Predominant role of msr(D) over mef(A) in macrolide resistance in Streptococcus pyogenes

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In Japan, the number of patients with streptococcal toxic shock syndrome is reported to be increasing. mef(A) gene-positive macrolide-resistant emm1 strains are thought to possibly contribute to the rise in the frequency of STSS. Although analyses of macrolide-resistant mechanisms, including mef(A) resistance, have been performed mainly in Streptococcus pneumoniae, the role of this gene in Streptococcus pyogenes has not been completely investigated. Therefore, to the best of our knowledge, we established the first mef(A)-knockout strain using an emm1-type S. pyogenes strain, and tested its susceptibility to erythromycin, clarithromycin and azithromycin. We found that the antimicrobial susceptibilities were almost identical to those of the parental strain. Hence, we established a knockout strain for another gene, msr(D), that is located immediately downstream of mef(A). The macrolide resistances of the resulting strain significantly decreased, and were further altered when both mef(A) and msr(D) were knocked out. The introduction of the msr(D) gene into a macrolide-sensitive strain conferred more resistance than the introduction of the mef(A) gene. The erythromycin susceptibilities of knockout strains were further dissected using two additional emm4- and emm75-type S. pyogenes strains. We found almost identical results for both strains except for the mef(A) knockout emm4 type, whose susceptibility was altered, although the change was less than that for the msr(D) knockout. These results suggest that both mef(A) and msr(D) are involved in macrolide resistance in S. pyogenes, and that the msr(D) gene plays a more predominant role in macrolide resistance than mef(A).

INTRODUCTION

Streptococcus pyogenes is a Gram-positive bacterium that causes mild (e.g. pharyngitis, impetigo and scarlet fever), invasive [e.g. bacteraemia/septicaemia, necrotizing fascitis, and streptococcal toxic shock syndrome (STSS)] and non-suppurative (e.g. rheumatic fever and glomerulonephritis) infections (Cunningham, 2000).

Epidemiological analyses have revealed that emm1-type S. pyogenes is the most commonly detected type in patients in high-income continents, such as Europe and North America, and countries such as Japan (Steer et al., 2009).

Moreover, in Japan, the emm1 type is the most frequently isolated type in invasive S. pyogenes infections (Ikebe et al., 2003; Wajima et al., 2008) and the number of patients with STSS has increased recently (http://www.nih.go.jp/niid/ja/all-surveillance/2085-irwr/ydata/4405-report-ja2012.html). In particular, the rise in the incidence of STSS was reported to be attributable to an increase in the number of cases associated with specific mef(A)-positive emm1 isolates (Ikebe et al., 2015).

In Japan, macrolides are widely used for the treatment of S. pyogenes infection and several macrolide-resistant strains have been isolated (Hotomi et al., 2005; Giovanetti et al., 2005; Wajima et al., 2008). One of the mechanisms of erythromycin resistance is caused by target-site modification...
by an rRNA-methylating enzyme that is encoded by erm(B) or erm(A) (Weisblum, 1995; Sutcliffe et al., 1996; Seppälä et al., 1998), which results in resistance to macrolide, lincosamide and streptogram B antibiotics. Another mechanism is the active efflux of erythromycin. The mef(A) gene was first cloned from S. pyogenes and it was suggested that it encoded a membrane-associated antipporter protein, which pumps all 14- and 15-membered macrolides including erythromycin out of the cell (Clancy et al., 1996). Since then, a tight relationship between macrolide resistance and the presence of the mef(A) (Perez-Trallero et al., 1998; Orden et al., 1998), erm(B) and erm(A) genes (Kataja et al., 1999) has been reported. However, several studies have mainly investigated Streptococcus pneumoniae and the role of the mef(A) gene in macrolide resistance in S. pyogenes has not been fully revealed. In addition, we studied the msr(D) gene because it is located immediately downstream of the mef(A) gene, and co-exists in the conjugative prophage Φ1207.3 (Iannelli et al., 2014), Φ10394.4 (Banks et al., 2003), Φm46.1 (Brenciani et al., 2010) and other related elements (Vitali et al., 2015) in S. pyogenes. Furthermore, mef(A) and msr(D) genes were reported to be co-identified in erythromycin-resistant S. pyogenes (Brenciani et al., 2004; Rubio-López et al., 2012; Iannelli et al., 2014). In this study, we established mef(A)- and msr(D)-knockout strains, and examined their roles in macrolide resistance in S. pyogenes.

**Methods**

**Isolates.** The macrolide-resistant *emm*1-type S. pyogenes strain 10-85 isolated from an STSS patient in 2010 (Okada et al., 2014) was used for the knockout experiments. The genome data of 10-85 was deposited in DDBJ/GenBank (accession number SAMD00034931). The *emm*75-type NCU107 and *emm*4-type NCU115 strains were isolated from pharyngitis patients in 2008 and also used for knockout experiments. The macrolide-resistant *erm*(B)-positive *emm*1-type 14-T-4 strain and *erm*(A)-positive *emm*1-type NCU41 strain were used for PCR analyses. The 1529 STSS strain was used as a representative macrolide-sensitive strain (Hasegawa et al., 2010b).

**Culture conditions.** Bacteria were cultured in a brain heart infusion broth (Becton Dickinson) (BHI-YE) at 37 °C without agitation. The growth of the bacteria was monitored using a colorimeter (Asahi Science) at OD660.

**Assessment of growth of bacteria exposed to erythromycin.** An aliquot of 10-85 and its mef(A)-knockout strain stored solution stored frozen at −80 °C was inoculated into 3 ml BHI-YE and pre-cultured overnight at 30 °C without agitation. Then, 36 μl overnight culture was added to 3.6 ml BHI-YE with 1 mg erythromycin 1 °C and cultured at 37 °C without agitation. The turbidity of the strains was monitored at OD660 at the indicated time points (2, 3, 4, 5, 6, 7 and 24 h after starting the culture).

**Detection of macrolide resistance-related genes.** The mef(A), erm(B), erm(A) and msr(D) genes were detected by PCR using Ex Taq DNA polymerase (TaKaRa). The primers used (mef-n1/c1, msr-n4/c4, ermB-F/R and ermA-F/R) (Sutcliffe et al., 1996; Seppälä et al., 1998) are described in Table 1. PCR was performed with the following cycle profiles: 30 cycles annealing at 41 °C [mef(A), erm(B)], 36 °C [erm(A)] or 53 °C [msr(D)] and extension at 72 °C for 40 [mef(A), erm(B)], 30 [erm(A)] or 45 s [msr(D)].

**Drug susceptibility experiment.** The MICs of erythromycin, azithromycin and clarithromycin were determined using the Etest method. The Etest was performed in accordance with the manufacturer’s instructions (SYSMEX bioMérieux). In brief, the inoculum concentration was adjusted to 0.5 in the McFarland standard for the WT and mutant types. The diluted samples were inoculated onto Mueller–Hinton agar with 5 % sheep blood by swabbing over the entire surface in three directions. In the case of the analyses of complemented strains, the MICs were analysed using BHI-YE agar plates, without sheep blood, containing 100 mg kanamycin l−1 to keep the transformed plasmid. After a period of 15 min, three kinds of macrolide antibiotic Etest strips (erythromycin, azithromycin and clarithromycin) were applied. The plates were incubated at 35 °C in air containing 5 % CO2 and read after 24 h. The MICs and minimum concentrations in the complementation experiments of all strains were read where the ellipse of growth inhibition was intersected by the strip. The independent Etest experiment was repeated three times. The significances were determined using Student’s t-test.

**Establishment of knockout and complemented strains.** Non-polar inactivated mutants of the mef(A) and/or msr(D) gene were constructed through double-crossover allelic replacement in the chromosome of S. pyogenes *emm*1-type 10-85, *emm*75-type NCU107 and *emm*4-type NCU115. For the construction of the plasmid for the mef(A) knockout, the DNA fragment of mef(A) was amplified with oligonucleotide primers mef-n9/SmaI and mef-c3 (Table 1, Fig. 1) (fragment 1). The Smal/DraI-digested fragment 1 was subcloned into the Smal site of the pFW12 vector (Lukomski et al., 2000) (named pTH643). A second round of PCR was performed using mef-n8/NheI and mef-c9 (Table 1, Fig. 1) (fragment 2). Both the NheI-digested fragment 2 and the spc1 DNA fragment containing aadB (promoterless spectinomycin resistance gene) obtained from a Smal-digested fragment of pSL60-1 (Lukomski et al., 2000) were cloned into the NheI/Smal site of pTH643 (named pTH647). For the construction of the plasmid for the msr(D) knockout, the DNA fragment of msr(D) was amplified with oligonucleotide primers msr-n2/SmaI and msr-c2 (Table 1, Fig. 1) (fragment 3). The Smal-digested fragment 3 was subcloned into the Smal site of the pFW12 vector (named pTH676). The final round of PCR was performed using msr-n1/NheI and msr-c1 (Table 1, Fig. 1) (fragment 4). Both the NheI-digested fragment 4 and the spc3 DNA fragment containing aadB obtained from a Smal-digested fragment of pSL60-3 (Lukomski et al., 2000) were cloned into the NheI/Smal site of pTH676 (named pTH677). For the construction of the plasmid for both the mef(A) and msr(D) double knockout, both the NheI-digested fragment 2 and the spc3 DNA fragment were cloned into the NheI/Smal site of pTH676 (named pTH678). These plasmids, pTH647, pTH676 and pTH678, were suicide vectors for S. pyogenes.

To construct a plasmid for mef(A) complementation, the DNA fragment of mef(A) was amplified with oligonucleotide primers mef-n2 and mef-c3 with PrimeSTAR HS DNA Polymerase (TaKaRa). To construct a plasmid for msr(D) complementation, the DNA fragment of msr(D) was amplified with oligonucleotide primers msr-n1/NheI and msr-c2 with PrimeSTAR HS DNA Polymerase. The fragments were treated with T4 polynucleotide kinase and then inserted into the Smal site of the pLZ12-Km2 plasmid (Okada et al., 1998) with ligase. The method to prepare competent cells and the electroporation conditions were identical to those published previously (Hasegawa et al., 2010a; Ichikawa et al., 2011).
RESULTS

Establishment of the \textit{mef}(A)-knockout strain and analysis of drug susceptibility

Although many studies have described the relevance of \textit{mef}(A) in macrolide resistance in \textit{S. pyogenes}, we could not find studies in the literature using a \textit{mef}(A)-knockout \textit{S. pyogenes} strain. Hence, we attempted to establish a \textit{mef}(A)-knockout strain using the \textit{emm}1-type macrolide-resistant 10-85 strain, because it possesses a single copy of the \textit{mef}(A) gene and neither the \textit{erm}(B) nor the \textit{erm}(A) genes in the genome. We succeeded in establishing a \textit{mef}(A)-knockout strain and performed an Etest. No significant differences in MICs of erythromycin, clarithromycin and azithromycin between the parental strain and its \textit{mef}(A)-knockout strain were detected ($P > 0.5185$, 1 and 0.1161, respectively; Table 2), suggesting that other factor(s) could compensate for macrolide resistance by \textit{mef}(A). To confirm that the role of \textit{mef}(A) was fully compensated by other factor(s), we checked the growth of both the WT and its \textit{mef}(A)-knockout strain, with and without erythromycin (1 mg l$^{-1}$). As shown in Fig. 2, the growth curves of the WT and the \textit{mef}(A)-knockout strain.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Target gene} & \textbf{Primer name} & \textbf{Nucleotide sequences (5'→3')} \\
\hline
\textit{mef}(A) & mef-n1 & AGTATCATTAATCACTAGTGC \\
& mef-c1 & TTCTTCTGGTACTAAAGTGGA \\
& mef-n9Sma1 & TCCCCGGGCAGGAAAATTAAGCCCTGA \\
& mef-c3 & TCCATAACATTTCCTCGGT \\
& mef-n8NheI & GGTGACAGTGAAAGAAATCAACATTTG \\
& mef-c9 & GAGGCTCCTACGCACACCA \\
& mef-n2 & GGTGACGCCAAGTCTATACCGAGTGA \\
& msr(D) & msr-n1NheI & GGTGACGCAGAATCTATACCGAGTGA \\
& msr-n2Sma1 & TCCCCGGGCAGGAAAATTAAGCCCTGA \\
& msr-c1 & TGCCTATTGAAATTCACACT \\
& msr-c2 & AGAACCCTGAGAAGAAATCC \\
& msr-n4 & TTTGCCGGATGAACCTACG \\
& msr-c4 & TTCCGTGGTCAATTACCAAC \\
& \textit{erm}(B) & ermB-F & GAAAGGCTACCAACAAATA \\
& ermB-R & AGTAACGGGTACTTAAATTTTAC \\
& \textit{erm}(A) & ermA-F & GAAGTTAGCTTTCTAA \\
& ermA-R & GCTTCAGCACCCTGCTTAATTG \\
\hline
\end{tabular}
\caption{Oligonucleotides used in this study}
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Strategies for the establishment of \textit{mef}(A)-, \textit{msr}(D)- and \textit{mef}(A)–\textit{msr}(D)-knockout strains. The positions of primers are indicated. Similar shading within the diagram represents the same DNA regions.}
\end{figure}
strain, without erythromycin, were almost the same. Although the turbidity of the mutant strain reached the same level as that of the WT strain at the 24 h time point in the presence of erythromycin, it was obvious that the growth of the mef(A)-knockout strain was slower than that of the WT, suggesting that the role of mef(A) was not completely compensated for by other factor(s).

Establishment of the msr(D)-knockout strain and analysis of drug susceptibility

The mef(A) gene is reported to be present with its adjacent msr(D) gene in the conjugative prophage Φ1207.3 (formerly Tn1207.3) (Santagati et al., 2003; Iannelli et al., 2014), Φ10394.4 (Banks et al., 2003) and Φm46.1 (Brenciani et al., 2010). The genome data for strain 10-85 showed the presence of a single copy of the msr(D) gene. Hence, we focused on msr(D) because this gene was shown to be involved in macrolide resistance in S. pneumoniae (Santagati et al., 2000; Ambrose et al., 2005; Gay & Stephens, 2001). We established the msr(D)-knockout strain and performed an Etest. The data from the Etest with erythromycin showed that the sensitivity of the msr(D) mutant was significantly different from the parental strain (P=0.0005; Table 2). The data from the Etest with azithromycin and clarithromycin also showed a striking difference between the parental strain and the msr(D) mutant (P=0.0007 and 0.0014, respectively; Table 2). To confirm these results, complementation experiments were performed. In these experiments, BHI-YE agar plates without sheep blood containing 100 mg kanamycin l⁻¹ were used. Hence, the MIC of erythromycin for 10-85 transformed with pLZ12 plasmid was different from the MIC for WT 10-85 in Table 2. As shown in Table 3, msr(D) complementation rescued the resistance in the msr(D)-knockout strain (P=0.0167), suggesting this gene is indispensable and that mef(A) and/or other factor(s) could not compensate for macrolide resistance by msr(D) knockout.

Table 2. Susceptibilities of the WT strain 10-85 and knockout strains by Etest

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (mg l⁻¹)</th>
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<tbody>
<tr>
<td></td>
<td>Erythromycin</td>
</tr>
<tr>
<td>10-85</td>
<td>29.33 ± 4.62</td>
</tr>
<tr>
<td>10-85 Δmef(A)</td>
<td>34.67 ± 12.22</td>
</tr>
<tr>
<td>10-85 Δmsr(D)*</td>
<td>1.50 ± 0.00</td>
</tr>
<tr>
<td>10-85 Δmef(A)–msr(D)†</td>
<td>0.10 ± 0.02</td>
</tr>
</tbody>
</table>

*P-values were determined using Student’s t-test for MICs of erythromycin, azithromycin and clarithromycin: *P=0.0005 (erythromycin), †P=0.0007 (azithromycin) and ‡P=0.0014 (clarithromycin) when compared with the WT strain 10-85; ‡P=0.0004 (erythromycin), †P=0.0006 (azithromycin) and ‡P=6.62 × 10⁻¹⁴ (clarithromycin) when compared with strain 10-85 Δmef(A); P=1.803 × 10⁻⁸ (erythromycin), P=0.0182 (azithromycin) and P=0.0076 (clarithromycin) when compared with strain 10-85 Δmsr(D).

Establishment of the mef(A)- or msr(D)-complemented strains and analysis of drug susceptibility

We introduced the mef(A) or msr(D) gene into a 1529 macrolide-sensitive strain and analysed the drug sensitivity of the complemented strains using the Etest. In this experiment, we also used BHI-YE agar plates without sheep blood containing 100 mg kanamycin l⁻¹ to analyse the function of these genes. Hence, the values are not the real MICs assayed by the standard method. As shown in Table 4, the msr(D)-complemented strain was more resistant to erythromycin than the parental strain (P=0.0022);
We screened many macrolide-resistant isolates by PCR, and found several isolates that possessed both mef(A) and msr(D) genes, but no erm(B) and ermA genes. We tried to establish mef(A)-, msr(D)- and mef(A)-msr(D)-knockout strains, and succeeded in the establishment of all three knockout strains from emm75-type NCU107 and emm4-type NCU115 strains. We checked that the amino acid sequences of Msr(D) of these strains deduced from nucleotide sequences were completely identical to that of 10-85, but aa 209 from the start methionine of Mef(A) in both strains was lysine instead of the glutamine in 10-85. The erythromycin Etest was performed and the same results were obtained from NCU107 (Table 5). The sensitivity of the NCU115 mef(A) knockout was altered, albeit the change in msr(D) was more significant (Table 5), suggesting that the predominance of msr(D) was confirmed in other emm-type S. pyogenes examined. However, compensation mechanisms in the absence of mef(A) could be different amongst the strains with different emm types.

### DISCUSSION

Since the mef(A) gene was first cloned from S. pyogenes (Clancy et al., 1996), the tight relationship between macrolide resistance and presence of the mef(A) gene has been reported (Perez-Trallero et al., 1998; Orden et al., 1998), and a crucial role of mef(A) in macrolide resistance is irrefutable. Clancy et al. (1996) concluded that mef(A) was responsible for erythromycin resistance because (1) an Escherichia coli strain containing mef(A) maintained a lower level of intracellular erythromycin compared with the isogenic E. coli strain and (2) the intracellular accumulation of \(^{14}\)C-erythromycin in erythromycin-resistant S. pyogenes strains.

### Table 3. Susceptibilities of the complemented strains by Etest

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC of erythromycin (mg l(^{-1}))</th>
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<tbody>
<tr>
<td>10-85 + pLZ</td>
<td>4.50 ± 2.38</td>
</tr>
<tr>
<td>10-85 Δmsr(D) + pLZ*</td>
<td>0.13 ± 0.00</td>
</tr>
<tr>
<td>10-85 Δmsr(D) + pLZmsr(D)</td>
<td>1.13 ± 0.48</td>
</tr>
<tr>
<td>10-85 Δmef(A)–msr(D) + pLZ†</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>10-85 Δmef(A)–msr(D) + pLZmef(A)‡</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>10-85 Δmef(A)–msr(D) + pLZmsr(D)</td>
<td>0.23 ± 0.03</td>
</tr>
</tbody>
</table>

Statistical P-values were determined using Student’s t-test: *P=0.0267 and P=0.0167 when compared with strains 10-85 + pLZ and 10-85 Δmsr(D) + pLZmsr(D), respectively; †P=0.0244, P=0.0123 and P=0.0004 when compared with strains 10-85 + pLZ, 10-85 Δmef(A)–msr(D) + pLZmef(A) and 10-85 Δmef(A)–msr(D) + pLZmsr(D), respectively; ‡P=0.0006 when compared with strain 10-85 Δmef(A)–msr(D) + pLZmsr(D).

### Table 4. Susceptibilities of 1529 and its mef(A)- and msr(D)-complemented strains by Etest

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC of erythromycin (mg l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1529 + pLZ</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>1529 + pLZmef(A)*</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>1529 + pLZmsr(D)†</td>
<td>0.11 ± 0.02</td>
</tr>
</tbody>
</table>

P-values were determined using Student’s t-test: *P=0.05 when compared with strain 1529 + pLZ; †P=0.0022 when compared with strain 1529 + pLZ.

### Assessment of the results from the emm1 type in other emm-type S. pyogenes

We tried to assess the results obtained from the 10-85 emm1-type S. pyogenes in other emm-type S. pyogenes. We screened many macrolide-resistant isolates by PCR, and found several isolates that possessed both mef(A) and msr(D) genes, but no erm(B) and ermA genes. We tried to establish mef(A)-, msr(D)- and mef(A)-msr(D)-knockout strains, and succeeded in the establishment of all three knockout strains from emm75-type NCU107 and emm4-type NCU115 strains. We checked that the amino acid sequences of Msr(D) of these strains deduced from nucleotide sequences were completely identical to that of 10-85, but aa 209 from the start methionine of Mef(A) in both strains was lysine instead of the glutamine in 10-85. The erythromycin Etest was performed and the same results were obtained from NCU107 (Table 5). The sensitivity of the NCU115 mef(A) knockout was altered, albeit the change in msr(D) was more significant (Table 5), suggesting that the predominance of msr(D) was confirmed in other emm-type S. pyogenes examined. However, compensation mechanisms in the absence of mef(A) could be different amongst the strains with different emm types.

### Table 5. Susceptibilities of the WT strains NCU107 and NCU115 and their derivative knockout strains by Etest

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC of erythromycin (mg l(^{-1}))</th>
</tr>
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<tbody>
<tr>
<td>NCU107</td>
<td>15.00 ± 2.00</td>
</tr>
<tr>
<td>NCU107 Δmef(A)*</td>
<td>14.67 ± 2.31</td>
</tr>
<tr>
<td>NCU107 Δmsr(D)†</td>
<td>5.33 ± 2.31</td>
</tr>
<tr>
<td>NCU107 Δmef(A)–msr(D)‡</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>NCU115</td>
<td>13.33 ± 2.31</td>
</tr>
<tr>
<td>NCU115 Δmef(A)§</td>
<td>3.67 ± 0.58</td>
</tr>
<tr>
<td>NCU115 Δmsr(D)‖</td>
<td>0.92 ± 0.14</td>
</tr>
<tr>
<td>NCU115 Δmef(A)–msr(D)¶</td>
<td>0.09 ± 0.08</td>
</tr>
</tbody>
</table>

P-values determined using Student’s t-test: *P>0.05 and P=0.0004 when compared with the WT strain NCU107 and strain NCU107 Δmef(A)–msr(D), respectively; †P=0.0019 and P=0.0167 when compared with the WT strain NCU107 and strain NCU107 Δmef(A)–msr(D), respectively; ‡P=5.52 × 10^{-5} when compared with the WT strain NCU107; §P=0.0022 and P=0.0004 when compared with the WT strain NCU115 and strain NCU115 Δmef(A)–msr(D), respectively; ‖P=0.0007, P=0.0010 when compared with the WT strain NCU115 and strain NCU115 Δmef(A)–msr(D), respectively; ¶P=0.0006 when compared with the WT strain NCU115.
was always lower than that observed in sensitive \textit{S. pyogenes} strains. Furthermore, they stated that their hypothesis should be substantiated by creating \textit{mef}(A)-knockout \textit{S. pyogenes} strains (Clancy \textit{et al.}, 1996). To the best of our knowledge, however, there have been no such published reports that directly confirmed the relationship between \textit{mef}(A) and macrolide resistance in \textit{S. pyogenes}, although \textit{mef}(E) was analysed using \textit{S. pneumoniae} (Ambrose \textit{et al.}, 2005).

In this study, first, we examined the roles of \textit{mef}(A) in macrolide resistance in an \textit{emm1}-type \textit{S. pyogenes} clinical isolate by using a derivative knockout strain and found that the MICs of the \textit{mef}(A)-knockout strain for macrolides were almost the same as that of its parental strain, but growth in the presence of erythromycin was suppressed. Second, the macrolide susceptibility of the \textit{msr}(D)-knockout strain was significantly different from that of its parental strain. Finally, introduction of \textit{msr}(D) into a macrolide-sensitive strain conferred drug resistance, unlike \textit{mef}(A). From these results, we suggest that the \textit{msr}(D) gene can almost compensate for the \textit{mef}(A) function; however, the reverse is not true. Thus, \textit{mef}(A) does not play dominant roles in macrolide-resistant \textit{emm1}-type \textit{S. pyogenes}, but \textit{msr}(D) is predominant. By using other \textit{emm}-type \textit{S. pyogenes} strains, we confirmed that the conclusion obtained from the data from \textit{emm1} is universal across all \textit{S. pyogenes} strains.

\textit{msr}(D) has been mainly studied in \textit{S. pneumoniae}. In \textit{S. pneumoniae}, the \textit{msr}(D) gene is located adjacent to the \textit{mef} gene as ORF5, and \textit{mel} in Tn1207.1, and the \textit{mega} element (Santagati \textit{et al.}, 2000; Gay & Stephens, 2001). This has homology with the \textit{msr}(A) gene cloned from \textit{Staphylococcus epidermidis} (Ross \textit{et al.}, 1990). The common regulatory mechanism is considered to be conserved in \textit{mef}–\textit{msr}-containing elements in a wide variety of Gram-positive bacteria (Chancey \textit{et al.}, 2015). An \textit{msr} pump was reported to potentially contribute to the efflux phenotype (Daly \textit{et al.}, 2004). Ambrose \textit{et al.} (2005) showed that macrolide efflux in \textit{S. pneumoniae} was mediated by a dual-efflux pump, \textit{mef}(E) and \textit{mel} (\textit{msr}). Etest erythromycin MICs were significantly reduced for both independent \textit{mef}(E) (13-fold) and \textit{mel} (22-fold) deletion mutants compared with those of the parental strains (Ambrose \textit{et al.}, 2005). The significant reduction for independent \textit{mef}(E) may be coincident with our result from the \textit{emm4}-type NCU115 strain. However, it is not coincident with our result from \textit{emm1}-type and \textit{emm75}-type \textit{S. pyogenes}. In these two \textit{S. pyogenes} strains, the erythromycin susceptibilities between the parental strain and the \textit{mef}(A) knockout were not significantly different, but the erythromycin susceptibilities of \textit{msr}(D) were significantly different. Although the growth of the \textit{emm1}-type \textit{mef}(A)-knockout strain was suppressed in the presence of a low concentration of erythromycin, the ability to compensate for factor(s) other than \textit{mef}(A) could be more potent in \textit{S. pyogenes} than \textit{S. pneumoniae}. As the nucleotide sequences of \textit{msr}(D) of \textit{emm1}-type 10-85, \textit{emm4}-type NCU115 and \textit{emm75}-type NCU107 strains are identical, the existence of different uncharacterized factor(s) that may contribute or support the \textit{msr}(D)/\textit{mef}(A) system in \textit{S. pyogenes} is a possibility.

In conclusion, we present data showing that \textit{msr}(D) plays a predominant role over \textit{mef}(A) in macrolide resistance in \textit{S. pyogenes}. Further analyses are necessary to completely reveal the mechanism of macrolide resistance in \textit{S. pyogenes}, particularly the different mechanisms between \textit{S. pyogenes} and \textit{S. pneumoniae}.

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