

Structure and Dissemination of a Chromosomal Insertion Element Encoding Macrolide Efflux in *Streptococcus pneumoniae*

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Macrolide resistance associated with macrolide efflux (*mef*) has rapidly increased in *Streptococcus pneumoniae*. We defined the genetic structure and dissemination of a novel *mefE*-containing chromosomal insertion element. The *mefE* gene was found on the 5' end of a 5.5- or 5.4-kb insertion designated as the macrolide efflux genetic assembly (*mega*), which is found in ≥ 4 distinct sites of the pneumococcal genome. The element was transformable and conferred macrolide resistance to susceptible *S. pneumoniae*. The first 2 open-reading frames (ORFs) of the element formed an operon composed of *mefE* and a predicted adenosine triphosphate-binding cassette homologous to *msrA*. Convergent to this efflux operon were 3 ORFs with homology to stress response genes of Tn5252. *Mega* was related to the recently described *mefA*-containing element Tn1207.1 but lacked the genes necessary for transposition and had unique termini and insertion sites. In metropolitan Atlanta, macrolide resistance due to *mega* rapidly increased in *S. pneumoniae* by clonal expansion and horizontally by transformation.

Streptococcus pneumoniae is a leading cause of bacterial pneumonia, acute otitis media, and acute sinusitis. The treatment of infections due to *S. pneumoniae* has become increasingly complicated because of the rapid emergence of penicillin resistance. Macrolides offer treatment alternatives; however, resistance to these agents also has emerged [1–5].

There are 2 known mechanisms of macrolide resistance in *S. pneumoniae*, target modification and macrolide efflux. The pneumococcal *ermAM* gene product methylates highly conserved adenine residues in the peptidyl transferase center of newly synthesized 23S rRNA [6]. This methylation blocks the binding of macrolides (M), lincosamides (L), and streptogramin B (S_B), which thereby confers the MLS_B phenotype of antibiotic resistance [7]. The pneumococcal *ermAM* gene is associated with conjugative transposons that harbor other antimicrobial resistance determinants (e.g., *tetM*) [8].

In 1996, a newly detected macrolide efflux mechanism, *mefE*,

was identified in macrolide-resistant strains of *S. pneumoniae* that lack an *ermAM* determinant [9]. Pneumococcal strains containing *mefE* were reported to express resistance only to 14- and 15-membered macrolides (M phenotype). A 3.7-kb pneumococcal fragment containing *mefE* when cloned in *Escherichia coli* was reported to encode a proton motive force-driven transporter sufficient to confer the M phenotype [10]. *mefE* and the related determinant *mefA*, originally described in *S. pyogenes*, are ~90% identical and have been placed in a single class of macrolide efflux genes [11, 12]. Both *mefE* and *mefA* are now found in *S. pneumoniae* [13, 14] and are known to be transferable [15].

In Europe, the *ermAM* determinant is reported to account for recent increases in macrolide resistance of *S. pneumoniae*, whereas *mefA* and *mefE* are found less often [16]. In contrast, most macrolide-resistant pneumococcal strains in North America harbor *mefE* [13, 17–19]. In metropolitan Atlanta from 1994 through 1999, macrolide resistance of invasive pneumococcal isolates increased from 16.4% to 31.5% [13]. By 1999, *mefE* was found in 26% of all invasive *S. pneumoniae* isolated in metropolitan Atlanta [13].

Materials and Methods

S. pneumoniae isolates. Erythromycin-resistant *S. pneumoniae* isolates were obtained as a part of an active, population-based surveillance for invasive bacterial pathogens in metropolitan Atlanta. The specific collection methods have been described elsewhere [13, 20]. In brief, all sterile-site isolates of *S. pneumoniae* ($n = 4148$) were collected from the 8-county metropolitan Atlanta area, Georgia Health District 3 (population of 2.7 million, according to 1997 census estimate), from 1994 through 1999. Laboratory audits were conducted to assess accuracy, and case report forms were generated on all cases.

A subset of 336 erythromycin-resistant isolates (MIC ≥ 4 $\mu\text{g/mL}$),

Received 9 January 2001; revised 23 March 2001; electronically published 31 May 2001.

Presented in part: 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, September 2000 (abstract 1929).

Financial support: Centers for Disease Control and Prevention (H50/CCH413121 to D.S.S.); Veterans Affairs Merit Review Grant from the Office of Research and Development, Department of Veterans Affairs, Atlanta.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF274302).

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The Journal of Infectious Diseases 2001;184:56–65

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0022-1899/2001/18401-0009\$02.00

identified consecutively by the surveillance in 1994–1996 and in 1998, was studied for the presence of *mefE* and *ermAM* [13]. Eighty-nine *mefE*-containing isolates (MIC ≥ 4 $\mu\text{g}/\text{mL}$) recovered from patients with invasive pneumococcal disease from January 1994 through February 1996 and the 129 *mefE*-containing isolates recovered in 1998 formed the collection of isolates used in this study. These isolates included GA3488 (serotype 6A), GA2551 (serotype 14), GA2254 (serotype 14), GA4375 (serotype 19F), and GA4175 (serotype 19A). The erythromycin-sensitive (*mefE*⁻ and *ermAM*⁻) pneumococcal laboratory strain R6 also was used. Isolates were grown in Todd-Hewitt broth with 0.5% yeast extract. Antimicrobial susceptibility of isolates collected from this surveillance was determined by guidelines established by the National Committee for Clinical Laboratory Standards [21]. Serotyping was done as described elsewhere [13, 20].

DNA isolation. Genomic DNA was isolated from *S. pneumoniae* by one of 2 methods. Crude preparations of DNA were obtained by boiling bacterial pellets for 10 min in 100 mM NaCl, 10 mM Tris-HCl (pH 8.3), 1 mM EDTA, and 1% Triton X-100. Higher-purity DNA was prepared by sequential incubations of cell suspensions in lysozyme and RNase (Sigma), phenol/chloroform purification, and ethanol precipitation, as described by Nath [22].

Polymerase chain reaction (PCR)-based detection of *mefE*. Nucleotide primers used in PCR and DNA sequencing are shown in table 1. Primer set KG5F and KG5R2 was used to define 345 bp of *mefE* [12]. KG17R also was paired with KG5F to amplify *mefE*, resulting in a 1355-bp product. PCR amplification consisted of 35 cycles with a 30-s denaturation at 95°C, a 30-s anneal at 60°C, and extension for 1 min at 72°C in a thermal cycler (GeneAmp PCR System 9600; Perkin-Elmer Applied Biosystems). Each reaction contained 500 ng of template DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μM of each deoxynucleotide triphosphate (dNTP), 2.5 U of Taq DNA polymerase (Perkin-Elmer Applied Biosystems), 1.5 mM MgCl₂, and 1.5 μM each primer. PCR products were visualized by 1.2% agarose gel electrophoresis and ethidium-bromide staining.

Single specific primer PCR (SSP-PCR). Restriction digests of chromosomal DNA from GA3488 were ligated with *SmaI*-restricted pUC18. Twenty units of *HaeIII* (New England Biolabs) was used to digest 100 μg of chromosomal DNA at 37°C for 2 h.

The vector was prepared by digesting 2 μg of pUC18 with 10 U of *SmaI* (New England Biolabs) at room temperature for 1 h. One unit of shrimp alkaline phosphatase (USB) then was added, and the vector was incubated for an additional hour at 37°C. Both the vector and chromosomal insert were heat-inactivated at 70°C for 20 min. The ligation reaction consisted of the pUC18 vector and chromosomal insert in a 1:4 ratio, incubated at 4°C with 400 U of T4 ligase (New England Biolabs). Unsequenced regions of DNA flanking *mefE* were amplified, using a single insert-specific primer paired with a primer, CM7, specific to the cloning site of pUC18, as described above. KG7 and KG8 initially were used as the *mefE*-specific primers on the basis of GenBank accession number SPU83667. Further characterization of the region upstream from *mefE* was accomplished with primers KG9 and KG12. Additional SSP-PCR of the downstream sequence used primers KG16, KG18, KG20F, KG24, and KG26F.

DNA sequence analysis. PCR products were purified using QiaQuick columns, according to the manufacturer's instructions (Qiagen). Automated sequencing was done, using Applied Biosystems PRISM 377 equipment and the same primers as those used for PCR. Sequence analysis was conducted with GeneJockey II (BioSoft) and BLAST version 2.0 (preliminary pneumococcal sequence data were obtained from The Institute for Genomic Research [TIGR]: <http://www.tigr.org>).

RNA isolation and reverse-transcription (RT) PCR. Total RNA was obtained from strains GA3488 and GA2551, using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RT was performed with Gene Amp (Perkin-Elmer Applied Biosystems), which was used according to the manufacturer's protocol with primer KG5F. PCR was performed as described above, using KG5F as the forward primer and KG5R2 or KG18R as the reverse primer.

Pulsed-field gel electrophoresis (PFGE) and Southern blot hybridizations. Pneumococci from overnight cultures were suspended in low melting-point agarose at a concentration of ~0.5 McFarland units to form plugs, were incubated in 500 mM EDTA/1% *N*-lauroylsarcosine overnight at 55°C, and were digested with *SmaI* (New England Biolabs) at room temperature for 2 h, according to the protocol described by McEllistrem et al. [23]. PFGE

Table 1. Oligonucleotide primers used in polymerase chain reaction and sequencing.

Primer	Sequence (5'→3')	Sequence used for primer design	Position in mega	Primer paired with
KG5F	AGTATCATTAATCACTAGTGC	ORF 1 (<i>mef</i>)	1181–1201	KG5R2; KG17R
KG5R2	TTCTTCTGGTACTAAAAGTGG	ORF 1 (<i>mef</i>)	1506–1525	KG5F
KG17R	CTTCACGGTCTAAATGGCTCG	ORF 2 (<i>mel</i>)	2841–2861	KG5F
CM7	CCAGTCACGACGTTGTAAAACG	pUC19		KG7-26F
KG7	TAGACAAGACCATCGCAGATCCT	ORF 1 (<i>mef</i>)	1244–1266	CM7
KG8	GTATCATGTCACTTGCTATGCC	ORF 1 (<i>mef</i>)	2182–2203	CM7
KG9	CCTCACCGTAACTAATGAATGCTC	Upstream from <i>mef</i>	911–934	CM7
KG12	CATAGACTGTAACGCTCTGG	Upstream from <i>mef</i>	653–672	CM7
KG16	CACTTGTAGGCAAGCTAGGTGT	ORF 2 (<i>mel</i>)	2715–2736	CM7
KG18	CATACCCTATAGTCGGTGCAG	ORF 2 (<i>mel</i>)	3342–3361	CM7
KG20F	CTGGTTCTGGTTGGCGACTC	Downstream from <i>mel</i>	3955–3972	CM7
KG24	GTATCCTGGTACTCTCTTGCTG	ORF 4	4646–4667	CM7
KG26F	TCTACTGCACCAGAGGTG	ORF 5	5284–5301	CM7
KG25F	GGATACCCAGTCTCTGAAG	TIGR SP-66		KG33R
KG33R	GACTGGCAATGCTAGCGGC	TIGR SP-66		KG25F

NOTE. mega, Macrolide efflux genetic assembly; ORF, open-reading frame; TIGR, The Institute for Genomic Research.

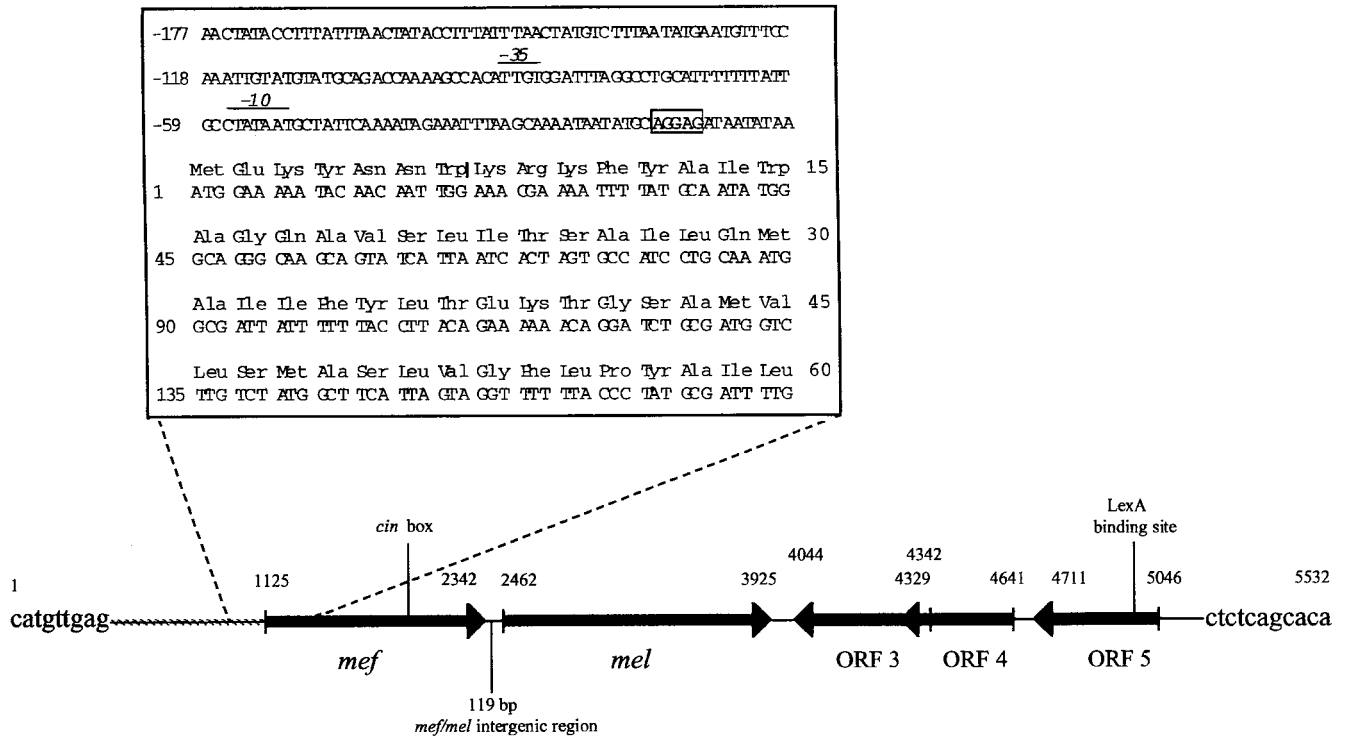


Figure 1. Genetic organization of macrolide efflux genetic assembly (mega), a chromosomal insertion element 99 bp in *Streptococcus pneumoniae* strain GA3488. This was designated a mega Class I insert. The sequence of mega insert Class II is identical, except that the *mef/mel* intergenic region is truncated by 99 bp. *Box*, Nucleotide sequence of the first 180 bases of open-reading frame (ORF) 1, *mef*, with the predicted promoter region and the amino acid sequence of *mefE*. A Shine-Dalgarno sequence is outlined. Lines are drawn over predicted -35 and -10 promoter sequences.

then was done in a 1% agarose (SeaKem GTG agarose; FMC Bioproducts) 0.5× Tris-borate-EDTA buffer, using an electrophoresis system (CHEF-DR II; Bio-Rad Laboratories) at 6 V/cm (1–20-s switch time) for 22 h. The gel then was stained with ethidium bromide and photographed. Dendograms were constructed with an electrophoresis analysis system and software package (BioImage) [24].

Gels that were used for Southern blot transfer were subjected to a 30-min acid depurination (0.25 M HCl), followed by a 30-min base treatment (1.5 M NaCl and 0.5 M NaOH) and neutralization (1.5 M NaCl and 0.5 M Tris-HCl). Gels were equilibrated in transfer buffer (2× standard saline citrate [SSC; 1× SSC is 0.15 M NaCl and 0.015 M sodium citrate]) before overnight capillary blotting [25]. DNA was fixed to the membrane by UV cross-linking and was prehybridized for ≥ 2 h at 65°C. Membranes were hybridized with a *mef* probe in 5× SSC, 1% blocking reagent, 0.1% *N*-lauroylsarcosine, and 0.02% SDS at 65°C overnight. Chemiluminescent detection was performed with the Genius 3 system (Boehringer Mannheim), according to the manufacturer's instructions.

Random prime labeling with digoxigenin was done using Genius 2 (Boehringer Mannheim), according to the manufacturer's instructions. A 1680-bp template spanning most of *mefE* and *mel* was generated by PCR, using primers KG17R and KG5F. PCR amplification was performed, as described above.

Transformation. A 6278-bp PCR product containing a chro-

mosomal insertion element designated as the macrolide efflux genetic assembly (mega) and flanking chromosomal sequence was generated with primers KG25F and KG33R. The unencapsulated laboratory strain R6, which was grown to an optical density of 1.8 at 650 nm in C + Y media, was transformed with 10 μ g/mL of PCR product in the presence of 50 ng/mL of competence-stimulating peptide (courtesy of D. A. Morrison, University of Illinois at Chicago) at 30°C for 45 min [26, 27]. Transformants were selected on trypticase soy agar plus 5% sheep blood agar plates with 1 μ g/mL of erythromycin.

Results

The *mefE*-containing genetic locus. Erythromycin-resistant isolates of *S. pneumoniae* confirmed by PCR to contain *mefE* were studied (see Materials and Methods). The isolates were obtained from patients with invasive pneumococcal disease in metropolitan Atlanta. Southern hybridizations using a *mefE* probe and *Sma*I digestion of 15 randomly chosen erythromycin-resistant isolates indicated that *mefE* was present as a single band (data not shown). A 5532-bp element containing *mefE* was defined in a pneumococcal isolate (GA3488) recovered from a patient with pneumococcal bacteremia in 1995 (figure

epidermidis (SWISS-PROT accession no. P23212; figure 2). A 119-bp intergenic region was between *mefE* and *mel* in GA3488. This region contained a consensus Shine-Dalgarno sequence upstream from the predicted start codon for *mel*. RT-PCR demonstrated that *mefE* and *mel* were cotranscribed (figure 3).

At nucleotide positions 4044–4342 and 4329–4641, 3' from *mel*, there were 2 overlapping ORFs (ORF 3 and 4), which were oriented opposite to *mefE* and *mel*. The predicted proteins of these ORFs had 52% and 38% identity, respectively, to the predicted proteins of ORFs 11 and 12 of Tn5252 of *S. pneumoniae* (GenBank accession no. L29324). The first 4 ORFs of mega and Tn1207.1 ORFs 4–7 had >94% identity at the nucleotide level. A fifth ORF, also with a reverse orientation relative to *mefE*, was located between positions 4711 and 5046 of mega. ORF 5 of mega and ORF 8 of Tn1207.1 had 92% identity at the nucleotide level. ORF 5 of mega had 59% nucleotide identity with ORF 13 of Tn5252 and 45% identity with ORF U of a lactococcal plasmid (GenBank accession no. U36837). ORF 5 of mega, ORF 13 of Tn5252, and ORF U of *Lactococcus lactis* are predicted homologues of *umuC* of *E. coli* (figure 4) [28, 29]. However, the intergenic region between ORFs 5 and 4 of mega was different from that of Tn5252 and Tn1207.1. A 20-bp segment homologous with the *E. coli* LexA binding site was associated with ORF 5 (beginning at nt position 5449) [30]. About 150 bp of the 3' end, including the LexA binding sequence, were unique to mega. The 5' and 3' ends of the mega element had imperfect inverted terminal repeats that also were unique (figure 1).

Mega insertion sites (figure 5). The pneumococcal nucleotide sequences adjacent to the terminal inverted repeats of mega in isolate GA3488 (serotype 6A) were 92.5% homologous (817/883-bp identity) with the TIGR *S. pneumoniae* contig SP-66. The 5.5-kb mega element had inserted into an ORF predicted to encode a homologue of the phosphomethyl pyrimidine kinase of *Bacillus subtilis* in an orientation opposite to the chromosomal ORF. In this genomic location, mega was designated as a Class I mega insert.

A second insertion site for mega was defined in pneumococcal isolate GA2551 (serotype 14). The second site was located in the TIGR *S. pneumoniae* contig SP-101 at bp 1003. The sequence flanking the mega insert in GA2551 showed 91.8% nucleotide homology (460/501-bp identity) with this TIGR *S. pneumoniae* sequence. At this location, a 5433-bp mega insert was found disrupting an ORF predicted to encode a homologue of the DNA-3-methyladenine glycosidase of *Haemophilus influenzae*. This second mega insertion, designated Class II, also was oriented in a direction opposite to the chromosomal ORF. The nucleotide sequence of the 5.4-kb Class II mega element was identical, except for a 99-bp deletion in the region between *mefE* and *mel* that eliminated the consensus Shine-Dalgarno sequence upstream from the *mel*-predicted start codon.

Two additional *mefE*-containing clinical isolates, GA4175 (serotype 19A) and GA4375 (serotype 19F), which did not have

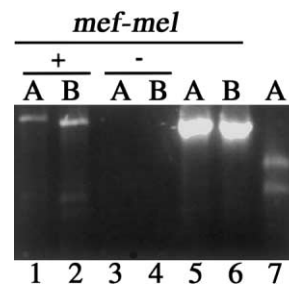


Figure 3. Cotranscription of *mefE* and *mel* of the macrolide efflux genetic assembly (mega). Reverse-transcriptase (RT) polymerase chain reaction (PCR) of pneumococcal RNA extracted from strains GA3488 (A) and GA2551 (B) are shown. Bacterial RNA was purified from log-phase liquid cultures, and RT-PCR was performed, as described in Materials and Methods. In brief, cDNA was generated with the *mef*-specific forward primer KG5F. *mef-mel* PCR products were amplified, using KG5F and the *mel*-specific reverse primer KG18R. Samples were analyzed by pulsed-field gel electrophoresis in a 1.5% agarose gel stained with ethidium bromide. Reactions were performed with (lanes 1 and 2) or without (lanes 3 and 4) RT. DNA controls (lanes 5 and 6) and total RNA (lane 7) from GA3488 are shown.

Class I or II mega insertions, were located in distinct sites (insertion sites Class III and IV). The 260-bp sequence 5' of mega in the Class III insert site showed 93% identity to the nucleotide sequence in contig SP-15 of the pneumococcal genome. The mega element was found in the same orientation as an ORF predicted to encode a protein with homology to Cap5D of *Staphylococcus aureus*. In the Class IV insert site, mega had inserted in the pneumococcal genome contig SP-28 at position 1329 and was in the same orientation as an ORF predicted to encode a protein with homology to an RNA-methyltransferase of *B. subtilis*. The intergenic region between ORFs 1 and 2 in the Class I and III mega elements was 119 bp. In the Class II and IV mega alleles, the region was truncated by 99 bp.

The 5' and 3' ends of the 4 different mega insertions and the pneumococcal genomic sequences immediately adjacent were compared (figure 5). Mega had identical 7-bp imperfect inverted repeats at the ends of each insertion. The *S. pneumoniae* genomic nucleotides immediately adjacent to mega in the 4 insertion sites were related (figure 5).

Mega confers macrolide resistance in *S. pneumoniae*. To show that mega confers M phenotype macrolide resistance in *S. pneumoniae*, we amplified a 6.3-kb PCR product composed of mega and 747 bp of flanking DNA from the Class I insert in GA3488. This PCR fragment was transformed into the erythromycin-sensitive (*mefE*⁻) strain R6. Erythromycin-resistant transformants were obtained at a frequency of $\sim 2 \times 10^{-4}$ μ g/DNA. The control parent strain, R6, grew only on nonselective plates. The Class I chromosomal location of mega in the transformants was confirmed by PCR and nucleotide sequencing.

Mega insertions in the *S. pneumoniae* population. The population-based collection of *mefE*-containing invasive *S. pneumoniae* isolates from Atlanta (see Materials and Methods)



Figure 4. Alignment of the predicted amino acid sequence of macrolide efflux genetic assembly (mega) open-reading frame (ORF) 5 with the predicted amino acid sequence of ORF 13 of Tn5252 (ORF13/Tn5252; AAC98439), ORF U of *Lactococcus lactis* (ORFU-LL; AAB52385), UvrA of *Enterococcus faecalis* (UvrA-EF; BAA23799), and UmuC of *Escherichia coli* (UmuC-EC; P04152).

was used to study mega insertions within individual pneumococcal isolates (table 2). PCR primers specific to the sequences flanking the Class I–IV mega insert sites were used to define the location of mega and adjacent DNA in 89 *mefE*-containing invasive pneumococcal strains isolated in Atlanta from 1994 through 1996. A Class I product was detected in 14 (15.7%) of the 89 *mefE*-positive strains. The termini of 4 Class I inserts (3 serotype 6A and 1 serotype 19A) and adjacent chromosomal DNA were confirmed to have nucleotide sequences identical to those of GA3488. All Class I inserts contained the 5.5 kb-size mega element.

Class II mega inserts were found in 55 (62%) of 89 *mefE*-containing invasive isolates. The 5.4-kb mega allele was present in each of the Class II strains. The nucleotide sequences of the Class II insertion sites and mega termini of 7 of the serotype 14 strains were sequenced and were found to be identical to the prototype strain GA2551 (serotype 14), as were 2 serotype 6B isolates. Of the 20 isolates *mefE*-containing invasive isolates not having Class I or Class II mega inserts, 3 were Class III, one was a Class IV, and 16 contained *mefE* of undefined insert types.

PFGE analysis of 56 of the 89 mega-containing isolates showed 19 distinct PFGE types. Isolates were related (e.g., having PFGE band differences ≤ 3 [31], an identical location and

size of the mega insertion, and identical nucleotide sequence adjacent to the insert) or were unrelated, as determined by PFGE. For example, 5 serotype 14 isolates recovered from 1 January 1994 through 2 July 1995 from epidemiologically distinct patients in metropolitan Atlanta were found to be indistinguishable or closely related by PFGE analysis (figure 6). These isolates contained the 5.4-kb mega allele in a Class II insertion site. The pneumococcal chromosomal sequence adjacent to the Class II mega insert was indistinguishable in all of these isolates. In contrast, other serotype 14 isolates containing the Class II mega insert were unrelated by PFGE, although the genomic location and size of the mega insert and adjacent nucleotide sequence were indistinguishable (figure 7). Similar results were seen with isolates of other serotypes. These data suggest that mega has spread in *S. pneumoniae* in Atlanta by both clonal expansion and horizontal transfer.

Discussion

Macrolide resistance associated with *mefE* has rapidly emerged in *S. pneumoniae* [13]. We found that *mefE* is part of a novel genetic insertion element associated with the increase of macrolide resistance in *S. pneumoniae* in North America.

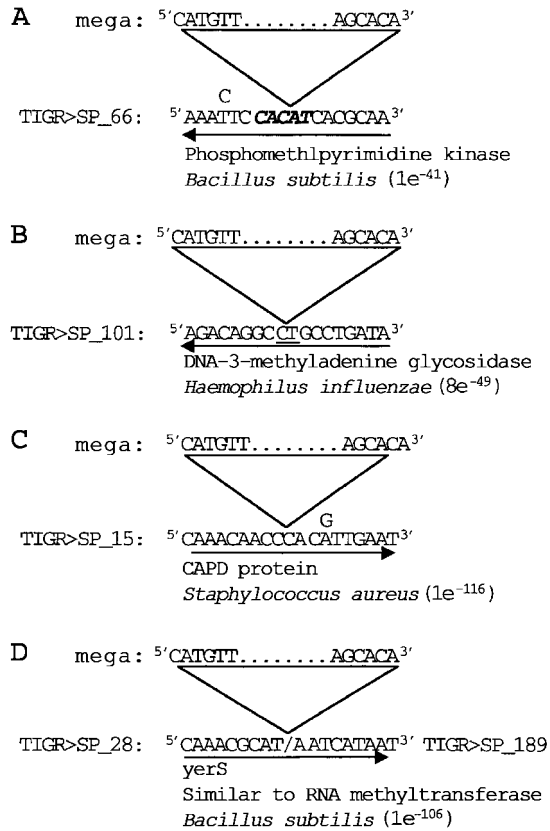


Figure 5. Macrolide efflux genetic assembly (mega) insertion sites. *A*, Class I insert site (e.g., GA3488) CACAT (in boldface italic) is duplicated at the ends of the mega insert. *B*, Class II insert site (e.g., GA2551). The underlined CT is missing in the chromosomal sequence containing mega. *C*, Class III insert site. *D*, Class IV insert site.

This element contains an operon with 2 ORFs, *mefE* and *mel*. The 3 other ORFs (ORFs 3–5) of mega share homology with ORFs 11–13 of Tn5252 and are convergent to *mef* and *mel*. The functions of these ORFs are unknown, but recent evidence suggests that ORF 13 of Tn5252 is involved in the SOS response in pneumococci [29]. Two other potential regulatory components of mega, a LexA binding domain in ORF 5 and a *cin* box within *mefE*, further suggest that this element might be regulated by competence and SOS stress response events [32, 33]. Mega has imperfect terminal inverted repeats that are not duplicated at the site of insertion, and the element is found in ≥ 4 distinct sites within the pneumococcal genome.

Mega contains an operon potentially encoding 2 distinct classes of efflux pumps. The first ORF in this operon, *mefE*, shares $\sim 90\%$ homology with *mefA* at the nucleic acid level [12]. *mefA* of *S. pyogenes* is predicted to encode a protein with 12 membrane-spanning regions that can be divided into 2 domains similar to those of the tetracycline protein motive force transporters found in gram-negative bacteria [10]. Experimental evidence supporting a proton motive function of MefE was dem-

onstrated by a decrease in the efflux of [14 C]-erythromycin in the presence of the proton motive pump inhibitors carbonylcyanide-*m*-chlorophenylhydrazone and arsenate [9, 10]. A 3.7-kb fragment of pneumococcal DNA containing the 1.2-kb *mefE* gene was reported to be sufficient for conferring erythromycin resistance when cloned into susceptible strains of *E. coli* [34], but the additional DNA contained in the fragment was not characterized. We demonstrate that mega can confer macrolide resistance when transformed into *S. pneumoniae*.

The second ORF of the efflux operon, *mel*, is a homologue of *msrA* that encodes an ATP-binding cassette (ABC) that provides the energy for macrolide and streptogramin B efflux in staphylococci [35]. The 1.46-kb *msrA* gene of *S. epidermidis* confers active [14 C]-erythromycin efflux when cloned into *S. aureus* [36]. MsrA is predicted to interact with as yet unidentified transmembrane proteins, to allow for erythromycin efflux [36]. The deduced 488 aa of MsrA have motifs that are common to the ABCs of gram-negative bacteria and eukaryotes [37]. Each of the 2 ABC transporter domains consists of 2 ATP-binding motifs [38]. Between these ATP-binding motifs lies a highly conserved SGG protein sequence that forms loop 3 of the predicted tertiary structure and is predicted to interact with the cell membrane [39]. MsrA is the prototype of a subfamily of ABCs that are characterized by an interdomain "Q-linker" [35, 40]. The position of *mefE* and *mel* in the mega element and their cotranscription as an operon suggest that the encoded proteins are a dual efflux system in *S. pneumoniae*.

The amino acid sequence homology between ORFs 5, 4, and 3 of mega and ORFs 11, 12, and 13 in Tn5252 suggest that mega is related to conjugative transposons. Conjugative transposons (e.g., Tn5252) insert by a nonduplicative, site-specific recombination mechanism. Although mega lacks enzymes required for such DNA transposition, the similar insertion site sequence, the nonduplicative insertions, and evidence of carryover bases suggest that mega was introduced initially into the pneumococcal chromosome by a site-specific event. In support of this hypothesis, a genetic element, designated Tn1207.1, which is related to mega, recently was described by Santagati

Table 2. Macrolide efflux genetic assembly (mega) insertions in clinical isolates of macrolide-resistant *Streptococcus pneumoniae* containing *mefE* ($n = 89$).

Serotype	Class I ($n = 14$)	Class II ($n = 55$)	Class III ($n = 3$)	Class IV ($n = 1$)
6A	11	6	0	0
6B	0	12	0	0
14	1	27	1	1
19A	1	2	2	0
19F	0	1	0	0
23F	0	1	0	0
NT	1	6	0	0

NOTE. The *mefE*-containing invasive pneumococcal strains were isolated in Atlanta, 1994–1996. Classes I–IV refer to genomic locations of mega in the pneumococcal chromosome (figure 5). Sixteen of 89 isolates were of undefined insert type by polymerase chain reaction. NT, nonserotypeable.

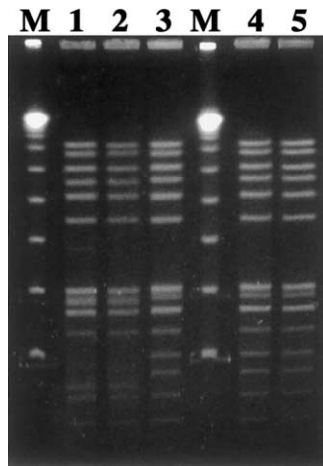


Figure 6. Pulsed-field gel electrophoresis of chromosomal DNA of serotype 14 *Streptococcus pneumoniae* isolates recovered from patients with invasive pneumococcal disease in Atlanta, 1994–1995, and containing macrolide efflux genetic assembly Class I inserts. Closely related (lanes 1 and 2) and identical isolates (clones; lanes 3–5) are shown. Strains GA5196, GA4313, GA4988, GA2092, and GA2551 are shown (lanes 1–5, respectively). Lane M, 50-kb λ -ladder.

et al. [14]. Tn1207.1 is a 7244-bp defective transposon containing *mefA* and 4 downstream ORFs with homology and position similar to those of the corresponding ORFs of mega. However, we did not detect a *mefE(A)*-containing element of this size in any of the pneumococcal isolates that we studied, and the sequence 5' of *mefA* in Tn1207.1 contains 3 additional ORFs with recombinase and integrase homology. Furthermore, the 944-bp and 150-bp 5' and 3' termini, including the LexA binding sequence, are unique to mega. Also, the insertion site of the *mefA*-containing Tn1207.1 is a transformation-specific locus of the pneumococcal chromosome [14] that we have not found as a mega insertion site.

ORF 13 of Tn5252 and ORF 5 of mega have homology to UmuC. In Tn5252, ORF 13 is followed by ORF 14, a UmuD homologue. The UmuC and D homologues of Tn5252 restore error-prone repair in *S. pneumoniae* [29], and the expression of the Tn5252 *umuCD* operon is regulated by the RecA and LexA proteins. Although a UmuD homologue is absent in mega, ORF 5, the mega UmuC homologue, has a consensus LexA binding sequence. In addition to ORFs 3–5, the complement of the strand encoding *mefE* contains a TACGAATA sequence. Campbell et al. [32] postulate that this sequence is part of a core promoter consensus recognized by an alternative sigma factor critical for the expression of competence-induced elements. This sequence, which Claverys and Martin [41] refer to as a *cin* box, is found in 9 putative competence loci of the pneumococcal genome.

The dissemination of mega in *S. pneumoniae* was studied in pneumococcal isolates obtained from a defined human population undergoing active population-based surveillance for *S.*

pneumoniae invasive disease. In 6 years of surveillance (1994–1999), rapid increases in macrolide resistance rates were linked with mega [13]. In this study, we show identical mega insertions and adjacent chromosomal DNA in epidemiologically and genetically unrelated pneumococcal strains obtained from the surveillance. These data indicate that transformation and homologous recombination have spread mega in *S. pneumoniae*. This hypothesis is further supported by serotype data from our 6-year surveillance showing the appearance of mega in new serotypes [13] and, experimentally, by transformation of mega and induction of macrolide resistance in susceptible *S. pneumoniae*.

Evidence for dissemination of mega by clonal expansion also was found in this study. Serotype 14 isolates identical by PFGE, mega insertion, and DNA adjacent to mega was recovered from geographically and temporally unrelated pneumococcal isolates in metropolitan Atlanta. Spread of drug-resistant pneumococcal clones previously has been documented for serotypes 23 and 14 [42, 43]. The pressure of increased macrolide use may select for the clonal expansion of pneumococci that harbor mega and for the transformation events that spread mega to other pneumococcal strains.

In summary, *S. pneumoniae* in our population is rapidly developing resistance to macrolide antibiotics. This is associated with the dissemination of the novel 5.5- or 5.4-kb genetic element designated mega. The element contains genes that may encode 2 distinct classes of efflux pumps. Mega has features of a defective conjugative transposon and may be influenced by the induction of competence and the SOS network. Mega ap-

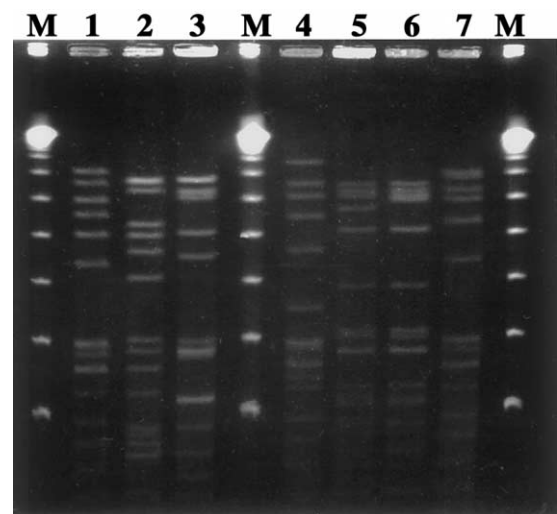


Figure 7. Pulsed-field gel electrophoresis showing chromosomal DNA of serotype 14 *Streptococcus pneumoniae* isolates recovered from patients with invasive pneumococcal disease in Atlanta, 1994–1995, and containing macrolide efflux genetic assembly Class II inserts; possibly related (lanes 5 and 6) and unrelated isolates (lanes 1–4 and 7) are shown. Strains GA2566, GA4672, GA2572, GA2073, GA4796, GA5248, and GA2551 are shown (lanes 1–7, respectively). Lane M, 50 kb λ -ladder.

pears to have entered the pneumococcal genome on ≥ 4 occasions by site-specific recombination events but has expanded rapidly in the pneumococcal population by the selection of mega-containing clones and has spread horizontally by transformation. The emergence of mega in *S. pneumoniae* demonstrates how rapidly selection of a genetic element can occur in vivo in this important human pathogen and further urges the judicious use of antibiotics.

Acknowledgments

We thank Monica Farley, Fred Tenover, Wendy Baughman, the hospitals, staff, and surveillance personnel of Georgia Emerging Infections Program for helpful assistance. We also thank Lane Pucko for administrative assistance and Larry Martin and Linda McDougal for technical support.

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