·基础实验研究论著·

Point mutation in regulatory region of *msrA* gene contributes to the telithromycin resistance induced by erythromycin in *Staphylococcus aureus* *

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Abstract: *msrA* encodes an efflux protein in *Staphylococcus spp.* that confers inducible resistance to macrolides, such as erythromycin(ERY), but not telithromycin(TEL). Thus, *msrA*+ *S. aureus* remain susceptible to TEL. Production of *msrA* is regulated by a 320-bp upstream region, presumably by translational attenuation, similar to regulation of *erm*. We investigated whether ERY could induce TEL resistance in *msrA*+ *S. aureus*. MICs of ERY and TEL were determined by broth microdilution(BMD). Inducible TEL resistance was detected by a BMD checkerboard panel containing combinations of ERY and TEL and by a D-test where an ERY disk was placed 15 mm from a TEL disk on a blood agar plate. Blunting of the TEL zones of inhibition proximal to the ERY disk indicated inducible resistance and no blunting indicated no inducible resistance. Approximately 400 bp of the upstream regulatory region and 200 bp of *msrA* were amplified by PCR and sequenced. The TEL MICs for 10 *msrA*+ isolates ranged from 0.06－2 g/mL (MIC_{90}＝0.25 g/mL); ERY MICs were all ≥32 g/mL. All isolates showed an increase in TEL MICs of >2 doubling dilutions (MIC_{90}>4 g/mL) in the presence of ERY; All were also positive by D-test. For 9 isolates(initial TEL MIC<1 g/mL), constitutive TEL resistance was selected by plating cells on Mueller-Hinton agar with 2 g/mL TEL. All 9 isolates grew on the selective media and were resistant to TEL(MIC ranged 2－8 g/mL). Mutation to constitutive resistance occurred at a rate of 8×10^{-7} to 2.4×10^{-6}. Eight of the 9 resistant isolates had a single nucleotide substitution in the upstream regulatory region relative to the parent strain. Specifically, six isolates had a G to T mutation at position －222; one had a C to A mutation at －228; And one had a C to A mutation at －247. Transformation of the *msrA* gene with above point mutation in regulatory region into *S. aureus* RN4220 conferred the strain resistant(MIC＝8 g/mL) to the telithromycin while transformants with the wild-type *msrA* gene remained susceptible(MIC＝1 g/mL) to telithromycin.

Key words: telithromycin; induction; msrA; Staphylococcus. aureus
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Introduction

Staphylococcus aureus (S. aureus) is significant cause of both healthcare- and community- acquired infections. Multidrug resistance in *S. aureus* is common in the United States and worldwide. For many hospitals, methicillin-resistance *S. aureus* (MRSA) rate are below 50%[1]. MRSA isolates are often resistant to other antimicrobials including macrolides, tetracycline, and trimethoprim/sulfamethoxazole[2]. Vancomycin-resistant(vanA-mediated) isolates were also reported from thirteen patients in the U. S. to date[3].

Telithromycin is a ketolide, chemically altered macrolide, and belongs to a new class of antibiotics. Ketolides have a second site of interaction with the ribosome at domain II on the 23S rRNA of the 50S ribosomal subunit in addition to the interaction at domain V where 14- and 15-membered ring macrolides act[4-5]. This drug inhibits protein synthesis and is active against *S. aureus* that are resistant to beta-lactams, macrolides, and fluroquinolones. Macrolides, lincosamides, and streptogramin B(MLSB) are mainly used against MRSA or in penicillin-related allergic patients. The telithromycin has been used for treatment of bronchitis, acute bacterial sinusitis and community-acquired pneumonia.

There are mainly two mechanisms of resistance to MLSB antibiotics among staphylococci: One is the active efflux mechanism mediated by *msrA*, the other one is the ribosomal target modification mechanism mediated by *erm* genes(mainly *ermA*, and *ermC*)[6-7]. The *msrA* gene was first identified in Staphylococcus epidermidis and encodes an efflux protein that confers resistance to 14- and 15-membered ring macrolides and streptogramin type B(MSB phenotype) such as erythromycin(ERY), but not telithromycin(TEL). Thus, *msrA*+ *S. aureus* remain susceptible to TEL, while the *erm* genes-mediated resistance genotype is associated with resistance to macrolides, lincosamides, and streptogramin B(MLSB phenotype). This latter

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mechanism can be erythromycin induced(iMLSB) or constitutive(cMLSB)[8-11].

Production of *msrA* is regulated by a 320-bp upstream region, presumably by translational attenuation, similar to regulation of *erm*. The previous report by Davis et al.[12] demonstrated the telithromycin resistance was inducible by erythromycin in the isolates of macrolide-resistant *Staphylococcus spp*. However, the data did not discriminate if the mechanism of a positive macrolide induction test with telithromycin was due to the *erm* or *msrA* genes. The purpose of this study was to further investigate the mechanism of inducible resistance in isolates of *msrA* positive *Staphylococci spp*.

1 Materials and methods

1.1 Bacteria, media and antibiotics Strains and plasmids used in this study are listed in the Table 1. 10 *msrA* positive *S. aureus* isolates from the culture collections of the Centers for Disease Control and Prevention were used for telithromycin induction studies. *S. aureus* isolates were grown on tryptic soy agar plates(TSA) with 5% sheep blood(Becton Dickinson and Company, Cockeysville, MD). Escherichia coli Max Efficiency DH10B(Invitrogen) was used for plasmid transformation. Antibiotics, when required, were added at the following concentrations: ampicillin, 100 μg/mL for *E. coli*, and chloramphenicol, 10 μg/mL for *S. aureus*.

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
TOP10	F-*mcrA*△(*mrr-hsdRMS-mcrBC*)Φ80*dlacZ*△*M*15△*lacX*74 *recA*1 *araD*139(*ara- leu*)7697*galU galK rpsL*(*StrR*)*endA*1 *nupG*	Invitrogen
DH10B	*mcrA*△(*mrr-hsdRMS-mcrBC*)Φ80*dlacZ*△*M*15? *lacX*74 *deoR recA*1 *araD*139(*ara*,*leu*)7697*galU galK rpsL nupG*	Invitrogen
S. aureus		
RN4220	An efficient plasmid transformation strain	Dr. Novic
Plasmids		
pCR2. 1TOPO	AT direct cloning vector for pcr products, Ampr, Kanr	Invitrogen
pCN50	*E. coli*-*S. aureus* shuttle vector, Ampr, Cmr	From Dr. Novic
pMIN2	1. 9kb PCR product of *msrA* with a C to A mutation at position −228	This study
pMIN3	1. 9kb PCR product of *msrA* with a C to A mutation at position −247	This study
pMIN4	1. 9kb PCR product of *msrA* with a G to T mutation at position −222	This study
pMIN8	1. 9kb PCR product of the wild type *msrA*	This study
pCN501	pCN50 with 1. 9 kb *PstI*/*BamHI* fragment from pMIN2	This study
pCN502	pCN50 with 1. 9 kb *PstI*/*BamHI* fragment from pMIN3	This study
pCN503	pCN50 with 1. 9kb *PstI*/*BamHI* fragment from pMIN4	This study
pCN504	pCN50 with 1. 9 kb *PstI*/*BamHI* fragment from pMIN8	This study

1.2 Inducible experiments Inducible TEL resistance was detected by a limited broth microdilution(BMD) panel containing various combinations of ERY and TEL.

1.3 Susceptibility testing Susceptibility to telithromycin was determined by BMD with cation-adjusted Mueller-Hinton broth(BD Biosciences, Sparks, MD) according to procedures approved by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS).

1.4 D-Test The D-shaped zone test(D-test) was performed by placing ERY and TEL disks at an edge-to-edge distance of 15 to 20 mm apart on Mueller-Hinton agar that has been inoculated with a standardized(0. 5 MacFarland) suspension of *S. aureus* and looking for flattening of the telithromycin zone nearest the erythromycin disk.

1.5 DNA Preparations Total DNA was prepared by Qiagen protocol(Valencia, CA) for isolation of genomic DNA from gram-positive bacteria, but the method was modified for *S. aureus* by the inclusion of lysostaphin(Sigma) at a final concentration of 30 μg/mL in the cell lysis buffer.

1.6 Polymerase chain reaction(PCR) amplification The 590 bp upstream regulatory region of *msrA* gene[13] was PCR amplified using primers *msrA*F, 5′-GTG CTG CAT TTT ATA AGT ACA CTC T-3′(forward) and *msrA*R, 5′-GAA TTT CGC CAT TCG TTT CAA CTG-3′(reverse). Each PCR reaction was prepared in a total volume of 50 μL including 8 μL of 10 mmoL dNTP(Roche Applied Bioscience, Foster City, CA). 1 μL of each primer at 20 μmoL, 2 μL of DNA(equaling 100−500 ng), 5 μL of 10x buffer, 2 μL of 25 mmol/L $MgCl_2$, 0. 5 μL of ApliTaq Gold Enzyme(Roche), and 30. 5 μL of H_2O. PCR reactions were performed in a GeneAmp PCR System 9700(PE Applied Biosystems, Foster city, CA) with reaction cycles as follow: an initial denaturation step of 2 min at 94 ℃, 30 cycles

of 15 s at 95 ℃, 30 s at 55 ℃, 30 s at 72 ℃; and a final elongation at 72 ℃ for 7 min. PCR products were run on an agarose gel and purified with QIAquick PCR purification kit (QIAGEN, Valenica, CA) for DNA sequencing.

1.7 DNA sequencing and analysis PCR products were purified with QIA quick spin columns (Qiagen). The concentrations of the DNA were quantitated on an ND1000 spectrophotometer (NanoDrop, Inc., Rockland, DE) before sequencing. PCR products served as the template for sequencing reactions with the BigDye Terminator v. 3.1 cycle sequencing kits (Applied Biosystems). Centri-Sep 8 strips and 96-well plates (Princeton Separation, Adelphia, NJ) were used to remove excess dye terminators from the sequencing reactions before the sequence was determined on an ABI 3130xl automated DNA genetic Analyzer (Applied Biosystems). DNA sequences were aligned and compared using Sequencher software (GeneCodes Corp., Ann Arbor, MI).

1.8 Construction of the plasmids Plasmid DNA preparation, restriction endonuclease analysis and ligations were carried out by standard methods. PCR amplifications were performed by using pfu DNA polymerase (Stratagene, La Jolla, CA). Oligonucleotides were synthesized by the CDC core facility. A 1.9 kb *msrA* gene with different point mutation in the regulatory region was amplified using the primers containing engineered restriction sites: *msrA*9F (PstI), 5′-CGC TGC AGG TAC ACT CTA AAT TAA CAA GAT G (forward) and *msrA*10R(BamHI), 5′-CGG GAT CCA TTC AAT ATA AAT TAG TGT ATA G-3′(reverse). Poly(A) overhangs were generated by the addition of 1 μm dATP and 1 μL of Taq polymerase to the completed PCR mixture and incubation at 72 ℃ for 15 min. The 1.9 kb PCR products were cloned into the pCR2.1 TOPO Ⅱ vector (Invitrogen) and then subcloned into the pCN50 shuttle vector by *Bam*HI and *Pst*I digestion and religation.

1.9 Plasmid transformation The shuttle plasmids contain wild type *msrA* gene and the *msrA* genes with point mutation in regulatory region were introduced into wild-type *S. aureus* RN4220 by electroporation, respectively. Bacteria suspension in 10% glycerol electroporation buffer with 1 μg of DNA were subjected to a single pulse using the Bio-Rad Gene Pulser (Bio-Rad Laboratories) set at 2.5 kV, 25 μF, with the pulse controller set at infinity. The content of the cuvette was diluted into 3 mL of BHI and incubated for 2 hours with shaking. After incubation, aliquots were plated onto TSA plates containing 10 μg/mL of chloramphenicol for selection of transformants.

2 Results

2.1 Induction of telithromycin resistance by erythromycin in *msrA* positive *S. aureus* In order to test if telithromycin resistance can be induced by erythromycin, we performed the inducible experiments with a limited BMD checkerboard panel containing various combinations of ERY and TEL. Ten *msrA* positive isolates with TEL MICs ranged from 0.06 to 2 μg/mL (MIC_{90} = 0.25 g/mL) were examined. The results showed that all isolates had an increase in TEL MICs of >2 doubling dilutions (MIC_{90} >4 μg/mL) in the presence of ERY; All were also positive by D-test. For 9 isolates (initial TEL MIC<1 μg/mL, Tables 1), constitutive TEL resistance was selected by plating cells on Mueller-Hinton agar containing 2 μg/mL of TEL. One isolate did not grow on the telithromycin-containing agar plates and was not further analyzed. The remaining 9 isolates grew on the selective media and were resistant to TEL (MIC = 2 − 8 g/mL, Table 2). Mutation to constitutive resistance occurred at a rate of 8×10^{-7} to 2.4×10^{-6}.

Table 2 **MIC results for erythromycin and telithromycin and frequency of mutation rates for strains of *S. aureus***

ID number	Erythromycin MIC(μg/mL)	Telithromycin MIC(μg/mL)	Frequency of mutation rate**
5091	64	0.06	1×10^{7}
5091-C*	64	2.00	
5078	32	0.06	1×10^{7}
5078-C	64	8.00	
1519	64	0.12	4×10^{7}
1519-C	64	4.00	
5114	64	0.12	1×10^{7}
5114-C	32	2.00	
3571	64	0.12	2×10^{7}
3571-C	64	4.00	
1397	64	0.12	5×10^{7}
1397-C	64	4.00	
2654	64	0.06	2×10^{7}
2654-C	64	4.00	
4220	128	2.00	3×10^{8}
4220-C	64	4.00	
5373	128	0.25	1×10^{7}
5373-C	128	8.00	

*: "C" indicates the daughter strain that was grown on telithromycin-containing agar, becoming constitutively resistant to telithromycin; **: Frequency of mutation rate is the number of colony-forming units (cfu)/mL on telithromycin-containing agar plates.

2.2 Point mutation found in the regulatory region of *msrA* gene To understand the mechanism of telithromycin resistance, approximately 400 bp of the upstream regulatory region and 200 bp of *msrA* gene from above 9 constitutive TEL resistant isolates and their parent strains were amplified by PCR using the primer *msrA*F and *msr*AR. DNA sequence analysis of PCR products indicated that eight of the 9 resistant isolates had a single nucleotide substitution in the upstream regulatory region relative to the parent strain. The three types of point mutation were observed. Specifically, six isolates (1519C, 3571C, 1379C, 2654C, 5373C, 5078C) had a G to T mutation at position −222, one (5091C) had a C to A mutation at −228, and one (5114C) had a C to A mutation at -247. One isolate, 4220C, that lacked a point mutation had an initial TEL MIC= 2 μg/mL.

2.3 Cloning of the *msrA* genes 1.9 kb DNA fragment including 0.4 kb upstream nucleotide of *msrA* gene was PCR amplified from the wild-type and strains containing 3 different types of the point mutation using primers *msrA*9f and *msrA*10R, respectively. PCR products were cloned into the pCR2.1 TOPO Ⅱ vector to generate plasmids pMIN2, pMIN3, pMIN4 and pMIN6(Table 1). The nucleotide sequences of the PCR products were confirmed by DNA sequencing, and they were then subcloned into the shuttle vector pCN50 to generate plasmids pCN51, 52, 53 and 54, respectively. The resulting plasmids were examined with restriction digestion before transforming into the *S. aureus* RN4220.

2.4 The point mutation of *msrA* gene confers *S. aureus* telithromycin To investigate if the point mutation in *msrA* gene regulatory region plays a role in telithromycin resistance, we transformed the wild type and point mutated *msrA* genes into the *S. aureus* RN4220 by the electroporation and the transformants were selected on the TSA plates containing 10 μg/mL of chloramphenicol. Colonies resistant to chloramphenicol were tested for susceptibility to telithromycin using the broth microdilution method. The results indicated that all three types of point mutated *msrA* gene in regulatory region conferred the wild-type *S. aureus* RN4220 resistant to telithromycin(MIC=8 μg/mL) while the wild type *msrA* gene did not significantly change the pattern of telithromycin susceptibility in *S. aureus* RN4220(MIC=1 μg/mL, Table 3). In order to further confirm that telithromycin resistance in *S. aureus* is the result of the point-mutated *msrA* genes in the regulatory region, we recovered the plasmids from the tested transformants of *S. aureus* and performed restriction enzyme(BamHI/PstI) digestion and DNA sequencing; The data showed the identity of the recovered plasmid was identical to the previously transformed plasmid. These results clearly indicated that the point mutation in *msrA* regulatory region is the cause of *S. aureus* resistance to telithromycin.

Table 3 MIC results for telithromycin in *S. aureus* RN4220 transformants

Experiments	MICs(μg/mL)
No plasmid	0.12
pCN50(vecctor control)	0.12
pCN51(*msrA* mutation 1)	8.00
pCN52(*msrA* mutation 2)	8.00
pCN53(*msrA* mutation 3)	8.00
pCN54(*msrA* wild-type)	1.00
*S. aureus*29213(quality control)	0.12

3 Discussion

Telithromycin is effective against the most frequently isolated bacterial causes of pharyngitis, otitis media and sinusitis. In comparison with clarithromycin and azithromycin, telithromycin is two- to eight-fold more active against erythromycin-susceptable strains of *S. aureus*. Telithromycin maintains activity against macrolide-resistant strains of *S. aureus* that have an inducible MLSB gene but not against strains in which resistance in constitutively expressed[14]. Telithromycin is also more active in vitro against *S. pneumonia* and has activity against strains that are macrolide resistant[15]. For example, in one study, the MIC_{90} for telithromycin against *S. pneumonia* strains with *mefA* gene was 0.25 mg/L or less, compared with 1 to 4 mg/L for macrolides. Against strains expressing the *ermB* gene, telithromycin had an MIC_{90} of 0.5 mg/L, whereas the macrolides had an MIC_{90} of more than 64 mg/L[16]. For penicillin-intermediate and penicillin-resistant pneumococcal strains, telithromycin MIC_{90} increased from 0.015 mg/L to 0.25 mg/L and 0.5 mg/L, respectively[17].

The *msrA* gene is controlled by a regulatory region containing several inverted repeat sequences and an open reading frame encoding a short leader peptide. Deletion of this control region resulted in constitutive resistance to macrolides and type B streptogramins(MSB) in *S. aureus*[18]. However, the MICs of telithromycin for *S. aureus* RN4220 carrying the deletion of the control region of *msrA* gene were not affected by the induction of erythromycin, indicating that the *msrA* control region may also be required for inducible telithromycin resistance[19]. Our data clearly showed that the telithromycin resistance is inducible by erythromycin in the *msrA* positive *S. aureus* isolates as demonstrated by D-test and BMD experiments(Table 2). Our results also indicated that point mutation in the regulatory region resulted in the constitutive telithromycin resistance and transformation of the *msrA* gene with different point mutation in regulatory region into *S. aureus* RN4220 conferred the strain resistant(MIC=8 g/mL) to the telithromycin while transformants with the wild-type *msrA* gene remained susceptible (MIC=1 g/mL) to telithromycin. Our data supported previous hypothesis[18-19].

To our knowledge, this is the first report on the genetic mechanism of inducible telithromycin resistance in *msrA* positive *S. aureus* isolates. The prevalence of the erythromycin resistance determinants such as *msrA* gene among staphylococci varies in different geographical areas and species. In a survey of the macrolide resistance genes in clinical *S. aureus* and coagulase-negative staphylococci(CoNS)[20], the frequency of *msrA* was 10.2% among *S. aureus*(n=46) and *msrA* was 41% in CoNS(n=71). Another report about the distribution of erythromycin genes indicated the frequency of *msrA* was 28.6%, *ermA* was 22.8%, *ermB* was 45.7%, and *ermC* 17.1%[21]. However, a French report indicated that erythromycin resistance among *S. aureus* strains was due to the presence of *ermA* or *ermC* genes, with *msrA* gene rarely encountered(2.1%); While among CoNS, macrolide resistance *msrA*-mediated gene was most commonly found in *S. haemolyticus* strain[9]. Likewise, from a study in the Czech Republic, a high frequency of *msrA*-positive strains was detected among methicillin-resistant CoNS (53%), predominantly in *S. haemolyticus* strains (69%)[10]. A more recent report also detected 14 out of 15 erythromycin-resistant *S. haemolyticus* isolates that carried the

msrA gene. This indicates that macrolide resistance among *S. haemolyticus isolates is mainly due to the presence of the msrA* resistance genotype. Nevertheless, the results of this study, induction of telithromycin resistance by erythromycin in *msrA* positive isolates and transfer of telithromycin resistance in *S. aureus*, may indicate a need for the routine detection of inducible telithromycin resistance in clinical laboratories, and the impact of this issue on the clinical use of telithromycin should be investigated, to avoid therapeutic failures.

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