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Lysogenic Transfer of *mef*(A) and *tet*(O) Genes Carried by Φm46.1 among Group A Streptococci[▽]

Maria Chiara Di Luca,[1] Stefania D'Ercole,[1] Dezemona Petrelli,[2] Manuela Prenna,[2] Sandro Ripa,[1] and Luca A. Vitali[1]*

School of Pharmacy, Microbiology Unit, University of Camerino, Camerino, Italy,[1] and School of Biosciences and Biotechnology, University of Camerino, Camerino, Italy[2]

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We report the ex vivo lysogenic transfer of erythromycin and tetracycline resistance genes among group A streptococci (GAS). Of 42 susceptible strains, 69% acquired erythromycin/tetracycline resistance when infected with purified supernatants from strain m46 culture containing the phage Φm46.1. A significant *emm*-type-dependent barrier to lysogenic transfer was not observed. The *emm*12 strains were the only strains susceptible to the lytic action of the bacteriophage preparation.

In *Streptococcus pyogenes* (a group A streptococcus [GAS]), the analysis of available genome sequences has revealed a surprising number of prophages (1). Owing to their extreme flexibility, prophages can incorporate important virulence and resistance genes into their own genomes. The acquisition of genes that confer efflux-mediated macrolide resistance in GAS is a typical example. This resistance is due to a H^+-driven protein pump and an ATP-binding protein [encoded by the *mef*(A) and *msr*(D) genes, respectively] and results in low-level resistance to 14- and 15-membered macrolides, with MICs ranging from 4 to 16 mg/liter. This resistance is known as "M phenotype resistance" (17).

M phenotype resistance has been extensively investigated in several countries (9, 15) and shows a mean prevalence of 30 to 40% across GAS macrolide-resistant isolates (14). The genes conferring efflux-mediated macrolide resistance in GAS are harbored in chimeric genetic elements that result from the insertion of a transposable element within the genome of a carrier prophage (19). In this context, the recently fully characterized phage Φm46.1 represents one of the most common variants (2). Additionally, Φm46.1 carries the *tet*(O) gene, which confers tetracycline resistance mediated by ribosomal protection. The horizontal transfer of these genes has been shown to occur by conjugative transposition (7), whereas phage-mediated transfer has not yet been conclusively demonstrated. Moreover, transduction-mediated transfer of resistance to various antibiotics (including erythromycin) has previously been observed in general virology experiments, but neither the genes nor the phages involved were characterized (10, 12, 18).

In this study, we sought to demonstrate that the transfer of erythromycin and tetracycline resistance to a set of susceptible clinical GAS strains can be sustained by the GAS phage Φm46.1.

In accordance with previous reports (2, 8), mitomycin C treatment of GAS strain m46 resulted in the release of mature phage particles into the culture supernatant. The m46 strain was grown overnight at 37°C in brain heart infusion (BHI) medium (Oxoid, Ltd., Basingstoke, United Kingdom), diluted with BHI supplemented with 5% horse serum (Oxoid), and grown to an optical density at 600 nm (OD_{600}) of 0.2. The inducer mitomycin C (Sigma Chemical Co., St. Louis, MO) was added to the culture to give a final concentration of 0.2 μg/ml. After 3 h at 37°C, the cell suspension was incubated with 0.1 μg/ml DNase and RNase (Sigma) for 1 h and centrifuged at 5,500 × *g* for 15 min and the supernatant filtered through a 0.22-μm-pore-size membrane (Millipore Corporation, Bedford, MA). Possible bacterial contamination of the filtrate was excluded by inoculating a part of the supernatant in liquid medium. The presence of a DNase-resistant Φm46.1 genome was confirmed by PCR detection of *mef*(A) and *tet*(O), while a nonamplification of the *gyrA* gene was an indication of the absence of contaminating chromosomal DNA.

Table 1 lists the primer pairs used to amplify the *mef*(A), *tet*(O), and *gyrA* genes, while the PCR conditions were from references 16, 7, and 13, respectively. Mitomycin C-uninduced m46 cells did not release any PCR-detectable amount of Φm46.1.

Lysogenization experiments were performed to study whether the phage released from donor strain m46 can convert *mef*(A)-*tet*(O)-negative strains into *mef*(A)-*tet*(O)-positive strains. The indicator strain K56 (ATCC 14919; *emm* type 12) (11) and 41 erythromycin-susceptible clinical GAS isolates belonging to different *emm* types, including some of the more prevalent GAS isolates in Italy (23), were used as recipients (Table 2). Supernatants of mitomycin C-treated m46 cultures were mixed with recipient cultures grown to an OD_{600} of 0.4 to 0.5 and further incubated for 15 min at 37°C with agitation. Aliquots of the mixture were then plated on BHI agar containing erythromycin (4 mg/liter; Sigma) and incubated overnight at 37°C. As shown in Table 2, lysogenic clones were successfully selected and their erythromycin- and tetracycline-resistant phenotype was subsequently verified by a disc (Oxoid) diffusion susceptibility test in accordance with standard procedures (4). A mean number of three erythromycin-positive ly-

* Corresponding author. Mailing address: School of Pharmacy, Microbiology Unit, University of Camerino, I-62032 Camerino (MC), Italy. Phone: 39-0737-403282. Fax: 39-0737-403281. E-mail: luca.vitali@unicam.it.

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TABLE 1. Oligonucleotide primer pairs used in this work

Designation	Sequence (5′–3′)	Source or reference
VUF	AAACCGTATCTTTGACGCACTCGAGG ACACAATTTGCGAGATTAG	This study
MEFA1	AGTATCATTAATCACTAGTGC	16
MEFA2	TTCTTCTGGTACTAAAAGTGG	16
TETO1	AACTTAGGCATTCTGGCTCAC	7
TETO2	TCCCACTGTTCCATATCGTCA	7
gyrA1	GAAGTGATCCCTGGACCTGA	13
gyrA2	CCCGACCTGTTTGAGTTGTT	13
ins-f	CTTTTCCAGCAGTGGTGTCT	This study
ins-r	GCGCAAAACTAAAGGAATCA	This study
MEF-r	GCAATCACAGCACCCAATAC	This study

sogens were selected, and their genomic DNA was analyzed by PCR to determine the presence of the *mef*(A) and *tet*(O) genes. The presence of the specific *emm* type gene prior to and after the lysogenization experiments was determined by PCR M typing using reverse primers described by Zampaloni et al. (23) and the universal forward primer VUF (Table 1). The chromosomal DNA from the phage donor strain m46 and the purified phage DNA from mitomycin C-induced m46 cells were used as positive controls for the amplification reactions, while the chromosomal DNA from the recipient strains and the indicator strain K56 were used prior to lysogenization as negative controls. The chromosomal insertion site within the lysogens was also investigated. The primers ins-f and ins-r amplified the Φm46.1 attachment site prior to insertion, while ins-f and MEF-r amplified the left junction and TETO1 and ins-r amplified the right junction after insertion (Table 1). All lysogens had Φm46.1 inserted into the 23S rRNA uracil methyltransferase gene as it is in the donor strain (2).

In this study, 28 strains, belonging to nine different *emm* types, were successfully lysogenized (Table 2). In addition to strain K56, all of the representatives of *emm*1, *emm*12, and *emm*75 received Φm46.1, as did the single isolates representing the *emm* 18 and *emm*94/13W types. In the remaining strains, we observed a mixed response, with lysogenization percentages ranging from 71% for *emm*3 to 33% for *emm*4. Finally, no lysogen was isolated when *emm*77, *emm*78, and *emm*89 were used as recipients.

The study of the effect of GAS *emm* type on lysogenic capability has previously been addressed, and it was found that the M protein is not a key factor in the primary recognition and attachment of lytic phages to the GAS cell surface (6). The same conclusion has been drawn for the temperate phage Φ149 carrying the *ssa* gene (21). Also in the case of Φm46.1, its ability to lysogenize seems to be not influenced by the strain *emm* type. The confirmed low M-type specificity might be one explanation for the wide diffusion of Φm46.1 in GAS strains showing various *emm* types (20). An exception is represented by the strains belonging to *emm*77, *emm*78, and *emm*89. However, in view of the limited number of strains representative of these *emm* types, a general conclusion cannot be drawn.

Giovanetti et al. previously reported that Φm46.1 [formerly known as the *tet*(O)-*mef*(A) element] was able to transfer *mef*(A) and *tet*(O) to a GAS recipient strain by conjugation (7). However, the determined frequency of transfer was considerably high (10^{-4}) compared to the mean values that are usually

TABLE 2. Susceptibility of the strains under study to lysogenic transfer and lytic action by phage preparation

emm type	No. of strains		
	Total	Lysogenized	Positive by plaque assay
1	7	7	0
3	7	5	0
4	3	1	0
5	2	1	0
6	7	4	0
12	6	6	6
18	1	1	0
75	2	2	0
77	1	0	0
78	2	0	0
89	2	0	0
94	1	1	0

measured for conjugation. In a subsequent work, the authors also hypothesized that a phage-based transfer of the *mef*(A) and *tet*(O) genes could be mediated by the same chimeric element (8). Their deduction was based on the detection of phage DNA in the culture supernatant of mitomycin C-induced cells. The high conjugation frequency reported recently (7) is consistent with the low frequency of lysogenic transfer (10^{-3} to 10^{-2}), which we measured as the ratio of the number of lysogens to the number of recipient cells. The low frequency of lysogenic transfer is more likely the consequence of a very low infecting phage titer, which is a common feature of temperate GAS bacteriophages (21, 22).

In light of our work, lysogenic transfer can finally be considered a mechanism of *mef*(A) and *tet*(O) transfer. This might represent at least one of the reasons for the wide dissemination of GAS isolates positive for Φm46.1 and therefore resistant to erythromycin and tetracycline (3, 5).

Finally, we evaluated the ability of the released phage particles to sustain a lytic cycle. GAS recipient strains were grown to an OD_{600} of 1.0 and mixed with the filtrate of mitomycin C-treated phage donor culture. The mixtures were incubated for 15 min at 37°C with agitation, added to molten top agar (BHI plus 0.4% agar) with 5% horse serum, and then poured over BHI agar plates and incubated overnight at 37°C. Lysis was confirmed by the presence of visible plaques. The strain K56 was used as the control indicator recipient (11).

The obtained plaques were turbid, and the bacteriophage titer was estimated to be approximately 10^3 PFU/ml. Visible plaques were obtained only for *emm*12 clinical isolates (Table 2). The other 11 *emm* types did not produce plaques, at least under the experimental conditions used, indicating poor efficiency in the lytic cycle.

Subsequently, we attempted to verify if the observed lytic properties of the prepared supernatants were due to Φm46.1. Lysogenized *emm*12 strains were treated with mitomycin C. Each filtered supernatant was confirmed as *mef*(A)-*tet*(O) positive and used to infect the strain K56 in the soft agar plaque assay. Plaques were not detected under these experimental conditions, suggesting that the supernatant from mitomycin C-treated m46 cultured cells probably contains at least one additional phage whose lytic capabilities are higher than those of Φm46.1.

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