A composite transposon associated with erythromycin and clindamycin resistance in group B *Streptococcus*

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Group B *Streptococcus* (GBS) resistant to erythromycin and clindamycin has been isolated with increasing frequency since the mid-1990s. This work studied GBS isolates from three US cities to determine the genetic basis of the macrolide resistance phenotype. *ermB* genes were amplified from five isolates collected in Boston, Pittsburgh and Seattle from infant and adult sources. Gene-walking methods were used to determine the chromosomal location of *ermB* and to identify associated genes. Southern mapping and random amplified polymorphic DNA (RAPD) analyses were used to distinguish the isolates. The *ermB* gene was present on the chromosome within a composite Tn917/Tn916-like transposon similar to one identified in *Streptococcus pneumoniae*. Four strains from Boston and Pittsburgh were serotype V and identical by Southern hybridization and RAPD analysis. The Seattle isolate was serotype Ib, with different patterns on RAPD analysis and Southern mapping. The composite transposon was integrated at an identical chromosomal site in all five isolates. The presence of this composite transposon in both GBS and pneumococci suggests that *ermB*-mediated macrolide resistance in streptococci may be due to the horizontal transfer of a mobile transposable element, and raises concern for further dissemination of high-grade erythromycin and clindamycin resistance among streptococcal species.

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INTRODUCTION

In 1996, the US Centers for Disease Control and Prevention (CDC) issued guidelines for the use of intrapartum antibiotic prophylaxis to prevent neonatal early-onset group B streptococcal disease (Centers for Disease Control and Prevention, 1996). Over the past 10 years, coincident with the implementation of these guidelines, multiple investigators have reported an increase in the incidence of erythromycin and clindamycin resistance among both group B *Streptococcus* (GBS) colonizing and invasive disease isolates. Rates of erythromycin resistance

ranging from 7 to 25 % have been reported (Andrews *et al.*, 2000; Fernandez *et al.*, 1998; Pearlman, *et al.*, 1998). This resistance is present primarily, but not exclusively, in serotype V GBS (Andrews *et al.*, 2000; Croak *et al.*, 2003; Diekema *et al.*, 2003). Due to the rising incidence of macrolide resistance, in 2002 the CDC revised their recommendations for intrapartum GBS prophylaxis to include antibiotic susceptibility testing on colonizing isolates obtained from penicillin-allergic women (Centers for Disease Control and Prevention, 2002).

Relatively little is known about the genetic origins of antibiotic resistance in GBS. These organisms remain largely sensitive to β -lactam antibiotics, although there are rare case reports of penicillin-tolerant GBS, as well as cefotaxime-resistant GBS (Betriu *et al.*, 1994; Morikawa *et al.*, 2003). As allergy to β -lactam antibiotics is common, the emergence of widespread resistance to clindamycin and the macrolide antibiotics poses a serious clinical problem.

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Abbreviations: GBS, group B *Streptococcus*; RAPD, random amplified polymorphic DNA.

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We have reported a case of early-onset neonatal sepsis due to resistance of the invasive isolate to the antibiotic (clindamycin) administered to the mother for intrapartum antibiotic prophylaxis against GBS (Puopolo *et al.*, 2005). We have also shown that erythromycin and clindamycin resistance was present in an increasing proportion of neonatal invasive disease cases occurring in a large delivery service following the implementation of a screening-based approach to intrapartum antibiotic prophylaxis (Chen *et al.*, 2005). The purpose of this study was to determine a genetic mechanism for *ermB*-associated erythromycin resistance in GBS strains.

METHODS

Bacterial strains. Nineteen neonatal blood culture GBS isolates were obtained from the microbiology laboratory at the Brigham and Women's Hospital, Boston, USA. Fifty GBS isolates, obtained from adults in Pittsburgh and Seattle, were generously provided by Dr Patricia Ferrieri (University of Minnesota, MN, USA). Bacteria were grown in Todd-Hewitt broth (THB) or on THB agar supplemented with 5% sheep blood. All incubations (other than those for MIC determination) were done in ambient air at 37 °C.

Susceptibility testing and serotyping. MIC testing was performed with Epsilon strips (AB Biodisk) using Mueller-Hinton agar with 5% defibrinated sheep blood. Incubations were done at 35 °C in 5% CO₂, following Clinical and Laboratory Standards Institute (NCCLS) methods and interpretative guidelines. *Streptococcus pneumoniae* ATCC 49619 was used for quality control. Disc diffusion testing was also performed with erythromycin (15 µg) and clindamycin (2 µg) discs, following NCCLS guidelines. Capsule serotyping was performed using serological methods and standard reference antisera (Paoletti *et al.*, 1999). Typing for the major GBS surface C proteins was performed using PCR methods and primers designed to detect unique regions of the genes for the alpha C protein, Alp1, Alp2, Alp3, Rib and beta C protein (L. C. Madoff, unpublished results).

DNA manipulation, PCR amplification and DNA sequencing. Bacterial genomic and plasmid DNA was isolated using commercial kits (Qiagen), with the additional step of pre-treatment of the GBS to facilitate bacterial lysis prior to preparing DNA with each kit. GBS was suspended in 10 mM Tris/HCl (pH 8.0), 1 mM EDTA and 25% glucose (glucose/TE), and mutanolysin (200 µg ml⁻¹) and lysozyme (3 mg ml⁻¹) were added and the bacteria were incubated at 37 °C for 60 min. The bacteria were then spun down and DNA preparation proceeded following the manufacturer's instructions. The primers used for identification of the *ermA*, *ermB*, *ermC* and *mefA* genes, primers designed to transposons Tn916 (GenBank accession no. NC_006372) and Tn917 (GenBank accession no. M11180), *recA* control primers and primers for chromosomal localization are listed in Table 1. PCRs were performed with genomic DNA or protoplast preparations (Chaffin & Rubens, 1998) using a standard thermocycler and Platinum PCR Supermix (Invitrogen) with the following program: 95 °C for 5 min and 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30–60 s. For DNA sequencing, PCR products were purified with a PCR purification column (Qiagen), mixed with 0.2 µM oligonucleotide primer and sequenced by the Brigham and Women's Hospital Sequencing Facility.

Identification of the chromosomal location of *ermB* genes. Chromosomal DNA flanking *ermB* was identified using the TOPO Walker kit (Invitrogen). Southern blot analysis revealed an approximately 2 kb *KpnI* fragment containing the *ermB* structural gene that

would be amenable to sequencing using this protocol. Genomic DNA was digested with *KpnI*, subjected to primer extension with an *ermB* gene-specific primer and ligated to commercial linkers. PCR was performed using *Taq* polymerase and *ermB*-specific and linker-specific primers. The subsequent amplicons were sequenced and analysed using BLAST analysis against both the GBS genomes and the entire GenBank database.

An inverse PCR strategy was used to identify the chromosomal insertion site of the composite transposon. We identified an *EcoRI* site near the 5' end of the Tn916 sequence. The inverse PCR strategy was based on the idea that if an *EcoRI* site were present in the chromosomal DNA flanking the transposon insertion site, then a circular DNA molecule could be generated containing the chromosomal flanking DNA and transposon DNA. PCR amplification outward from the transposon sequence could then be performed to identify the flanking DNA. Genomic DNA from strains KMP104 and PF48 was digested with *EcoRI* and then incubated with T4 DNA ligase for 3 h at room temperature to generate circular DNA molecules. One microlitre of ligated DNA was used as template for PCRs using primers Tn916997R and Tn9161996F or Tn9161996F and Tn916_5'R. Products were separated on a 1% agarose gel. Bands were excised and purified using the SNAP Gel Purification kit (Invitrogen) and sequenced using the Tn916_5'R primer.

PCR amplification was used to confirm the genomic location of the Tn916/917 composite transposon. Primers A909_Tn916juncF and A909_Tn916juncR were generated from the genomic sequence surrounding the putative site of integration. PCR was performed with each *ermB* isolate and GBS strains A909, 2603V/R and KMP102 as positive and negative controls. Primer pairs used were: A909_Tn916juncF/A909_Tn916juncR; A909_Tn916juncF/Tn916_3'R; A909_Tn916juncR/Tn916_5'R; and control primers recA4R/recA7F.

Random amplified polymorphic DNA (RAPD) analysis. Primer H2 (Table 1) has been shown previously to discriminate between unrelated strains of both *Streptococcus pyogenes* and GBS via RAPD mapping (Culebras *et al.*, 2002; Seppala *et al.*, 1994). PCRs were performed as described earlier, using 40 cycles and an annealing temperature of 38 °C.

Southern blotting and hybridization. Genomic DNA was digested with restriction enzymes without sites in the GBS *ermB* sequence. Agarose electrophoresis and Southern blotting were performed using standard methods (Sambrook *et al.*, 1989). Probe labelling, hybridization and high-stringency washes were carried out using an enhanced chemiluminescent system (Amersham). Blots were exposed to autoradiography film for 5–30 min.

Filter mating. *Enterococcus faecalis* strain RH101 was generously provided by Dr Craig Rubens (Children's Hospital & Regional Medical Center, WA, USA) and Dr Michael Wessels (Children's Hospital, Boston, MA, USA). This strain harbours a Tn916 transposon with the *tet* gene replaced with an *erm* gene and has been used previously to create transposon insertions into the GBS genome at high frequency (Weiser & Rubens, 1987). Cultures of donor (*ermB*-resistant clinical strains KMP104 and PF19, or control strain RH101) and recipient (erythromycin-sensitive, spectinomycin-resistant strain A909/pDL278; LeBlanc *et al.*, 1992) were grown in THB to an OD₆₅₀ of 0.6–0.7. Cultures were centrifuged, resuspended in THB, spread on sterile 0.22 µm pore size nitrocellulose discs on blood agar plates containing no antibiotic and incubated for 20 h at 37 °C. The filters were washed with 500 µl THB and the resulting suspension was spread on blood agar plates containing 100 µg erythromycin ml⁻¹ and 100 µg spectinomycin ml⁻¹. Plates were incubated for 24 h at 37 °C and inspected for the presence of drug-resistant colonies.

Table 1. PCR primers used in this study

Primer	Sequence (5'→3')
ermAF	ATGAAACAGAAAAACCCGAAAAATACG
ermAR	ATTGAAATAATTTGTAACATTTGAAAAACAG
ermBF	ATGAACAAAAATATAAAATATTCTCAAAAC
ermBR	ATTTCTCCCGTTAAATAATAGATAAC
ermB5end	GTTCCAATTTTCGTAAACGGTATCG
ermB3end	GCCAAAGTAAACAATTTAAGTACCG
ermCF	ATGAACGAGAAAAATATAAAACACAGTCAA
ermCR	ACTTATTAATAATTTATAGCTATTGAAAA
H2	CCTCCCGCCACC
mefAF	ATGGAAAAATACAACAATTGGAAACTTAA
mefAR	ATTTTAAATCTAATTTTCTAATCTCA
recA4R	AAAGTGCACGCGCACTTTATGGTC
recA7F	TGCAAGCTCGTATGATGAGTCAAG
Tn9167F	GCATAAAAATCTAGTTATCCGCAT
Tn916997R	GTGTTGAGCTTTGAATGAAGTAG
Tn9161996F	GCAAAAATTATGCTTACTTAGGT
Tn9162045R	TTTCATGAAAGCGACGTATTCAT
Tn9163991F	GCGTGAGTTTAGCAAACTGATTGG
Tn9164025R	TAAGCCAACCGCAATAATCGCAAT
Tn9166003F	GAAATCTTCTACTACCAGCAACAG
Tn9166034R	TCATTGACGTATCAATCGGGAATG
Tn9167999F	GGAAGCCTATTCTTAGACTTCAT
Tn9168041R	TAAGGTCTGCATATTCTTACCAATG
Tn9169996F	GCCAAAGAATATGGCGTTGAAGAAT
Tn91610044R	TAATCGCAAGAAGTATGTTGACAT
Tn91612314F	GTGGGAAAATACGAAGGTGAACATCAT
Tn91612361R	GAACGATATACTTCTGCTAAGAAAT
Tn91613308R	GCAACAGTGCATAATCACTTCCAT
Tn91614420R	TCCTTAATGGAATCTTTAGCCAGCG
Tn91614611F	TTAGTTAAGTACCGTGGGTCAATT
Tn91617002F	GAAGTATTGACAGTATTAAGCCATCAG
Tn91617030R	TCATTCTAATAGCCATTCTTTAGCG
Tn91617958R	GTAAAGCCTTATTCTATGTGCTTTTCG
Tn916_917_F	CGATGTTTCTTGCTTCGTTG
Tn916_917_R	TGAACAAGTGGGAGCATTGA
Tn916_5'R	AGAGTGGGGAACGACCTTTT
Tn916_3'R	ACAGGAGCGTCTTGTTGCTT
Tn9171001F	GTTGGGAATATTCCTTACCATT
Tn9171999F	GAATTTACGATCAACTAAACAT
Tn9172500R	GCAAATCCTAGTTTATTGACCT
Tn9173003F	GACATTCCAGGTAAGGCAAAAT
Tn9173499R	GCTTTATTTTGAACCATTTCTAAAT
Tn9173990F	GTACACCTAGCGAAGCAGAAAT
Tn9174501R	ATGAGTAGCTTCCCTTGTATTAGTTG
Tn9174998F	GAACTATTTTTTGGGCGACGCGG
A909_Tn916juncF	GGGGTATGGACTTGTTCCTCA
A909_Tn916juncR	CGAAACAGCATATCAAATTCCA
Linker primer 1	AGGCACAGTCGAGGACTTATCCTA

RESULTS AND DISCUSSION

Identification of erythromycin-resistant GBS isolates

Two classes of resistance genes mediate macrolide resistance in streptococci. The *erm* genes modify the ribosome

by methylation and inhibit erythromycin binding, whilst the *mef* genes encode membrane proteins that act as drug efflux pumps. *ermA*, *ermB* and *mefA* are the most common genes reported among streptococcal species (Roberts *et al.*, 1999). Both *erm* and *mef* genes have been identified in GBS colonizing and invasive isolates by PCR, using primers designed to conserved areas of these genes (Culebras *et al.*,

2002; de Azavedo *et al.*, 2001; Heelan *et al.*, 2004). The sequence of *mefA* and *mefE* genes has been reported from GBS (Arpin *et al.*, 1999), but no sequence data are available for any of the *erm* genes.

We used the primers listed in Table 1 to identify erythromycin-resistance genes among 69 GBS isolates collected from neonatal and adult sources in three US cities (Boston, Pittsburgh and Seattle) between 1995 and 2001. The *ermB* gene was identified in five isolates; *ermA* was identified in three isolates and *mefA* in two isolates (Table 2). All five *ermB*-containing isolates demonstrated high-level, constitutive resistance to both erythromycin and clindamycin (MIC >256 µg ml⁻¹ for both antibiotics). Because of the clinical importance of high-grade resistance to both erythromycin and clindamycin, these isolates were chosen for detailed study. The characteristics of each isolate are summarized in Table 2. The 738 bp PCR product amplified from all five *ermB*-positive isolates was sequenced. In each case, the sequence was identical at the nucleotide level to *ermB* present on enterococcal transposon Tn917 and to that reported from *S. pyogenes* (GenBank accession no. AJ972607). This sequence has been deposited in GenBank (accession no. DQ355148).

Southern analysis of *ermB* isolates

erm genes are associated with both conjugative plasmids and transposons in enterococcal species (Takeuchi *et al.*, 2005; Tomich *et al.*, 1980), and on prophage elements and transposons in *S. pyogenes* (Banks *et al.*, 2003; Giovanetti *et al.*, 2005). Nothing is known about *erm*-associated genes in GBS, such as whether these genes are integrated on the GBS chromosome or present on extrachromosomal elements, information that is needed to begin to understand how macrolide antibiotic resistance is spreading among GBS. To determine the genetic associations of *ermB* in GBS, we isolated plasmids of variable size (ranging from 1.8 to 6 kb) from two *ermB* isolates (KMP104 and KMP106) and analysed them by Southern blotting, using

the *ermB* PCR product as probe. No signal was detected in any plasmid preparation (data not shown). Assuming then that *ermB* was integrated into the chromosome, the *ermB* isolates were characterized by Southern blotting to determine their relatedness and to aid in chromosomal localization of the genes. Four of the five *ermB*-containing isolates (KMP104, KMP106, PF19 and PF45) had identical patterns on Southern blots with four restriction enzymes, using the *ermB* gene as probe (Fig. 1). The fifth isolate, PF48, had a similar pattern with an increase in size of two bands by approximately 1–2 kb (Fig. 1).

RAPD mapping

The use of RAPD analysis to distinguish between related and unrelated strains has been validated for the epidemiological study of GBS (Chatellier *et al.* 1997; Limansky *et al.* 1998). To determine the overall relatedness of the *ermB* isolates, RAPD mapping was performed with a primer (H2) previously shown to distinguish unrelated strains of both *S. pyogenes* and GBS (Culebras *et al.*, 2002; Seppala *et al.*, 1994). The results are shown in Fig. 2. Control strains A909 and 2603V/R and the non-*ermB* erythromycin-resistant isolates KMP100, KMP102 and KMP103 all had differing patterns. *ermB* isolates KMP104, KMP106, PF19 and PF45 had identical patterns, whilst PF48 differed by a single band.

Chromosomal location of *ermB*

Gene walking was performed to determine the chromosomal location of the *ermB* gene, as described in Methods. The sequence obtained was analysed using BLAST analysis against both the GBS genomes and the entire GenBank database. Identical results were obtained for both KMP104 and KMP106, and are shown schematically in Fig. 3. The sequence flanking *ermB* was identical to that flanking *ermB* in transposon Tn917. Downstream of *ermB*, sequence was obtained up to a *KpnI* site in Tn917. Upstream of *ermB*,

Table 2. Bacterial isolates used in this study

Strain	Source	Year isolated	Polysaccharide serotype	Surface protein type	Antibiotic gene
A909	Lancefield collection	1927	Ia	ACP	None
2603V/R	Italy/blood isolate	2000	V	Rib	<i>tetR</i>
KMP100	Boston/neonatal blood	2001	V	Alp3	<i>ermA</i>
KMP102	Boston/neonatal blood	2001	Ia	Alp1	None
KMP103	Boston/neonatal blood	2001	III	Rib	<i>ermA</i>
KMP104	Boston/neonatal blood	2001	V	Alp3	<i>ermB</i>
KMP106	Boston/neonatal blood	2001	V	Alp3	<i>ermB</i>
PF19	Pittsburgh/rectal colonizing	1999	V	Alp3	<i>ermB</i>
PF33	Pittsburgh/rectal colonizing	2000	III	Rib	<i>mefA</i>
PF39	Pittsburgh/rectal colonizing	2000	Ia	Alp1	<i>mefA</i>
PF41	Pittsburgh/rectal colonizing	2000	V	ACP	<i>ermA</i>
PF45	Pittsburgh/rectal colonizing	2001	V	Alp3	<i>ermB</i>
PF48	Seattle/vaginal colonizing	1995	Ib	Beta	<i>ermB</i>

Fig. 1. Southern blots of *ermB* GBS isolates. Genomic DNA from each isolate digested with *Dra*I (lanes 2, 6, 11, 15, 19); *Hind*III (lanes 3, 7, 12, 16, 20); *Kpn*I (lanes 4, 8, 13, 17, 21); *Psi*I (lanes 5, 9, 14, 18, 22). Lanes 1 and 10, molecular mass markers. The full *ermB* gene sequence was used as the probe.

sequence was obtained up to Tn917 nt 90. An abrupt transition was then found to sequence that was an exact match to ORF9 of enterococcal transposon Tn916. The Tn917 insertion site interrupted the reading frame of ORF9, a putative transcriptional regulator gene. To determine whether the same integration was present in PF19, PF45 and PF48, PCR was performed with these strains using primers designed to the regions of Tn916 flanking the insertion site demonstrated in KMP104 and KMP106 (Tn916_917F and Tn916_917R). Sequencing of the amplicons revealed the same transition from Tn916 ORF9 to Tn917 sequence in each isolate.

Determination of the extent of transposon integration

A similar integration of Tn916 and Tn917-like sequences (designated transposon Tn3872) has been reported in four erythromycin-resistant *S. pneumoniae* strains (McDougal *et al.*, 1998). Tn917-like sequences were detected by DNA hybridization, and limited sequence data demonstrated the same insertion of Tn917 into *orf9* of Tn916, as found in this study. The composite transposon was designated Tn3872. A similar composite transposon comprising Tn916-like sequence with integration of Tn917-like sequence at Tn916 *orf9* has also been reported in an isolate of *Abiotrophia defectiva* (formerly designated *Streptococcus defectivus*) (Poyart *et al.*, 2000). In neither study was the full structure of the proposed element or its chromosomal location reported. To determine the extent of transposon integration in the *ermB* isolates, PCR primers were designed to the *E. faecalis* Tn917 and Tn916 sequences. Primers were designed to span the entire 5614 nt of Tn917 and the entire 18 032 nt of Tn916, in overlapping fashion. The sequenced GBS strain 2603V/R (GenBank accession no. NC_004116) was used as a positive control for Tn916 alone, as alignment of Tn916 with the 2603V/R genome demonstrated that all but 53 nt of the 18 032 nt Tn916 sequence are integrated into this genome at 98 % identity. Strain 2603V/R contains no Tn917-like sequences. The sequenced GBS strain A909 was used as a negative control, as neither Tn916 nor Tn917-like sequences are present in this strain. This analysis, combined with sequencing of the Tn916/Tn917 junction from each strain, suggested that a composite transposon containing all of Tn916 and nt 90–5355 of Tn917 was present in each *ermB* isolate, as shown schematically in Fig. 3.

Fig. 2. RAPD mapping of GBS isolates. H2 primer PCR products were electrophoresed on a 1.5% agarose gel. MW, Molecular mass markers.

Fig. 3. Schematic representation of the *ermB* chromosomal flanking region. The transposon representation is from published sequences from *E. faecalis* (Tn916, GenBank accession no. NC_006372; Tn917, GenBank accession no. M11180). The GBS chromosomal flanking region is as found in the sequenced genome of A909 (GenBank accession no. CP000114). Arrows represent PCR amplicons generated by the primer pairs noted here. For all amplicons except 5 and 6 (generated by gene walking), the predicted amplicon size is given in parentheses. The observed size on gel electrophoresis was the same as predicted in each case, except for amplicon 2 in strain PF48 (as explained in Results). 1, ErmBF/ErmBR (736 bp); 2, Tn9171001F/Tn9172500R (1517 bp); 3, Tn9171999F/Tn9173499R (1525 bp); 4, Tn9173003F/Tn9174501R (1523 bp); 5, linker primer 1/erm5end; 6, linker primer 1/ermB3end; 7, Tn9173990F/Tn916_917R (1811 bp); 8, Tn916_917F/ermBR (1569 bp); 9, Tn9167F/Tn916997R (1014 bp); 10, Tn9167F/Tn9162045R (2062 bp); 11, Tn9161996F/Tn9164025R (2029 bp); 12, Tn9163991F/Tn9166034R (2065 bp); 13, Tn9166003F/Tn9168041R (2062 bp); 14, Tn9167999F/Tn91610044R (2045 bp); 15, Tn9169996F/Tn91612361R (2389 bp); 16, Tn91612314F/Tn91613308R (1018 bp); 17, Tn91612314F/Tn91614420R (2130 bp); 18, Tn91614611F/Tn91617030R (2444 bp); 19, Tn91617002F/Tn91617958R (982 bp); 20, A909_Tn916juncF/Tn916_3'R (1101 bp); 21, A909_Tn916juncR/Tn916_5'R (718 bp). Amplicons 1, 2, 5, 6, 7, 8, 20 and 21 were fully sequenced.

Genomic localization of the composite transposon

Inverse PCR was performed to determine the exact genomic insertion site of the composite transposon. A 2 kb fragment was amplified from isolates KMP104 and PF48 and sequenced with primer Tn916_5'R. The sequences obtained were subjected to BLAST analysis; the results were identical for KMP104 and PF48. Tn916 sequence was obtained through the 5' end of the transposon, followed by sequence that was an exact match to chromosomal DNA found in the sequenced, erythromycin-sensitive type Ia GBS strain A909, beginning at nt 653909 of the published sequence (GenBank accession no. CP000114). Sequence was obtained to an apparent *EcoRI* site in the chromosome, and transition back to Tn916 sequence was again found, as expected from amplification of the circular ligation product. As shown in Fig. 3, the

insertion site is within a predicted non-coding, intergenic region of the chromosome.

The sequence obtained indicated that the composite transposon was inserted in the exact same position in KMP104 and PF48, isolates that are not clonal as evidenced by Southern mapping, serotyping and RAPD analysis. To determine whether the composite transposon is inserted at the same position in the other *ermB* isolates, we performed PCR using primers designed from the A909 genome sequence upstream and downstream of the predicted transposon insertion site, into the predicted flanking ORFs SAK_0719 and SAK_0720. Primer pair A909_Tn916juncF/A909_Tn916juncR should generate a 1346 bp product from strain A909, but should not successfully amplify anything from the *ermB* isolates due to the size of the predicted product (>26 kb). Primer pair A909_Tn916juncF/Tn916_3'R should generate an 1101 bp

product if the composite transposon was present in the predicted orientation; and primer pair A909_Tn916juncR/Tn916_5'R should generate a 718 bp product. No product would be amplified if the composite transposon was not present in the predicted genomic location. *RecA* primers were used as controls. The results of this study are given in Table 3. The predicted size products were generated by PCR using the genomic/transposon pairs from the *ermB* isolates, but not from the non-erythromycin-resistant control strains A909 or 2603V/R, nor from the non-*ermB*, erythromycin-resistant isolate KMP102. The genomic pair generated a product only from A909. The *recA* control product was successfully amplified from each strain.

The fact that the chromosomal DNA flanking the composite transposon in all five isolates was identical suggested that the acquisition of this resistance element is mediated by an integration 'hotspot' that exists within the genome of select GBS strains. Integration-favourable sites have been described for both transposons and viruses, and recombination-favourable sites are found in both eukaryotic and prokaryotic genomes (Junop & Haniford, 1997; Kohli *et al.*, 1999; Lavigne *et al.*, 2005; Snoek *et al.*, 1998). Tn916 itself is a well-characterized conjugative transposon that integrates preferentially in AT-rich regions (Scott *et al.*, 1994; Whittle & Salyers, 2002) and is thus frequently found in the AT-rich streptococcal genomes. It is possible that the composite transposon is not as readily transferred and integrated as Tn916 or Tn917 alone, and that an integration-favourable site is required for the acquisition of the composite element.

A requirement for a specific integration site may in part explain the relatively limited serotype background reported for *ermB*-containing strains (largely serotype V). The acquisition of erythromycin resistance in serotype V GBS may also have contributed to the recent emergence of this as an important serotype in both newborn and adult invasive GBS disease (Tettelin *et al.*, 2002). Four of the five *ermB* isolates in our study had identical capsular polysaccharide and surface protein serotypes, and appeared to be identical by Southern blot hybridization and by

RAPD mapping. Given that these isolates were obtained from different US cities in different years, isolated under different clinical circumstances from both adults and newborns, it is possible that these isolates represent widespread dissemination of a specific serotype V clone. This would be consistent with previous reports that have found little diversity among both macrolide-resistant and antibiotic-sensitive serotype V strains in the USA (Diekema *et al.*, 2003; Blumberg *et al.*, 1996). Our BLAST analysis of the eight sequenced GBS genomes (Tettelin *et al.*, 2005) revealed that the integration site identified within the clinical *ermB* isolates is present in six of the eight sequenced GBS genomes. The ORF immediately downstream of the integration site in the clinical isolates, a predicted IS256-like transposase, was present only in two sequenced genomes (A909 and CJB111). We speculate that this relatively small (3.6 kDa) transposase may itself play a role in the integration of the composite transposon, much as insertion sequence elements perform a role in the integration of class I transposons (Whittle & Salyers, 2002).

ermB isolate PF48

Although the composite transposon integration site is identical in all five *ermB* isolates, isolate PF48 has a different capsular polysaccharide serotype and surface protein serotype, and maps as a different strain by RAPD analysis. Southern blot mapping (Fig. 1) revealed that the *KpnI* and *PstI* fragments in isolate 48 were larger than those in the other *ermB* isolates. One PCR fragment (generated by primers Tn9171001F/Tn9172500R) was also larger than predicted from the Tn917 sequence. Sequencing of this fragment with primers *ermB3end* and Tn9172500R revealed an insertion of 854 nt not present in the other *ermB* isolates, beginning after Tn917 nt 1624. BLAST analysis identified this sequence as 93% identical to the known streptococcal insertion sequence IS1381 (GenBank accession no. AF064785), encompassing nt 35–889 of this element. Thus the composite transposon in PF48 was the same as that found in the other four isolates, with the exception of an IS1381 insertion in a non-coding section of Tn917.

Table 3. Genomic localization of composite transposon

Strain	A909_Tn916juncF/ A909_Tn916juncR	A909_Tn916juncF/ Tn916_3'R	A909_Tn916juncR/ Tn916_5'R	RecA7F/RecA4R
A909	+	–	–	+
2603V/R	–	–	–	+
KMP102	–	–	–	+
KMP104	–	+	+	+
KMP106	–	+	+	+
PF19	–	+	+	+
PF45	–	+	+	+
PF48	–	+	+	+

Filter matings

We demonstrated that the complete Tn916 and Tn917 sequences comprised the composite transposon associated with *ermB* erythromycin resistance in GBS. The presence of a similar composite structure in pneumococcus and *Abiotrophia* suggests that this is a functional mobile element responsible for *ermB*-mediated antibiotic resistance among streptococcal strains. Alternatively, in each organism, wild-type Tn917 may have inserted into existing chromosomally integrated Tn916 to create the erythromycin-resistant strains. McDougal *et al.* (1998) were unable to demonstrate *in vitro* transfer of their composite transposon between pneumococcal strains, and Poyart *et al.* (2000) could only demonstrate transfer of the composite element in *A. defectiva* to *E. faecalis* at low frequency. Conjugal transfer of transposable elements between GBS strains has never been demonstrated, although transfer of wild-type Tn916 from *E. faecalis* to GBS has been achieved (Weiser & Rubens, 1987). Integration of the wild-type non-conjugative transposon Tn917 into the GBS chromosome can be achieved *in vitro* only if the element is initially present on a plasmid that has been electroporated into GBS (Framson *et al.*, 1997). To determine whether the composite transposon present in our *ermB* strains could be readily transferred to another GBS isolate, filter-paper mating experiments were carried out using isolate KMP104 as the donor and A909/pDL278 as the recipient. As a control for conjugal transfer, *E. faecalis* strain RH101 containing wild-type Tn916 with the *tet* gene replaced with an *erm* gene was mated with A909/pDL278. Transfer of Tn916 from strain RH101 into A909 was accomplished at high frequency ($>1 \times 10^{-6}$ per donor). Transfer of the composite transposon from KMP104 or PF19 to A909 would be detected by isolation of a polysaccharide serotype Ia strain that is resistant to both spectinomycin and erythromycin. However, consistent with the findings in pneumococcus and *Abiotrophia*, attempts to isolate such a strain by filter mating were unsuccessful. It is possible that the composite transposon transfers with a very low frequency of transconjugation in GBS or that transconjugation occurs *in vivo* from other species (i.e. enterococci or viridans streptococci) to GBS rather than GBS to GBS. It is also possible that the composite transposon is carried initially on a plasmid that is later lost, or that the composite structure is in fact a mobilizable transposon that requires other genetic elements to be present (plasmids or other transposons) for transfer.

We conclude that a chromosomally integrated, composite transposon is associated with *ermB*-type macrolide resistance in GBS. Analysis of a larger number of strains from divergent sources will be needed to establish the contribution of this specific genetic element to the spread of macrolide resistance among GBS strains. Given the presence of this unusual transposon in both GBS and pneumococci, further study is also warranted to determine whether this element is responsible for *ermB*-type resistance in other streptococcal species.

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