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Correlation between genetic features of the *mef*(A)-*msr*(D) locus and erythromycin resistance in *Streptococcus pyogenes*

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ABSTRACT

We investigated the correlation between the genetic variation within *mef*(A)-*msr*(D) determinants of efflux-mediated erythromycin resistance in *Streptococcus pyogenes* and the level of erythromycin resistance. Twenty-eight *mef*(A)-positive strains were selected according to erythromycin MIC (4–32 μg/mL), and their *mef*(A)-*msr*(D) regions were sequenced. Strains were classified according to the bacteriophage carrying *mef*(A)-*msr*(D). A new Φm46.1 genetic variant was found in 8 strains out of 28 and named VP_00501.1. Degree of allelic variation was higher in *mef*(A) than in *msr*(D). Hotspots for recombination were mapped within the locus that could have shaped the apparent mosaic structure of the region. There was a general correlation between *mef*(A)-*msr*(D) sequence and erythromycin resistance level. However, lysogenic conversion of susceptible strains by *mef*(A)-*msr*(D)–carrying Φm46.1 indicated that key determinants may not all reside within the *mef*(A)-*msr*(D) locus and that horizontal gene transfer could contribute to changes in the level of antibiotic resistance in *S. pyogenes*.

1. Introduction

Macrolide resistance (MR) in *Streptococcus pyogenes* (group A *Streptococcus* [GAS]) is a global phenomenon with significant implication for the treatment of streptococcal infections when beta-lactams cannot be used. The high prescription and consumption of macrolides and the dissemination of MR clones have contributed to the high prevalence of resistance in several countries (Huang et al., 2014; Silva-Costa et al., 2006; Syrogiannopoulos et al., 2013; Wajima et al., 2014). Resistance to macrolides in GAS occurs by 2 main mechanisms, namely, the enzymatic modification of the target site onto the ribosome mediated by the product of the *erm* genes (Weisblum, 1995) and the active efflux of the drug by protein pumps encoded by the *mef*(A)-*msr*(D) system (Sutcliffe et al., 1996), which confers resistance to 14- and 15-membered macrolides. *mef*(A) and *msr*(D) are arranged in tandem and carried by mobile genetic elements. In GAS, 3 of these elements have been discovered: Tn1207.3 (Santagati et al., 2003), Φ10394.4 (Banks et al., 2003), and Φm46.1 (Brenciani et al., 2010). In all the cases, *mef*(A) and *msr*(D) are transferred together, either by conjugative transposition or by phage-mediated transfer (Di Luca et al., 2010; Santagati et al., 2003).

The phenotype of the *mef*(A)-*msr*(D)–mediated macrolide resistance is referred to as M phenotype and is associated with erythromycin MIC_{90} values of 8–16 μg/mL. Some studies on *Streptococcus pneumoniae* support the hypothesis that this resistance is able to negatively affect antibacterial therapy outcome (Lonks et al., 2002). Previous works have almost entirely focused on *mef*(A) only. Actually, both *mef*(A) and *msr*(D) play a key role in determining macrolide resistance in streptococci (Ambrose et al., 2005). Given that *mef*(A)-*msr*(D)–mediated resistance level is not uniform in GAS, with MIC ranging from 4–8 μg/mL to 32 μg/mL, some questions are still open: i) Which are the structural genetic bases of the observed phenotypic differences? ii) Which is the contribution, if any, of the carrying genetic element in the evolution of resistance? iii) Is the level of MR resistance strain dependent? To try to answer these questions, we investigated erythromycin-resistant isolates expressing the M phenotype due to the efflux pump MefA showing MICs for erythromycin ranging from 4 to 32 μg/mL. We studied the genetics of *mef*(A)-*msr*(D) locus aiming at understanding the bases of differences in erythromycin susceptibility and to eventually correlate them to sequence variations, carrying genetic elements, or strain specific genetic background.

2. Material and methods

2.1. Bacterial strains and antimicrobial susceptibility testing

S. pyogenes strains showing the M phenotype resistance toward macrolides were selected from our laboratory collection (Seppälä et al., 1993). They were originally isolated from symptomatic patients suffering from pharyngotonsillitis and previously characterized by different molecular methods such as *emm* typing and Pulsed Field Gel

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Electrophoresis (Vitali et al., 2002; Zampaloni et al., 2003). The latter information was used to select a subset of strains so as to avoid the inclusion of clonally related strains. A third-level selection was then done after the determination of the erythromycin MICs, which were assessed by the microdilution method following the guidelines of the CLSI (CLSI, 2011). Finally, 28 strains were considered for the analysis. At a second stage and to study the phenotype of erythromycin resistance upon lysogenic conversion by phage Φm46.1, additional 27 *S. pyogenes* strains previously generated in our laboratory were used (Di Luca et al., 2010). MIC determinations were repeated 3 times, in triplicate for each test, to have a confident value for each strain. PCRs specific for *erm*(B) and *erm*(A) were negative in all strains (Zampaloni et al., 2003).

2.2. Amplification and sequencing of the mef(A)-msr(D) locus

Oligonucleotide primers design was done with reference to the sequence of the Φm46.1 phage (acc. nr. FM864213). Their sequences are listed in Table S1 (Supplementary material). Four primers were designed to amplify the *mef*(A) and *msr*(D) coding (RAF1-mefA1F, RAF3-mefA3R, RAF4-mefA4F, RAF2-mefA2R) and the intergenic noncoding region. The pair up_CAM1 and up_mefA_R was used to amplify and sequence the *mef*(A)-*msr*(D) upstream region up to the insertion site of the Φm46.1-related genetic element into the chromosome. The 10500 series of primers has been designed after chromosome walking into the sequence of the newly recognized variant 5′-region found in a subset of strains (Table 1).

2.3. Phylogenetic reconstruction, tests for recombination, and statistical analysis

Phylogenetic, molecular evolutionary analyses, and tree construction were conducted using MEGA version 5 (Tamura et al., 2011). The evolutionary history was inferred using the maximum likelihood method based on the Tamura 3-parameter model for *mef*(A) alone and the entire locus and on the Hasegawa–Kishino–Yano model for *msr*(D) alone (Hasegawa et al., 1985). The maximum chi-square test was used to confirm suspected recombination events between pairs of *mef*(A)-*msr*(D) locus from different strains as it compares the distribution of polymorphic sites along sequences with those expected to occur by chance (Smith, 1999). The test was performed using the START package (Jolley et al., 2001), which is available at http://pubmlst.org/software/analysis/start/ (last accessed on 15 June 2015). Standard statistical analyses were performed using the software Statgraphics Centurion XV. Group to group comparisons were performed by the Kruskal–Wallis test for the medians and by chi-square test for the means. Significance threshold was set at $P = 0.05$ in all cases.

Table 1
List of strains used in this work with their susceptibility to erythromycin expressed as MIC and the PCR analysis of the mef(A)-msr(D) region. According to the expected size of the amplicon, the strain was associated to a known reference phage type (last column).

Strain		MIC_{ERY} (mg/L)	Approx. size of the amplicon (expected size)	Ref. phage type
	m46	16	3000 bp (3076 bp)	Φm46.1
VP_SPYO	9691	12	3000 bp (3076 bp)	Φm46.1
VP_SPYO	00211	12	3000 bp (3076 bp)	Φm46.1
VP_SPYO	12790	12	3000 bp (3076 bp)	Φm46.1
VP_SPYO	1049	12	3000 bp (3076 bp)	Φm46.1
VP_SPYO	20201	12	3000 bp (3076 bp)	Φm46.1
VP_SPYO	41201	12	3000 bp (3076 bp)	Φm46.1
VP_SPYO	82201	12	Negative	Φ10394.4 like
VP_SPYO	31311	8	Negative	Φ10394.4 like
VP_SPYO	04201	12	Negative	Φ10394.4 like
VP_SPYO	70411	16	Negative	Φ10394.4
VP_SPYO	20411	8	Negative	Φ10394.4
VP_SPYO	3179	8	Negative	Φ10394.4
VP_SPYO	34201	8	1500–2000 bp (1777 bp)	ΦMB56Spy045
VP_SPYO	84201	4	1500–2000 bp (1777 bp)	ΦMB56Spy045
VP_SPYO	11411	16	1500–2000 bp (1777 bp)	ΦMB56Spy045
VP_SPYO	40611	8	1500–2000 bp (1777 bp)	ΦMB56Spy045
VP_SPYO	11611	8	1500–2000 bp (1777 bp)	ΦMB56Spy045
VP_SPYO	40301	8	1500–2000 bp (1777 bp)	ΦMB56Spy045
VP_SPYO	11901	8	1500–2000 bp (1777 bp)	ΦMB56Spy045
VP_SPYO	6199	8	1500–2000 bp (1777 bp)	ΦMB56Spy045
VP_SPYO	0279	8	4000 bp	VP_00501.1
VP_SPYO	10201	8	4000 bp	VP_00501.1
VP_SPYO	53101	16	4000 bp	VP_00501.1
VP_SPYO	58101	32	4000 bp	VP_00501.1
VP_SPYO	00301	16	4000 bp	VP_00501.1
VP_SPYO	01301	12	4000 bp	VP_00501.1
VP_SPYO	61301	12	4000 bp	VP_00501.1
VP_SPYO	00501	8	4000 bp	VP_00501.1

3. Results and discussion

3.1. mef(A)-mediated erythromycin resistance

The results of the susceptibility testing for erythromycin are reported in Table 1. Twelve out of 29 strains (41.4%; 28 test strains plus the reference one) showed an MIC of 8 μg/mL; and 34.5% of the strains, a value of 12 μg/mL. The MIC against 4 strains (13.8%) plus the m46 reference strain was 16 μg/mL. Only strain VP_SPYO58101 showed a value above 16 μg/mL. A level below 8 μg/mL was recorded in 1 strain (VP_SPYO84201).

3.2. Genetic context of the mef(A)-msr(D) locus

A PCR screening was used to classify strains in respect to the known genetic elements carrying *mef*(A) or its homologs (Table 1). Using primers up_CAM1 and up_mefA_R (Table S1, Supplementary material), a PCR product of about 3 kb was expected if the macrolide efflux gene was carried by Φm46.1 phage type. Six strains were positive as well as the control strain m46. In 8 cases, *mef*(A) subclass was of the MB56Spy045 type (amplicon size 1.7 kb) (Blackman Northwood et al., 2009). An unexpected 4-kb fragment was obtained from 8 strains and was further investigated (see "Sequencing" section). At last, the remaining 6 strains were negative and then found positive to the methylase, and the R28-like genes carried by Φ10394.4 (Banks et al., 2003). They were assigned to the Φ10394.4 phage-type group (Table 1).

3.3. Sequencing

The *mef*(A)-*msr*(D) locus of each strain was sequenced and was composed by the coding regions of *mef*(A) and *msr*(D), the intergenic region between the 2 genes, and the 300 bp 5′ noncoding region upstream to *mef*(A). The length of the entire locus was in the range of 3112–3127 bp. The 16-bp difference is due to an indel present in the 5′ noncoding region upstream to *mef*(A) in Φ10394.4. The 4-kb amplicons from the left junction site of the element carrying *mef*(A) to the 5′ of the *mef*(A) itself obtained from 8 strains (Table 1 and "Genetic context of the *mef*(A)-*msr*(D) locus" section) were also sequenced. Comparative analysis showed a mosaic structure of the locus with contributions from both Φm46.1 and Φ10394.4-like phage-types. The new variant was called VP_00501.1 (Fig. 1). The chromosomal insertion site of the carrying element was the same as in Φm46.1 (GenBank: FM864213), i.e., a gene coding for a 23S rRNA uracil methyltransferase (*rum*A). Also, the sequence of the Φm46.1 *orf*1 was maintained, while the downstream sequence corresponding to *orf*2 was absent. The sequence continued with the *orf*6 and the 5′-end of *orf*7 carried by Φ10394.4 (alias SpyM6 *mef*A phage element, GenBank: AY445042). Downstream of the latter ORF, there was an 800 nt long sequence responsible for the difference in length between the VP_00501.1 and the other phage types (Fig. 1). XBLAST search found a match with a domain of the AlwI family of type II restriction endonucleases (59% identity, 82% similarity to *Lactobacillus delbrueckii*, GenBank: WP_003617961.1