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Prophage association of *mef*(A) elements encoding efflux-mediated erythromycin resistance in *Streptococcus pyogenes*

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***Objectives*: To compare different *mef*(A) elements of *Streptococcus pyogenes* for a possible chimeric genetic nature, i.e. a transposon inserted into a prophage.**

***Methods*: Eleven *S. pyogenes* isolates with efflux-mediated erythromycin resistance were used. The isolates were typed using several genotypic approaches. Gene detection was performed by PCR using specific primer pairs. The *mef*(A) elements of the test strains were induced with mitomycin C and phage DNA was extracted. Induction was monitored by PCR using primers targeting *mef*(A).**

***Results*: Six tetracycline-susceptible isolates had PCR evidence of all of the eight open reading frames (ORFs) of the Tn*1207.1* element; their *mef*(A) element was consistent with the Tn*1207.3* element in four isolates and with the 58.8 kb chimeric element in two. Five tetracycline-resistant isolates had no PCR evidence of *orf1* and *orf2* and showed variable patterns as to *orf3*, *orf7*, and *orf8*. Three ORFs placed along the conserved region downstream of Tn*1207.1* in the 58.8 kb *mef*(A) chimeric element were detected in the six tetracycline-susceptible, but not in the five tetracycline-resistant isolates. Induction assays with mitomycin C demonstrated that the *mef*(A) elements of all strains tested were present in culture supernatants in a DNAse-resistant form, such as a phage capsid.**

***Conclusions*: All recognized *mef*(A) elements of *S. pyogenes* appear to be prophage-associated. Whereas the two elements detected in tetracycline-susceptible isolates (Tn*1207.3* and the 58.8 kb one) were apparently inserted into the same prophage, the *tet*(O)-*mef*(A) element was inserted into a different prophage. Phage transfer is likely to play a critical role in the dissemination of erythromycin resistance in *S. pyogenes* populations.**

Keywords: *Streptococcus* phages, tetracycline resistance, typing

Introduction

During the last 15 years, the growing rates of erythromycin-resistant *Streptococcus pyogenes* reported in many countries largely reflected the emergence of active efflux and its progressive superseding of the more conventional methylase-mediated target site modification mechanism of macrolide resistance.[1] In *S. pyogenes* and other streptococci, efflux-mediated erythromycin resistance is encoded by the *mef*(A) gene and is associated with a particular resistance pattern (M phenotype) characterized by resistance only to 14- and 15-membered macrolides, usually at a low level.[2,3] A *mef*(A)-carrying element was first identified in *Streptococcus pneumoniae* as a 7.2 kb non-conjugative transposon (Tn*1207.1*) containing eight open reading frames (ORFs), of which *mef*(A) is the fourth.[4] Subsequently, Tn*1207.1* was also found in *S. pyogenes* as a part of larger mobile elements, such as a *ca.* 52 kb conjugative transposon (Tn*1207.3*), which contains Tn*1207.1* at its left end,[5] or a 58.8 kb genetic element, which contains Tn*1207.1* at a variable distance (usually *ca.* 6 kb) from its left end.[6] On the other hand, we have recently shown that tetracycline resistance, when present in *S. pyogenes* isolates with efflux-mediated erythromycin resistance, is encoded by the *tet*(O) gene, which is linked to *mef*(A) in a newly discovered mobile element.[7] Amplification and sequencing experiments demonstrated that, within this *tet*(O)-*mef*(A) element, *mef*(A) is part of an incomplete and variable Tn*1207.1*.[8]

Very recently, Banks *et al.*[6] first underscored the chimeric genetic nature—i.e. a transposon inserted into a prophage—of

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Table 1. Oligonucleotide primer pairs used

Gene	Primer		Reference	Product size (bp)
	designation	sequence (5′–3′)		
mef(A)	MEFA1	AGTATCATTAATCACTAGTGC	13	348
	MEFA2	TTCTTCTGGTACTAAAAGTGG	13	
orf1[a]	ORF1	TGATGAAGAGGAAAATTAG	14	266
	ORF1-rev	TACATCAACATTACCATCTG	15	
orf1–orf2[a]	ORF1	TGATGAAGAGGAAAATTAG	14	1066
	ORF2-rev	GATTGATGTTCCTGATGC	14	
orf2–orf3[a]	ORF2	GCATCAGGAACATCAATC	14	1119
	ORF3-rev	GACCTACCTGAACAATACC	14	
orf3[a]	ORF3-for	TATAGCATGACGGTG	14	199
	ORF3-rev	GACCTACCTGAACAATACC	14	
orf3–mef(A)[a]	ORF3	GGTATTGTTCAGGTAGGTC	14	1050
	OM18	TGCTTGCCCTGCCCATATT	16	
mef(A)–*orf5*[a]	MF4AR	TTCTTTGCTGATAAAATCGGTGT	14	440
	ORF5F-rev	GGCAAGTTCACCCAGATG	14	
orf5[a]	MSRA1	TGCCTATATTCCCCAGTT	15	708
	MSRA2	TTAATTTCCGCACCGACTA	15	
orf6[a]	ORF6-for	TGGATTCTCTTCTAAGGTTC	8	166
	ORF6-rev	CTACCGCTACTCCAACATG	14	
orf6[b]	ORF6-1	GATTAGTGGTTTCTC	8	223
	ORF6-2	TGGACTATTACAGCG	8	
orf6–orf7[a]	ORF6	CATGTTGGAGTAGCGGTAG	14	202
	ORF7-rev	CCAAGTCTGACCAAAGATTTC	14	
orf7[a]	ORF7	GAAATCTTTGGTCAGACTTGG	14	253
	ORF7-2	GCCATTTCAGTCAGCAAGAG	8	
orf7–orf8[a]	ORF7	GAAATCTTTGGTCAGACTTGG	14	297
	ORF8-rev	CGCTGGTGGATTGGAGGG	14	
orf8[a]	ORF8	CCCTCCAATCCACCAGCG	14	314
	ORF8-2	ATTGACCCAGCAAATCTTCC	8	
tet(O)	TETO1	AACTTAGGCATTCTGGCTCAC	17	519
	TETO2	TCCCACTGTTCCATATCGTCA	17	
speB	SPEB1	ACCGTGTTATTGTCTATTACC	6	1300
	SPEB2	TGCCTACAACAGCACTTTGG	6	
comEC	COMEC1	TGGTCTTAACAGGGCTCTTG	this study	327
	COMEC2	AGGTCACTTCATTGGTCAAA	this study	
comEC	MS54	CCTTTGACCAATGAAGTGACCTTT	5	453/6807[c]
orf1	MS34	TCTTCGCCGCATAAACCCTATC	5	
1[d]	R28-1	GAAATAGCACCCATGGAAAAAGAATAATC	6	2010
	R28-2	CAGAAATTGAATTCTGTTCTAAAGC	6	
2[d]	TSS1	TCTGTTATATGCGGATGGTG	this study	445
	TSS2	ATAAACAACTGGGTAGAACG	this study	
3[d]	CHPP1	CGTCGCTCTATTCTACCAGT	this study	283
	CHPP2	GCGTTGAAATGACCACCCCA	this study	

Table 1. (*Continued*)

Gene	Primer designation	Primer sequence (5′–3′)	Reference	Product size (bp)
4[d]	SSRec1	ACCAAGGTATGTGGGGAAAA	this study	260
	SSRec2	CAAGAGAGGTATGAAGGAAG	this study	
prtF1	PRTF1-1	TTTTCAGGAAATATGGTTGAGACA	18	variable[e]
	PRTF1-2	TCGCCGTTTCACTGAAACCACTCA	18	

[a]The primer pair was designed from the reported sequence of Tn*1207.1*[4] (accession no. AF227520).
[b]The primer pair was designed from a sequenced portion of the *tet*(O)-*mef*(A) element[8] (accession no. AJ715499).
[c]The expected amplicon size was 453 bp according to the reported organization of the Tn*1207.3* element,[5] whereas a larger size (6807 bp) was expected according to the reported sequence of the 58.8 kb *mef*(A) element[6] (accession no. AY445042).
[d]Specific primer pairs designed from the deposited sequence (accession no. AY445042) were used to target four ORFs of the 58.8 kb *mef*(A) element[6] encoding the following products: 1, R28-like protein; 2, terminase small subunit; 3, conserved hypothetical phage protein; 4, site-specific recombinase.
[e]Product size varied depending on the number of repeats in the *RD2* region of the *prtF1* gene.[18,19]

the *mef*(A) element they described in phylogenetically diverse clones of *S. pyogenes*. Given the high potential of phage transfer for the dissemination of virulence and resistance factors in streptococcal populations,[9,10] we compared different *mef*(A) elements of *S. pyogenes* for their association with prophages.

Materials and methods

Bacterial strains

Eleven *S. pyogenes* isolates with efflux-mediated erythromycin resistance (MIC range, 4–16 mg/L), isolated in different areas of Italy during 1997–2003, were used. Genotypically, all carried *mef*(A) as the only erythromycin resistance gene, whereas phenotypically they all shared the M phenotype, as demonstrated by their patterns of susceptibility to macrolide–lincosamide–streptogramin B antibiotics and the triple-disc (erythromycin, clindamycin and josamycin) test.[11] The 11 test strains were selected from a large collection of *S. pyogenes* clinical isolates with efflux-mediated erythromycin resistance, in that they represented a variety of resistance-related ORF patterns determined by PCR experiments.[8]

Susceptibility tests

Tetracycline was purchased from Sigma Chemical Co., St Louis, MO, USA. Broth microdilution MICs were determined using the NCCLS protocol and breakpoints.[12] *S. pneumoniae* ATCC 49619 was used for quality control.

PCR experiments and gene detection

Primer pairs used in PCR experiments are listed in Table 1. A scheme illustrating the location of the relevant amplicons is reported in Figure 1. The Ex Taq system (TaKaRa Bio, Shiga, Japan) was used when PCR products exceeding 3 kb in size were expected.

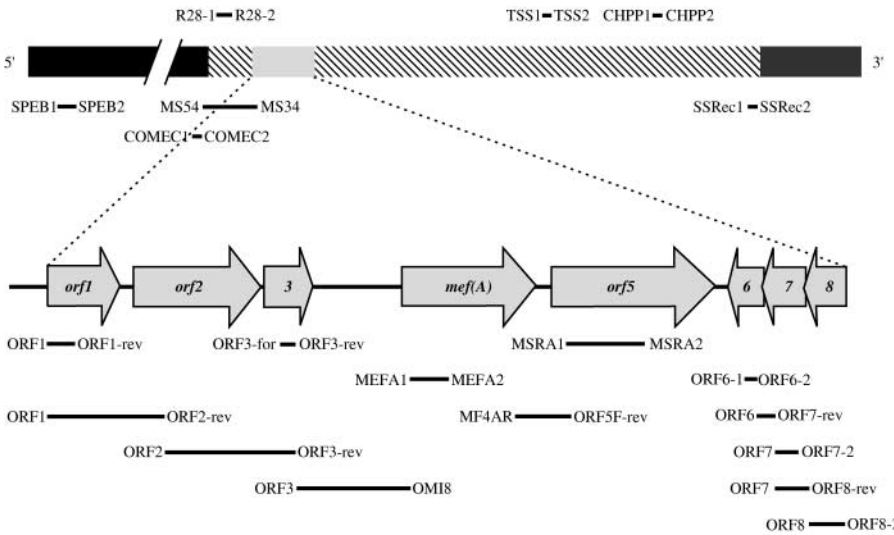

Figure 1. Schematic representation (not drawn to scale) of the primer pairs and PCR products listed in Table 1 (ORF6-for/ORF6-rev, TETO1/TETO2 and PRTF1-1/PRTF1-2 are not shown). The *mef*(A) element represents the 58.8 kb element.[6] Black bars, chromosome; cross-hatched bar, prophage-like region; light grey bar, Tn*1207.1*.

Typing assays

*Sma*I PFGE patterns were determined and analysed, as recently described in a survey of Italian erythromycin-resistant *S. pyogenes* isolates.[20] According to the scheme adopted in that study, the PFGE type was designated with a capital letter followed by an Arabic numeral for the subtype.[20] Random amplified polymorphic DNA (RAPD) analysis was performed by established methods[21] using primers M13 and H2.[22] The RAPD type was designated with an Arabic numeral preceded by m (M13 primer) or h (H2 primer). *emm* types were determined using a recently described method directed at amplifying the *N*-terminal region of the *emm* gene.[23] The internalization-related gene *prtF1* was detected by PCR using primers complementary to the flanking region of *RD2* as described elsewhere.[24] *prtF1*-positive isolates were further investigated by the *RD2* typing method, recently developed in our laboratory by combining PCR analysis of the *RD2* region and restriction analysis of PCR products using endonucleases *Hae*III, *Dde*I and *Hin*fI (New England Biolabs, Beverly, MA, USA).[19] The *RD2* type was designated with a lowercase letter, following the original scheme.[19] Multilocus sequence typing (MLST) was carried out following the procedure and using the seven housekeeping loci described by Enright *et al.*:[25] glucose kinase (*gki*), glutamine transporter protein (*gtr*), glutamate racemase (*murI*), DNA mismatch repair protein (*mutS*), transketolase (*recP*), xanthine phosphoribosyl transferase (*xpt*) and acetyl coenzyme A acetyltransferase (*yqiL*). The alleles of the seven housekeeping loci were obtained from the website http://www.mlst.net.

Induction of mef(A) elements with mitomycin C and their characterization in culture supernatants

The *mef*(A) elements of the test strains were induced with mitomycin C and phage DNA was extracted and purified as reported by Banks *et al.*[6] Induction was monitored by PCR using a primer pair targeting *mef*(A). Two chromosomal genes, *speB* and *comEC*, were monitored as negative controls to confirm that there was no contaminating chromosomal DNA in the DNA phage preparations.

Results

Strain typing

Typing data are summarized in Table 2. Of the 11 isolates, five were PFGE-untypeable, i.e. their DNA was not digested by *Sma*I. The remaining six isolates all belonged to different PFGE subtypes. RAPD analysis showed that the 11 isolates belonged to six types using primer M13 and that each constituted a distinct type using primer H2. *emm*4 was found in four isolates and *emm*2 and *emm*12 in two isolates each, whereas different *emm* types were determined for each of the remaining three isolates. Of the eight isolates found to carry the *prtF1* gene, two belonged to *RD2* type a, two to *RD2* type j and one each to *RD2* types e.g. i and k. Following the determination of the sequences of the seven housekeeping loci used for MLST, four pairs of strains with identical allelic profiles, thus assigned to identical sequence types, were recognized. One pair (due to a new *murI* allelic variant) and one of the three isolates with an individual profile (due to a new *xpt* allelic variant) yielded sequence types not previously identified.[25]

Tetracycline susceptibility and PCR detection of the ORFs of Tn1207.1

Of the 11 isolates tested, six—including all five *Sma*I-PFGE-untypeable isolates—were tetracycline-susceptible (MIC range, 0.125–1 mg/L), whereas five were tetracycline-resistant (MIC range, 32–64 mg/L) (Table 3) and carried the *tet*(O) gene. All six tetracycline-susceptible isolates had PCR evidence of all of the eight ORFs of Tn*1207.1*, whereas the five tetracycline-resistant isolates all carried *mef*(A) and *orf5*, but had no PCR evidence of *orf1* and *orf2* and showed variable patterns as to *orf3*, *orf7* and *orf8* (Table 3). Using the same pair of primers (MS54 and MS34) targeting the junctions between the left ends of Tn*1207.3*[5] or the 58.8 kb element[6] and the *S. pyogenes* chromosome, a PCR

Table 2. Typing of the 11 *S. pyogenes* strains tested

							MLST							
			RAPD type					assigned no. for allele						
Isolate	Year of isolation	PFGE type	M13	H2	*emm* type	*RD2* type	ST[a]	*gki*	*gtr*	*murI*	*mutS*	*recP*	*xpt*	*yqiL*
1	1997	ut[b]	m2	h2	12	g	36	5	2	2	6	6	2	2
2	1997	ut	m4	h5	6	j	374	5	2	66[c]	5	5	4	3
3	1998	C1	m1	h8	2	–[d]	55	11	9	1	9	2	3	4
4	1998	ut	m2	h3	12	k	36	5	2	2	6	6	2	2
5	1998	B2	m4	h6	4	j	374	5	2	66[c]	5	5	4	3
6	1998	B0	m5	h7	4	a	39	5	11	8	5	15	2	1
7	1999	G8	m4	h11	48	i	161	16	41	2	29	2	25	7
8	2001	ut	m3	h4	75	e	49	11	2	1	3	12	3	7
9	2001	ut	m1	h1	4	–	39	5	11	8	5	15	2	1
10	2002	C8	m6	h9	2	–	55	11	9	1	9	2	3	4
11	2003	B8	m6	h10	4	a	373	5	11	8	5	15	67[c]	1

[a]ST, sequence type.
[b]ut, untypeable.
[c]Not previously identified.
[d]–, *prtF1*-negative.

Table 3. Susceptibility to tetracycline and PCR evidence of specific sequences in the 11 *S. pyogenes* strains tested

Isolate	Tetracycline MIC (mg/L)	ORFs of Tn*1207.1*								Size (bp) of the amplicon obtained with primer pair MS54-MS34	ORFs of the 58.8 kb *mef*(A) element[a]			
		orf1	*orf2*	*orf3*	*mef*(A)	*orf5*	*orf6*	*orf7*	*orf8*		1	2	3	4
1	0.25	+	+	+	+	+	+	+	+	453	−	+	+	+
2	0.5	+	+	+	+	+	+	+	+	6807	+	+	+	+
3	32	−	−	+	+	+	+	+	−		−	−	−	−
4	0.25	+	+	+	+	+	+	+	+	453	−	+	+	+
5	1	+	+	+	+	+	+	+	+	6807	+	+	+	+
6	64	−	−	+	+	+	+	+	+		−	−	−	−
7	64	−	−	−	+	+	+	+	+		−	−	−	−
8	0.25	+	+	+	+	+	+	+	+	453	−	+	+	+
9	0.125	+	+	+	+	+	+	+	+	453	−	+	+	+
10	64	−	−	−	+	+	+	+	−		−	−	−	−
11	64	−	−	−	+	+	+	−	−		−	−	−	−

[a]Specific primer pairs designed from the deposited sequence (accession no. AY445042) were used to target four ORFs of the 58.8 kb *mef*(A) element[6] encoding the following products: 1, R28-like protein; 2, terminase small subunit; 3, conserved hypothetical phage protein; 4, site-specific recombinase.

product of the size (453 bp) expected for the Tn*1207.3* element was obtained from four of the six tetracycline-susceptible isolates, whereas a larger amplicon, of the size (6807 bp) expected for the 58.8 kb element, was obtained from the remaining two isolates (Table 3).

PCR detection of four phage-specific ORFs of the 58.8 kb mef(A) chimeric element

Using specific primers, the presence of four ORFs of the 58.8 kb *mef*(A) chimeric element[6]—one (encoding the R28-like protein) in the variable region upstream of Tn*1207.1* and three placed along the conserved region downstream of Tn*1207.1*—was investigated by PCR (Table 3). All six tetracycline-susceptible isolates yielded positive amplification reactions with the three primer pairs targeting the conserved region, whereas a positive amplification reaction with the primers targeting the R28-like protein was only obtained from the two isolates that yielded the larger (6807 bp) amplicon with primer pair MS54-MS34. All five tetracycline-resistant isolates yielded negative amplification reactions with the primer pairs targeting the four fragments of the 58.8 kb chimeric element.

Mitomycin C induction experiments

Induction assays by exposure of strains to mitomycin C demonstrated that the *mef*(A) elements of all strains tested—those of the tetracycline-susceptible isolates as well as the *tet*(O)-*mef*(A) elements of the tetracycline-resistant isolates—were present in culture supernatants in a DNAse-resistant form, such as a phage capsid. The results obtained with a tetracycline-resistant isolate are shown in Figure 2. No PCR fragments representing the amplification of chromosomal genes were observed after treatment with mitomycin C. In tetracycline-resistant strains, induction was monitored and confirmed by using primer pairs targeting *tet*(O) in addition to *mef*(A).

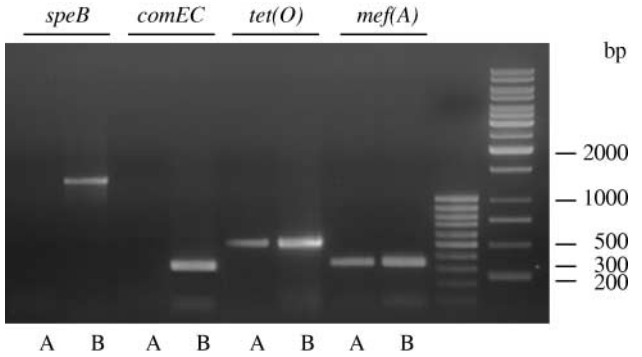

Figure 2. Agarose gel showing PCR products generated with DNA purified from the culture supernatant (lanes A) or purified chromosomal DNA (lanes B) of strain 6. The genes that were amplified are indicated above the lanes. GeneRuler 100 bp DNA Ladder (lane 1) and GeneRuler 1 kb DNA Ladder (lane 2) (both from Fermentas Life Sciences, Hanover, MD, USA) were used as molecular size markers.

Discussion

All the 11 *S. pyogenes* isolates tested were distinct strains, as demonstrated by their unique combinations of typing traits. In particular, all showed different RAPD types using primer H2. The finding that the DNAs not digested by *Sma*I were from five of the six tetracycline-susceptible isolates is in agreement with previous findings indicating that, among the *S. pyogenes* isolates with the M phenotype of macrolide resistance, most *Sma*I-PFGE-typeable cultures are tetracycline-resistant, whereas most untypeable cultures are tetracycline-susceptible.[20] In MLST assays, three isolates yielded sequence types not previously identified owing to new mutations in the *murI* allele (two isolates) or in the *xpt* allele (one isolate).

PCR assays suggested that the large fragments (over 45 kb) located to the right of Tn*1207.1* in both the Tn*1207.3* element[5] and the 58.8 kb element[6] are likely to overlap. Conversely, there

was no evidence of such a large fragment in the *tet*(O)-*mef*(A) element. In the tetracycline-susceptible strains, given that the left-hand region located upstream of Tn*1207.1* in the 58.8 kb *mef*(A) element has been reported to be *ca.* 6 kb long (i.e. approximately the difference between this element and Tn*1207.3*) and to be variable in size,[6] Tn*1207.3* could represent one extreme, completely lacking this region, of the variability range.

Most interestingly, our results not only confirmed the recent finding of a chimeric nature—i.e. a *mef*(A)-carrying transposon inserted into a prophage—of the 58.8 kb element,[6] but also showed that the same is true of both the related Tn*1207.3* element[5] and the newly discovered mobile element responsible for linked tetracycline resistance and efflux-mediated erythromycin resistance in *S. pyogenes*.[7] In other words, all recognized *mef*(A) elements of *S. pyogenes* appear to be prophage-associated. However, whereas the two elements detected in tetracycline-susceptible isolates (Tn*1207.3* and the 58.8 kb one) were apparently inserted into the same prophage, the *tet*(O)-*mef*(A) element was inserted into a different prophage.

It is well established that, in *S. pyogenes*, phages or phage-like elements are substantial contributors to bacterial virulence, genome diversification, and strain evolution and emergence.[10] In the 1970s, several experimental studies provided evidence that prophages might have participated in the dissemination of erythromycin resistance among *S. pyogenes* isolates.[9,10] The recent hypothesis that the resistance determinant involved in those transduction experiments was *mef*(A)[10] appears far sounder now that specific prophage-associated *mef*(A) elements have been identified. Although the molecular mechanisms mediating the horizontal transfer of *mef*(A) have not yet been clearly defined, several lines of evidence suggest that transduction can play a role.[6] The hypothesis has been put forward that an erythromycin-susceptible precursor strain containing a prophage, integrated into the chromosome, might have acquired the Tn*1207.1* transposon (from *S. pneumoniae* or from other streptococci present in the upper respiratory tract).[6] This event would have led to chimeric structures like Tn*1207.3* and the 58.8 kb element. Extension of this hypothesis, with a different prophage, to the origin of the *tet*(O)-*mef*(A) element would involve a more complex series of events, accounting for stepwise acquisition of the two resistance genes and the variability of the Tn*1207.1*-like transposon. Also in the case of *tet*(O), the source could be streptococci present in the upper respiratory tract, considering its >99% homology with the *tet*(O) genes of *S. pneumoniae* and *Streptococcus mutans*[8] (sequence deposited in EMBL-Bank under accession no. AJ715499). Both in the case of *mef*(A), primarily carried by a non-conjugative transposon such as Tn*1207.1*,[4] and in the case of *tet*(O), believed to be unable to move from one chromosome to another until the discovery of the *tet*(O)-*mef*(A) element,[7] a non-transferable resistance determinant of *S. pneumoniae* (or of other streptococci from the upper respiratory tract) could have become easily transferable once transfer to *S. pyogenes* and integration into the prophage had successfully occurred.

Phage transfer is likely to play a critical role in the dissemination of *mef*(A)-mediated erythromycin resistance in *S. pyogenes* populations and in the emergence of active efflux as the most widespread mechanism of macrolide resistance in this species.

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