGTATCATTAATCACTAGTGC-3′) and mef-R1 (5′-TTC TTCTGGTACTAAAAGTGG-3′).

mefE was identified by DNA sequencing as follows: chromosomal DNA was prepared from clinical isolates as described by Blue & Mitchell (2003) and used as a temperate for PCR. The *mefE* region (+10 to +1126 to the *mefE* translational start site) was amplified using the primer pair mef-F2 (5′-CCGGAATTCTACAACAATTGG-3′) and mef-R2 (5′-CACCAAGCTTTTACACCGAT-3′). The PCR product was digested with EcoRI–HindIII and the fragment was cloned into pUC18. The resulting plasmid was subjected to DNA sequencing.

Pulse-field gel electrophoresis (PFGE) analysis

PFGE analysis was performed as described previously (Yokoyama & Uchimura, 2006) with some modifications. Briefly, the plug containing bacteria from an overnight culture was made with Seakem gold agarose (Cambrex, Rockland, ME) using a sample plug caster (Bio-Rad, Hercules, CA). The plug was treated for 18 h at 50 °C with a solution of 1 mg proteinase K mL^{-1} (Roche). After incubation, the plug was treated twice for 20 min, each with Tris-EDTA (TE) buffer containing 4 mM Pefabloc (Roche) at 50 °C, and then washed twice on ice for 20 min, each with TE buffer. The plug was digested for 18 h at 37 °C with SmaI (Roche).

Table 1. Plasmids used in this study

Plasmids	Relevant properties	References and source
pTKY856	pUC18 with 2296-bp *mefE-mel* fragment	This study
pTKY857	pTKY856 carrying *mefE-mel*-disrupted genes, Sp	This study
pTKY858	pT7Blue with 618-bp *ermB* fragment	This study
pTKY859	pTKY858 carrying *ermB*-disrupted gene, Km	This study
pTKY862	pLZ12Km2 with Sp cassette	This study
pLZ12Km2	Km cassette	Okada *et al.* (1998)
pR350	Sp cassette	Dintilhac *et al.* (1997)
pUC18	Cloning vector	Yanisch-Perron *et al.* (1985)
pT7Blue	Cloning vector	Novagen

Transformation

Synthetic competence-stimulating peptide 1 or 2 and the method of Iannelli & Pozzi (2004) were used to transform *S. pneumoniae* clinical isolates into a transformation-competent state.

Construction of *mefE-mel* and *ermB* disruption mutants

The disruption of *mefE-mel* was constructed as follows: the region encoding *mefE* and *mel* was amplified from chromosomal DNA prepared from *S. pneumoniae* strain S88 by PCR using the forward primer (5′-ACTGGATCCGCGATGGT CTT-3′) and the reverse primer (5′-CCGGAAGCTTTT TTTGCCTTAG-3′). The PCR product was digested with BamHI–HindIII and the fragment was cloned into pUC18. The resulting plasmid pTKY856 was cleaved with AccI and PstI to eliminate the inter-*mefE-mel* region. The overhanging ends were blunted with T4 polymerase and then ligated to the fragment containing the spectinomycin resistance gene (Sp), generated from pTKY862 after digestion with BamHI, followed by blunting with T4 DNA polymerase. The plasmid pTKY862 is a derivative of pLZ12Km2, with the fragment encoding Sp amplified from pR350 using the primers SpcUP and SpcDO reported previously (Martin *et al.*, 2000). The resulting plasmid pTKY857 was used to replace Δ*mefE-mel*::Sp in clinically isolated TEL-susceptible strains.

The disruption of *ermB* was constructed as follows: the *ermB* region was amplified by PCR from chromosomal DNA of *S. pneumoniae* S88 with primers ermB-F and ermB-R, and the fragment was cloned into pT7Blue. The resulting plasmid pTKY858 was cleaved with StyI and then ligated, after blunting with T4 DNA polymerase, to the fragment carrying the kanamycin resistance gene (Km), generated from pLZ12Km2 after digestion with SalI, followed by blunting with T4 DNA polymerase. The resulting plasmid pTKY859 was used to replace Δ*ermB*::Km in clinically isolated reduced TEL-susceptibility strains. To construct the Δ*mefE-mel*::Sp, Δ*ermB*::Km double mutant, the Δ*ermB*::Km mutant strains originating from each clinical isolate were transformed with pTKY857 and selected by spectinomycin resistance. The double-crossover events in all constructed mutants were assessed by Southern hybridization.

Results and discussion

A total of 132 *S. pneumoniae* isolates collected between 2005 and 2006 at one hospital in Japan were examined for susceptibility to TEL (breakpoint; resistance $\geq 4\,\mu g\,mL^{-1}$, sensitivity $\leq 1\,\mu g\,mL^{-1}$) and EM (breakpoint; resistance $\geq 1\,\mu g\,mL^{-1}$, sensitivity $\leq 0.25\,\mu g\,mL^{-1}$). A total of 106 isolates were found to be resistant to EM. A total of 128

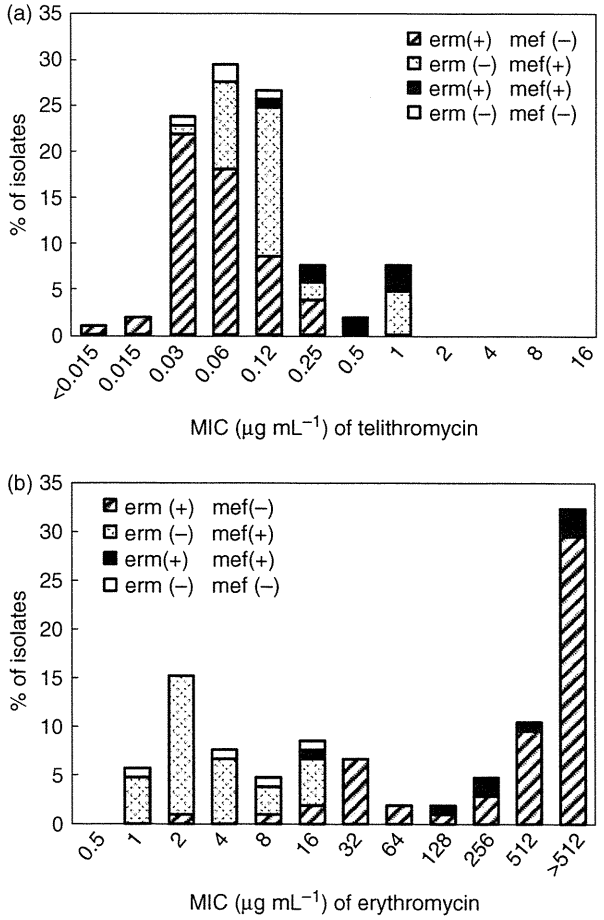

Fig. 1. MIC distribution and association with macrolide-resistant genes. MICs of telithromycin (a) and erythromycin (b) are shown. erm, *ermB*; mef, *mefA/E*.

isolates had low-level TEL susceptibility, with MICs of 0.03–1 $\mu g\,mL^{-1}$ (Fig. 1), suggesting that pneumococci with reduced TEL susceptibility have appeared without prior exposure to TEL, which has not been used in this hospital. The isolates included no TEL-resistant strains.

Distribution of macrolide-resistant determinants

To detect macrolide-resistant determinants in all isolates, PCR assays were performed for the rRNA methylase genes (*ermA*, *ermB* and *ermC*), macloride phosphotransferase genes (*mphA* and *mphB*), macrolide esterase genes (*ereA* and *ereB*) and genes encoding the macrolide efflux pump (*mefA* and *mefE*). All isolates were negative for *ermA*, *ermC*, *mphA*, *mphB*, *ereA* and *ereB* (data not shown). The distribution of *ermB* and *mef* is shown in Fig. 1. The rates of *ermB*-positive, *mef*-positive and double *ermB* and *mef*-positive isolates were 55.2%, 33.3% and 7.6%, respectively. Interestingly, all the isolates exhibiting reduced TEL susceptibility

(0.5–1 μg mL^{-1}) harbored *mef*. Two variants of *mef*, *mefA* and *mefE*, have been identified with high sequence homology (Roberts *et al.*, 1999). Because the initial PCR for detecting *mef* could not distinguish between these two variants, we performed DNA sequencing analysis to discriminate *mefA* and *mefE* in eight reduced TEL-susceptibility isolates (MIC 0.5–1 μg mL^{-1}) as described in Materials and methods. Consequently, all *mef*s in these isolates were assigned to *mefE*. It has been reported that *mefA* is the predominant efflux-associated gene found in *S. pneumoniae* in Japan (Isozumi *et al.*, 2007; Ikenaga *et al.*, 2008). In contrast, the present results demonstrated that *mefE* is also distributed with a high frequency in Japan and possibly generated the reduced-TEL-susceptibility *S. pneumoniae*.

These low-TEL-susceptibility isolates were analyzed by serotyping, multilocus sequence typing (MLST) and PFGE. Five isolates grouped to serotype 6B showed the same sequence type, which was ST2983 with MLST numbers 5-6-1-2-6-1-271 for *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl*, respectively. PFGE showed that five isolates (serotype 6B) were closely related (Fig. 2). On the other hand, the sequence types of strains S43 (serotype 15A), S88 (serotype 19F) and S120 (serotype 19F) were ST361 (7-13-8-6-6-6-8), ST558 (18-12-4-44-14-77-97) and ST1464 (4-16-19-15-6-20-106), respectively. PFGE also clearly distinguished these three strains (Fig. 2). In a recent study, the most frequently occurring serogroups and serotypes of clinical pneumococcal strains isolated from children in Japan were six (32.8%), 23 (21.7%), 14 (13.2%) and 19 (12.7%) (Ikenaga *et al.*, 2008).

Decreased susceptibility to TEL in clinically isolated *S. pneumoniae* is associated with mutations in the L4 and L22 riboproteins and domains II or V of the 23S rRNA gene, and the presence of *ermB* and *mefA/E* (Faccone *et al.*, 2005; Reinert *et al.*, 2005; Al-Lahham *et al.*, 2006; Wolter *et al.*, 2007). Although a combination of these mechanisms could be responsible for TEL susceptibility in clinical isolates, the exact contribution of *mefA/E* or *ermB* to TEL susceptibility has not been revealed previously using isogenic pneumococcal strains.

Mutational analysis of reduced TEL susceptibility in *S. pneumoniae*

To ascertain the contribution of *mefE* to the reduced TEL susceptibility of *S. pneumoniae* isolated clinically in the present study, an independent insertion mutation in *mefE* was constructed by allelic replacement in five clinical isolates (MIC 0.5–1 μg mL^{-1}). *mefE* is a part of the macrolide efflux genetic assembly (mega), which includes the downstream gene *mel* (Gay & Stephens, 2001). In *S. pneumoniae*, *mefE* and *mel* are predicted to be a dual efflux pump (Ambrose *et al.*, 2005). In this study, both *mefE* and *mel* were disrupted

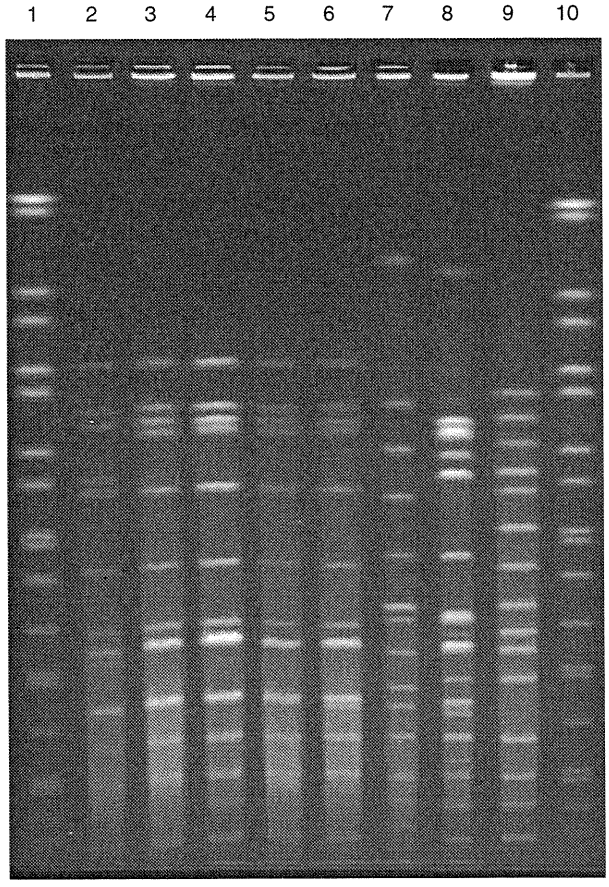

Fig. 2. PFGE profiles of SmaI restriction digests of eight TEL-reduced susceptible isolates of *Streptococcus pneumoniae*. Lanes 1 and 10, universal standard *Salmonella* serotype Braenderup H9812 (Hunter *et al.*, 2005); lane 2, strain S15; lane 3, strain S36; lane 4, strain S89; lane 5, strain S105; lane 6, strain S125; lane 7, strain S43; lane 8, strain S88; and lane 9, strain S120.

by eliminating the central 1409-bp fragment of the *mefE-mel* operon, followed by insertion of an Sp cassette. As shown in Table 2, mutations in *mefE-mel* of the serotype 6B strains S15 and S125 resulted in a significant decrease in TEL-MIC to the level of ATCC 49619 (< 0.015 μg mL^{-1}), which is used as a standard drug-susceptible strain. EM-MICs were also reduced to the level of ATCC 49619 (< 0.5 μg mL^{-1}). It is therefore concluded that *mefE-mel* is the determinant solely responsible for reduced TEL susceptibility and EM resistance in these clinical isolates.

The *mefE-mel* mutation in strain S88 (TEL-MIC 1 μg mL^{-1}), harboring both *mefE-mel* and *ermB*, resulted in a moderate reduction in TEL-MIC to 0.12 μg mL^{-1}. Independent disruption of S88 *ermB* resulted in a similar effect on TEL susceptibility (MIC 0.12 μg mL^{-1}). In contrast, disruption of both the *mefE-mel* and the *ermB* determinants further reduced TEL-MIC to the level of ATCC 49619 (MIC < 0.015 μg mL^{-1}). Similar results were obtained when

Table 2. MICs of the Δ*mefE-mel* and/or Δ*ermB* mutants derived from TEL low-susceptible isolates of *Streptococcus pneumoniae**

Strain	Serotype†	ST‡	Characteristics¶	MIC_{TEL} (μg mL^{-1})	MIC_{EM} (μg mL^{-1})
S15	6B	2983	*mefE-mel*$^+$	1	16
			Δ*mefE-mel*	< 0.015	< 0.5
S125	6B	2983	*mefE-mel*$^+$	1	16
			Δ*mefE-mel*	< 0.015	< 0.5
S43	15A	361	*mefE-mel*$^+$ *ermB*$^+$	0.5	512
			Δ*mefE-mel ermB*$^+$	0.06	246
			mefE-mel$^+$ Δ*ermB*	0.06	4
			Δ*mefE-mel* Δ*ermB*	< 0.015	< 0.5
S88	19F	558	*mefE-mel*$^+$ *ermB*$^+$	1	> 512
			Δ*mefE-mel ermB*$^+$	0.12	> 512
			mefE-mel$^+$ Δ*ermB*	0.12	4
			Δ*mefE-mel* Δ*ermB*	< 0.015	0.5
S120	19F	1464	*mefE-mel*$^+$ *ermB*$^+$	1	> 512
			Δ*mefE-mel ermB*$^+$	0.12	> 512
			mefE-mel$^+$ Δ*ermB*	0.12	4
			Δ*mefE-mel* Δ*ermB*	< 0.015	0.5

*ATCC49619 (MIC_{TEL} < 0.015 μg mL^{-1}, MIC_{EM} < 0.5 μg mL^{-1}) was used as a susceptible strain.

†Serotyping was performed by the Quellung reaction with antisera from Statens, Serum Institute (Copenhagen, Denmark).

‡Sequence type.

¶See Materials and methods for mutations in the Δ*mefE-mel* and Δ*ermB* mutants.

the mutants were constructed independently from strains S120 and S43, which carry both *mefE-mel* and *ermB* elements. Taken together, the results suggest that reduced TEL susceptibility (TEL-MIC 1 μg mL^{-1}) in *S. pneumoniae* may be caused by the acquisition of the *mefE-mel* element only and conferred additionally by the *ermB* element.

The disruption of *ermB* resulted in drastic decreases in resistance to EM; MIC declined from > 512 to 4 μg mL^{-1}. However, the *mefE-mel* mutations did not significantly affect resistance. Additional *mefE-mel* mutations in the *ermB* mutants reduced EM-MICs to the level of ATCC (MIC 0.5 μg mL^{-1}). These results suggest that *ermB* is a predominant mechanism for high resistance to EM in the pneumococcal isolates harboring both *ermB* and *mefE-mel* determinants, although the efflux assembly confers low-level resistance.

Sequence analyses of the five isolates revealed no mutations in 23S rRNA gene domains II or V. There were no mutations in the L4 ribosomal protein from any isolate, except that from strain S43, in which the S20N mutation was found (data not shown). No mutations were found in the L22 ribosomal protein from any isolate.

It has been demonstrated that the *mefE* and *mel* carried by mega may be a part of Tn*2009*, a composite element in which mega is integrated into a Tn*916*-like transposon carrying *tetM* (Franke & Clewell, 1981; Del Grosso *et al.*, 2004). The presence of *tetM* has been examined in isolates S15, S36, S89, S105 and S125, which express tetracycline resistance (MICs 16 μg mL^{-1}), using PCR with the primers TETM1 and TETM2 (Del Grosso *et al.*, 2004). This primer set produced an amplicon of approximately 2.0 kb, indicating the presence of *tetM*. The linkage between *mefE-mel* and *tetM* in these strains was investigated by Southern hybridization based on the restriction cleavage map constructed from the sequence (accession number AF376746). In these five isolates, *mefE-mel* and *tetM* were in close proximity, as shown in Tn*2009* (data not shown). Although the whole sequence of the composite transposon has not been revealed in the present study, it is speculated that transposition of a Tn*2009*-like element may be responsible for generating the reduced-TEL-susceptibility streptococci S15, S36, S89, S105 and S125. On the other hand, a recent report demonstrated that a different composite element, designated Tn*2010*, is similar to Tn*2009*, but also contains *ermB* (Del Grosso *et al.*, 2006). The presence of *tetM* in *S. pneumoniae* isolates S43, S88 and S120 was confirmed by DNA sequence analyses of PCR products of 2.0 kb amplified using the primer pair TETM1 and TETM2. Strain S43 expressed tetracycline resistance (MIC 16 μg mL^{-1}), but S88 and S120 showed a tetracycline-intermediate phenotype (MICs 4 μg mL^{-1}). In these isolates, Southern hybridization revealed a linkage between *mef-mel* and *tetM* and one between *ermB* and *tetM*, which are in Tn*2010* (data not shown).

Concluding remarks

The present study suggests that low-TEL-susceptibility pneumococci have appeared clinically in Japan without prior exposure to TEL. Mutational analysis with isogenic strains revealed that the acquisition of *mefE-mel* may reduce the susceptibility of pneumococci to TEL. It was demonstrated previously that high-level TEL resistance was easily generated from macrolide-resistant *S. pneumoniae* harboring *ermB* and *mefA* (Walsh *et al.*, 2003). It is therefore worth mentioning that the reduced TEL susceptibility clones demonstrated in the present study may have the potential to generate TEL-resistant pneumococci and spread further.

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References

Al-Lahham A, Appelbaum PC, van der Linden M & Reinert RR (2006) Telithromycin-nonsusceptible clinical isolates of

Streptococcus pneumoniae from Europe. *Antimicrob Agents Ch* **50**: 3897–3900.

Ambrose KD, Nisbet R & Stephens DS (2005) Macrolide efflux in *Streptococcus pneumoniae* is mediated by a dual efflux pump (mel and mef) and is erythromycin inducible. *Antimicrob Agents Ch* **49**: 4203–4209.

Blue CE & Mitchell TJ (2003) Contribution of a response regulator to the virulence of *Streptococcus pneumoniae* is strain dependent. *Infect Immun* **71**: 4405–4413.

Bozdogan B, Appelbaum PC, Kelly LM *et al.* (2003) Activity of telithromycin and seven other agents against 1034 pediatric *Streptococcus pneumoniae* isolates from ten central and eastern European centers. *Clin Microbiol Infec* **9**: 653–661.

Del Grosso M, Scotto d'Abusco A, Iannelli F, Pozzi G & Pantosti A (2004) Tn*2009*, a Tn*916*-like element containing *mef*(E) in *Streptococcus pneumoniae*. *Antimicrob Agents Ch* **48**: 2037–2042.

Del Grosso M, Camilli R, Iannelli F, Pozzi G & Pantosti A (2006) The *mef*(E)-carrying genetic element (mega) of *Streptococcus pneumoniae*: insertion sites and association with other genetic elements. *Antimicrob Agents Ch* **50**: 3361–3366.

Dintilhac A, Alloing G, Granadel C & Claverys J (1997) Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. *Mol Microbiol* **25**: 727–739.

Douthwaite S, Hansen LH & Mauvais P (2000) Macrolide–ketolide inhibition of MLS-resistant ribosomes is improved by alternative drug interaction with domain II of 23S rRNA. *Mol Microbiol* **36**: 183–193.

Faccone D, Andres P, Galas M, Tokumoto M, Rosato A & Corso A (2005) Emergence of a *Streptococcus pneumoniae* clinical isolate highly resistant to telithromycin and fluoroquinolones. *J Clin Microbiol* **43**: 5800–5803.

Farrell DJ & Felmingham D (2004) Activities of telithromycin against 13,874 *Streptococcus pneumoniae* isolates collected between 1999 and 2003. *Antimicrob Agents Ch* **48**: 1882–1884.

Fogarty CM, Kohno S, Buchanan P, Aubier M & Baz M (2003) Community-acquired respiratory tract infections caused by resistant pneumococci: clinical and bacteriological efficacy of the ketolide telithromycin. *J Antimicrob Chemoth* **51**: 947–955.

Franke A & Clewell D (1981) Evidence for a chromosome-borne resistance transposon (Tn*916*) in *Streptococcus faecalis* that is capable of 'conjugal' transfer in the absence of a conjugative plasmid. *J Bacteriol* **145**: 494–502.

Gay K & Stephens DS (2001) Structure and dissemination of a chromosomal insertion element encoding macrolide efflux in *Streptococcus pneumoniae*. *J Infect Dis* **184**: 56–65.

Hansen LH, Mauvais P & Douthwaite S (1999) The macrolide-ketolide antibiotic binding site is formed by structures in domains II and V of 23S ribosomal RNA. *Mol Microbiol* **31**: 623–631.

Hirakata Y, Mizuta Y, Wada A *et al.* (2007) The first telithromycin-resistant *Streptococcus pneumoniae* isolate in Japan associated with *erm*(B) and mutations in 23S rRNA and riboprotein L4. *Jpn J Infect Dis* **60**: 48–50.

Hunter SB, Vauterin P, Lambert-Fair MA *et al.* (2005) Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: converting the national databases to the new size standard. *J Clin Microbiol* **43**: 1045–1050.

Iannelli F & Pozzi G (2004) Method for introducing specific and unmarked mutations into the chromosome of *Streptococcus pneumoniae*. *Mol Biotechnol* **26**: 81–86.

Ikenaga M, Kosowska-Shick K, Gotoh K *et al.* (2008) Genotypes of macrolide-resistant pneumococci from children in southwestern Japan: raised incidence of strains that have both *erm*(B) and *mef*(A) with serotype 6B clones. *Diagn Micr Infec Dis* **62**: 16–22.

Isozumi R, Ito Y, Ishida T *et al.* (2007) Genotypes and related factors reflecting macrolide resistance in pneumococcal pneumonia infections in Japan. *J Clin Microbiol* **45**: 1440–1446.

Leclercq R & Courvalin P (2002) Resistance to Macrolides and Related Antibiotics in *Streptococcus pneumoniae*. *Antimicrob Agents Ch* **46**: 2727–2734.

Maglio D, Teng R, Thyrum PT, Nightingale CH & Nicolau DP (2003) Pharmacokinetic profile of meropenem, administered at 500 milligrams every 8 hours, in plasma and cantharidin-induced skin blister fluid. *Antimicrob Agents Ch* **47**: 1771–1773.

Martin B, Prudhomme M, Alloing G, Granadel C & Claverys J (2000) Cross-regulation of competence pheromone production and export in the early control of transformation in *Streptococcus pneumoniae*. *Mol Microbiol* **38**: 867–878.

National Committee for Clinical and Laboratory Standards (2007) *Performance and Standards for Antimicrobial Susceptibility Testing. Seventeenth Informational Supplement M100-S17*. National Committee for Clinical and Laboratory Standards Institute, Wayne, PA.

Okada N, Tatsuno I, Hanski E, Caparon M & Sasakawa C (1998) *Streptococcus pyogenes* protein F promotes invasion of HeLa cells. *Microbiology* **144**: 3079–3086.

Reinert RR, van der Linden M & Al-Lahham A (2005) Molecular characterization of the first telithromycin-resistant *Streptococcus pneumoniae* isolate in Germany. *Antimicrob Agents Ch* **49**: 3520–3522.

Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Rood J & Seppala H (1999) Nomenclature for macrolide and macrolide–lincosamide–streptogramin B resistance determinants. *Antimicrob Agents Ch* **43**: 2823–2830.

Sutcliffe J, Grebe T, Tait-Kamradt A & Wondrack L (1996) Detection of erythromycin-resistant determinants by PCR. *Antimicrob Agents Ch* **40**: 2562–2566.

Walsh F, Willcock J & Amyes S (2003) High-level telithromycin resistance in laboratory-generated mutants of *Streptococcus pneumoniae*. *J Antimicrob Chemoth* **52**: 345–353.

Wolter N, Smith AM, Low DE & Klugman KP (2007) High-level telithromycin resistance in a clinical isolate of *Streptococcus pneumoniae*. *Antimicrob Agents Ch* **51**: 1092–1095.

Yanisch-Perron C, Vieira J & Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**: 103–119.

Yokoyama E & Uchimura M (2006) Optimal settings of fingerprint-type analysing computer software for the analysis of enterohaemorrhagic *Escherichia coli* pulsed-field gel electrophoresis patterns. *Epidemiol Infect* **134**: 1004–1014.

Zuckerman JM (2004) Macrolides and ketolides: azithromycin, clarithromycin, telithromycin. *Infect Dis Clin N Am* **18**: 621–649, xi-.