Co-blockade of *mecR1/blaR1* signal pathway to restore antibiotic susceptibility in clinical isolates of methicillin-resistant *Staphylococcus aureus*

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Abstract

Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA) is caused by the production of low-affinity penicillin-binding protein 2a and β-lactamases, which are encoded by *mecA* and *blaZ*, respectively. Expressions of the two key genes are mutually regulated by MecI and BlaI. The aim of this study was to design specific anti-*mecR1* and anti-*blaR1* deoxyribozymes and identify the restoration of susceptibility in MRSA isolates with *mecI* or *blaI* or no deletions by interfering with the mutual regulation of *mecA* and *blaZ*.

Material and methods: Specific deoxyribozymes were designed by using the program RNA structure 4.6. RNA substrates were obtained by transcription *in vitro* and used to assess the target cleavage of DNAzymes. Transcription of *mecR1-mecA* and *blaR1-blaZ* was analysed by real time RT-PCR. The susceptibility of MRSA was tested.

Results: Specific deoxyribozymes showed efficient catalytic activity to each own substrate *mecR1* or *blaR1 in vitro* and caused the reduction of *mecR1* and *blaR1* transcription *in vivo*. Furthermore, simultaneous administration of two DNAzymes to knockdown *mecR1* and *blaR1* resulted in increased susceptibility of all MRSA strains tested in this study.

Conclusions: These results demonstrated that combined use of the two specific phosphorothioate deoxyribozymes could be a viable and promising strategy to restore the susceptibility of almost all MRSA clinical isolates.

Key words: methicillin-resistant *Staphylococcus aureus*, *mecR1*, *blaR1*, phosphorothioate deoxyribozyme.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA), a multidrug resistant gram-positive bacterium, is one of the leading causes of hospital-associated infections [1-3]. Vancomycin is one of the few drugs that have remained effective against MRSA. However, recently the susceptibility of MRSA to vancomycin has decreased and vancomycin is losing potency against MRSA [4-6]. Linezolid (LZD) is typically used for the treatment of infections with Gram-positive bacteria, including MRSA and VRSA in the United States [7, 8]. The emergence of linezolid (LZD)-resistant MRSA infections has been reported from the USA, the UK, Brazil and Japan; it warns that we are

currently facing a growing shortage of effective antibiotics [7, 9, 10]. The emergence of MRSA resistant to the last-defence antibiotics (vancomycin and linezolid) has created an urgent need to discover alternative anti-MRSA approaches.

The antibiotic resistance to β-lactam in MRSA is mediated by *mecA*, which codes for the low-affinity penicillin binding protein 2a (PBP 2a), an enzyme working to allow cell-wall synthesis despite the presence of β-lactam antibiotics [11]. Transcription of *mecA* is regulated by a repressor, *mecI*, and a sensor/transducer, *mecR1* [12]. More than 90% of staphylococcal isolates also produce β-lactamases, the product of *blaZ*, and contain *blaZ* regulatory sequences (*blaI* and *blaR1*) that are similar in sequence and function to *mecA* regulators [13]. In addition to regulating *blaZ* transcription, BlaI also binds to *mecA-mecR1* promoter-operator (P-O) sequences and regulates their transcription. Co-regulation of *mecA* by both MecI and BlaI has been proved in clinical *S. aureus* isolates [14, 15].

The restoration of susceptibility in MRSA by respective blocking *mecR1* and *blaR1* is only applicable to strains that harbour wild *mecI* or *blaI* [16, 17]. But, mutations and deletions in *mecI* and deletions of both *mecI* and *mecR1* appear to be common in clinical *S. aureus* isolates. It has been shown that 96% of clinical isolates with mutant *mecI* or with a deletion of *mecI* contain *blaI*, while the isolates that do not contain *blaI* all have wild-type *mecI* sequences [12, 18, 19]. The co-egulation of *mecA* by both MecI and BlaI has been proved [14, 15, 20]. Therefore, MRSA must have at least one of the two functional *mecA* regulators. This mutual regulation of *mecA* by both MecI and BlaI plays a very important role in antibiotic resistance of MRSA.

Based on current understanding of the mechanism of *mecA* co-regulation, the complete restoration of β-lactam antibiotic susceptibility in MRSA clinical isolates should be achieved by simultaneous blockade of *blaR1* and *mecR1*. In this study, we explored the use of specific anti-*mecR1* and anti-*blaR1* phosphorothioated deoxyribozymes (PS-DRz1694 and PS-DRz1366) and identified the restoration of susceptibility in MRSA clinical isolates with *mecI* deletion or *blaI* deletion or no deletions by simultaneous blockage of MecR1 and BlaR1 mediated signal pathways.

Material and methods

Bacterial strains

Three clinical isolates, wild-type MRSA080302 with *blaI* only (Δ*mecI-blaI*), MRSA080305 with *mecI* only (*mecI*-Δ*blaI*) and MRSA080309 with both *mecI* and *blaI* (*mecI-blaI*) were obtained from cultures of sputum and catheter samples from patients in Xijing Hospital (Xi'an, China). MSSA type strain ATCC 29213 was used as a positive control.

Deoxyribozyme design

The program RNAstructure 4.6 was used to design anti-*mecR1* and anti-*blaR1* 10-23 deoxyribozymes. The deoxyribozymes were synthesized by Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China), and were partly phosphorothioated (Table I).

In vitro transcription

The two gene fragments (*mecR1* and *blaR1*) were constructed into pGEM-T vector by Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China), respectively. The two constructed plasmids were linearized by *NcoI* (Toyobo, Japan). The RiboMAX large scale production system (Promega, Madison, WI, USA) was used for the transcription under the guide of the manufacturer's instructions. The resulting RNA was purified and concentrated with sodium acetate and chilled ethanol.

PS-DRzyme cleavage assay

To assess the target cleavage of DNAzymes, RNA substrates and DNAzymes were mixed with 20 µl of reaction buffer (50 mM Tris, pH 8.0, 20 mM $MgCl_2$, 0.01% SDS) at 37°C. The reaction was quenched at various time intervals with 50 mmol/l EDTA and the samples were denatured for 10 min at 70°C, and then the uncleaved substrate and products were resolved by electrophoresis on 3% denaturing agarose gel. The gel was incubated in 1×SYBR Gold nucleic acid gel staining solution (Invitrogen, Carlsbad, CA, USA) for 40 min. The band densities were quantitated by the Alpha Imager system (Alpha Innotech, California, USA). The

Table I. Summary of sequences and cleavage sites of DNAzymes used

	DNAzyme sequence (5'-3')[a]	RNA substrate sequence (5'-3')	Cleavage site (nt)
DRz1694	ATTCGCAggctagctacaacgaTGTCTTCGCCTT	AAGGCGAAGACAAUGCGAAU	1694
DRz1366	CTTGAGTTGAGggctagctacaacgaCGCAGTAAT	AUUACUGCGACUCAACUCAAG	1366
DRz5491	CATAGGCAcgcatgctaacacgaTGTCCGCTTCT		

[a]*The 10-23 catalytic motifs of PS-DRzymes are indicated in lower case. The sequences of PS-DNAzymes for the target binding domains are in uppercase, which are designed to be complementary to mecR1 and blaR1 respectively and were all modified with phosphorothioates to increase nuclease resistance*

fraction of substrates cleaved by DNAzymes was calculated and plotted against time. All reported kinetic values are means ± SD of at least three independent experiments. V_{max} and K_M values were determined from the *y* intercept and slope, respectively, of the best-fit line to a Lineweaver-Burke plot of 1/*V* vs. 1/[S].

PS-DRzyme delivery and real time RT-PCR

Staphylococcus aureus was cultured overnight and 1 ml of culture was transferred to 100 ml broth medium. The medium was incubated at 37°C until the OD_{600} reached 0.55-0.65. Cells were centrifuged at 6000 rpm at 4°C for 10 min. The cell pellet was washed twice by re-suspending in 100 ml of sterilized, ice-cold water and centrifuging at 6000 rpm at 4°C for 10 min. Then the pellet was washed an additional four times with 40, 10, 2 and 1 ml of 10% cold glycerol. Finally, the pellet was resuspended in 1 ml of 10% cold glycerol, distributed into 50 ml aliquots. 10 mg/l anti-*mecR1* phosphorothioated deoxyribozyme1694 (PS-DRz1694) or 10 mg/l anti-*bla*R1 phosphorothioated deoxyribozyme1366 (PS-DRz1366) was introduced into competent MRSA strains by electroporation under the following conditions: 25 µf, 900 V, 200 Ω, time constant 3.6-4.2 ms.

The culture of *S. aureus* was centrifuged at 5000 rpm for 10 min at 4°C and supernatant was decanted. The cell pellet was suspended in 100 µl of lysis solution (50 mM Tris-HCl pH 7.5, 5 mM EDTA pH 8, 50 mM NaCl, 10 mM Tris) with 300 µg of lysozyme (Sigma) and 5 µg of lysostaphin (Sigma) for 30 min at room temperature. The total RNA was extracted from the bacterial lysis with Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's instructions, and the RNA samples were treated with DNase I to remove any genomic DNA contamination. The total RNAs were extracted from the bacterial lysis with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The cDNA was synthesized by reverse transcription from 1 µg of each RNA sample using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The nucleotide sequences for various primers were listed (Table II). PCR was run in a DNA Engine Opticon (MJ Research, Waltham, USA) using SYBR Green I. PCR reagents consisted of: 12.5 µl of SYBR Premix Ex TaqTM (TaKaRa, DRR041S, Japan), 0.5 µl of 50× ROX Reference Dye (TaKaRa, DRR041S, Japan), 0.75 µl of each primer (10 µM) and 1 µl of sample cDNA, in a final volume of 25 µl. Thermal cycling conditions: initial denaturation step at 95°C for 5 min, 50 cycles at 95°C for 10 s, 56°C for 20 s and 72°C for 20 s.

The cDNA of the control group was fold series diluted and the analysis was performed as follows: for each sample, the difference in Ct values (ΔCt) was calculated for target genes subtracting Ct for the reference RNA. $\Delta Ct = Ct_{(target\ gene)} - Ct_{(16SrRNA)}$. Relative expression of target genes mRNA was calculated using the comparative Ct method as previously described [17]. The ΔΔCt values were calculated by the following equation: $\Delta\Delta Ct = \Delta Ct_{(treatment)} - \Delta Ct_{(control)}$. The mean of these ΔΔCt measurements was then used to calculate expression of the test gene ($2^{-\Delta\Delta Ct}$) relative to the reference gene, *16SrRNA*, and normalized to the untreated control as follows: relative expression = $2^{-\Delta\Delta Ct}$. The evaluation of $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the untreated control.

Susceptibility testing

After electroporation, the cells were recovered in preheated broth medium containing 6 mg/l of oxacillin at 37°C with 150 rpm agitation for 1 h. The growth determination was carried out as follows: The cells were diluted 10^4-10^8 times. Fifty microlitres of diluted cells were spread onto plates of Mueller-Hinton agar containing 6 mg/l of oxacillin, and the plates were incubated for 48 h at 35°C. The colonies were counted for plates with > 10 and < 500 colonies and the total number of CFU (colony forming units) per sample was determined by correcting the colony count for the dilution factor.

To determine the growth curves for MRSA, 100 µl of cell dilution were added to the wells of a 96-well microtitre plate, and the plate was incubated at 35°C with agitation at 120 rpm. The optical density (OD_{600}) of each well was measured with a microplate reader (Bio-Rad Laboratories, Tokyo, Japan) at different time points.

According to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), the minimum inhibitory concentrations (MICs) of oxacillin for MRSA080302, MRSA080305, MRSA080309 and MSSA ATCC29213 were determined by the standard broth dilution method [17, 21].

Statistical analysis

Values are expressed as mean ± SD. One-way ANOVA analysis followed by Student-Newman-Keuls (SNK) test was performed for CFU and RNA expression variables. A value of $p < 0.05$ was considered statistically significant.

Results

Design and characterization of anti-*mecR1* and anti-*blaR1* deoxyribozyme

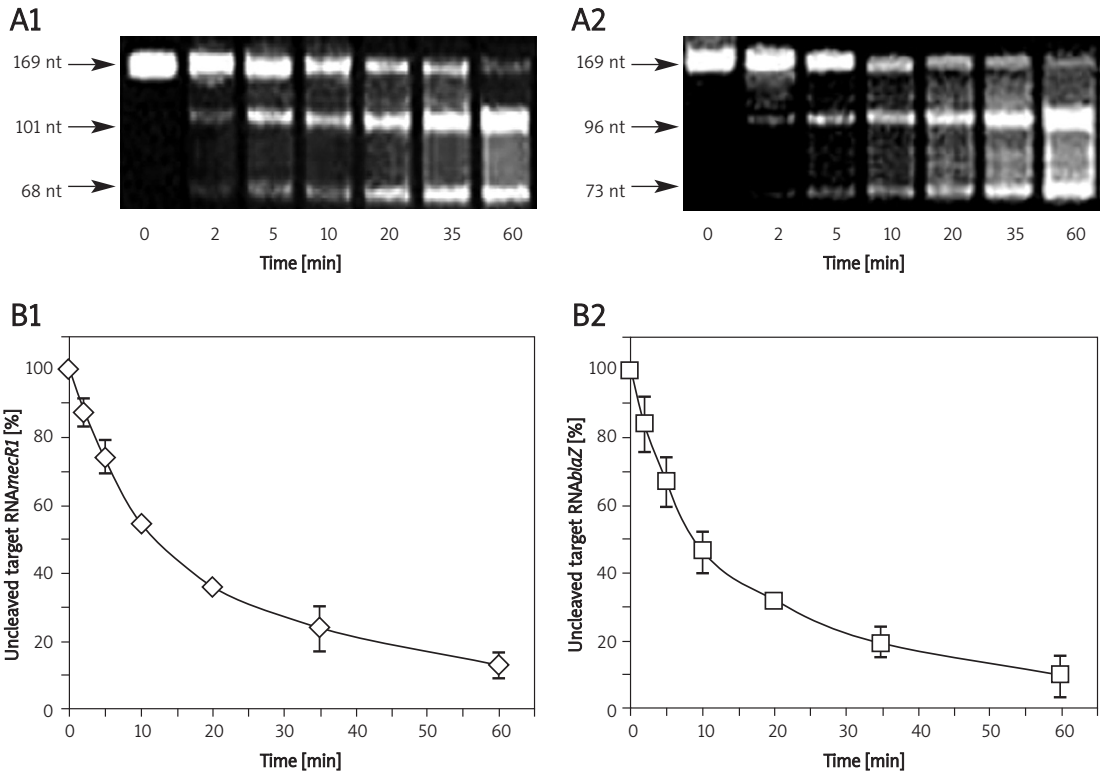
Based on analysis of target mRNA secondary structures by the program RNAstructure 4.6, two optimized deoxyribozymes, PS-DRz1694 targeting

mecR1 and PS-DRz1366 targeting *blaR1*, were generated. The design parameters of $\Delta G_{oligo\text{-}oligo}$, $\Delta G_{oligo\text{-}self}$, ΔG_{duplex}, $\Delta G_{overall}$ and Tm for PS-DRz1694 were –2.1, 0, –28.0, –17.3 kcal/mol, 75.3°C, respectively. The same parameters for PS-DRz1366 were –3, 0, –25.2, –20.6 kcal/mol and 73.9°C.

The target binding domain sequences of PS-DRz1694 and PS-DRz1366 are shown in Table I; they were designed to be complementary in sequence to nt 1682~1701 of *mecR1* (GenBank accession no. X63598) and nt 1357~1377 of *blaR1* (GenBank accession no. M62650) in *S. aureus* respectively. Both PS-DRz1694 and PS-DRz1366 are 34-mers consisting of a central 10-23 catalytic core domain (15 nts) flanked by target-specific binding arms (19 nts), which were modified with phosphorothioates to increase nuclease resistance. Mismatched sequences PS-DRz5491 had been randomly aligned with the same number of bases, in which binding arms were also phosphorothioated.

In vitro cleavage of *mecR1* and *blaR1*

The k_{cat} and K_M values for PS-DRz1694 or PS-DRz1366 were determined under a wide range of single turnover conditions with the respective *mecR1* or *blaR1* RNA substrates. The cleavage products of *mecR1* and *blaR1* were obtained respectively (Figures 1 A1, 1 A2). Both DNAzymes exhibited similar catalytic kinetics and the PS-DRz1694 or PS-DRz1366 was able to cleave nearly 90% of *mecR1* or *blaR1* within 60 min in a cell-free system respectively (Figures 1 B1, 1 B2). Cleavage of *mecR1* RNA by PS-DRz1694 proceeded with the efficiency of k_{cat}/K_M of 4.55×10^6 M^{-1} min^{-1} and V_{max} at 3.316 nM/min. PS-DRz1366 showed k_{cat}/K_M value at 6.22×10^6 M^{-1} min^{-1} and V_{max} at 3.598 nM/min (Figure 1 C). These results indicated that anti-*mecR1* PS-DRz1694 and anti-*blaR1* PS-DRz1366 could effectively cleave RNA_{mecR1} and RNA_{blaR1}, respectively, and the cleavage efficiency of PS-DRz1366 was higher than that of PS-DRz1694.



C

PS-DRzymes	V_{max} [nMmin^{-1}]	K_M [nM]	k_{cat} [min^{-1}]	k_{cat}/K_M [M^{-1}min^{-1}]
PS-DRz1694	3.316	26.682	0.1215	4.55×10^6
PS-DRz1366	3.598	21.176	0.1318	6.22×10^6

Figure 1. *In vitro* cleavage activities of PS-DRz1694 and PS-DRz1366. (**A1**) Cleavage of $RNAs_{mecR1}$ by PS-DRz1694. (**A2**) Cleavage of $RNAs_{blaR1}$ by PS-DRz1366. Time course of the cleavage of *in vitro*, transcribed mRNAs of *mecR1* and *blaR1* cleaved by PS-DRz1694 (**B1**) and PS-DRz1366 (**B2**), respectively. (**C**) Kinetic parameters of the PS-DRz1694 and PS-DRz1366 reactions

Real-time quantitation assays for *mecA*/*blaZ* and *mecR1*/*blaR1* transcription

To ascertain whether the blockage of *mecR1* or *blaR1* also inhibits the expression of downstream gene *mecA*/*blaZ* in MRSA isolates, MRSA080302 or MRSA080305 with *mecI* or *blaI* deletions, and MRSA080309 with wild types of *mecI*/*blaI*, the anti-*mecR1* PS-DRz1694 or anti-*bla*R1 PS-DRz1366 was applied to each clinical isolate of MRSA respectively. The transcriptions of *mecR1*/*blaR1* and *mecA*/*blaZ* were detected by real-time PCR.

Compared with the control groups, the relative transcription of *mecR1* in three MRSA isolates (MRSA080302, MRSA080305 and MRSA080309) was decreased to 33%, 21%, and 18% of the control values by PS-DRz1694 treatment, respectively (Figure 2 A). And the relative transcription of *blaR1* of those MRSA isolates was decreased to 14%, 31%, and 9% of the control values by PS-DRz1366 treatment, respectively (Figure 2 B). These results demonstrated that *mecR1* or *blaR1* was blocked specifically by PS-DRz1694 or PS-DRz1366 respectively.

Compared with the control group, anti-*blaR1* PS-DRz1366 treatment caused 85% reduction for *mecA* expression and 72% of reduction for *blaZ* expression respectively in the *mecI* deleted strain MRSA080302 (Figures 2 C, 2 D). However, the expression of *mecA* and *blaZ* was not altered by treatment with anti-*mecR1* PS-DRz1694 alone in MRSA080302 (Figures 2 C, 2 D). A similar inhibition pattern on *mecA*/*blaZ* expression was observed in the *blaI* deleted strain MRSA080305 after anti-*mecR1* PS-DRz1694 treatment. The expression of *mecA* and *blaZ* in *blaI* deleted strain MRSA080305 showed 56% (Figure 2 C) and 32% (Figure 2 D) reduction after PS-DRz1694 treatment. But the expression of *mecA* and *blaZ* in MRSA080305 was not altered by PS-DRz1366. Meanwhile, the expression of *blaZ* or *mecA* in MRSA080309, a strain harbouring wild type *mecI-blaI*, was inhibited only by PS-DRz1366 or PS-DRz1694 respectively (Figure 2 C, 2 D).

Restoration of susceptibility to antibiotic in MRSA clinical isolates

We found that the downregulation of *mecR1* and *blaR1* by combined administration of anti-*mecR1*

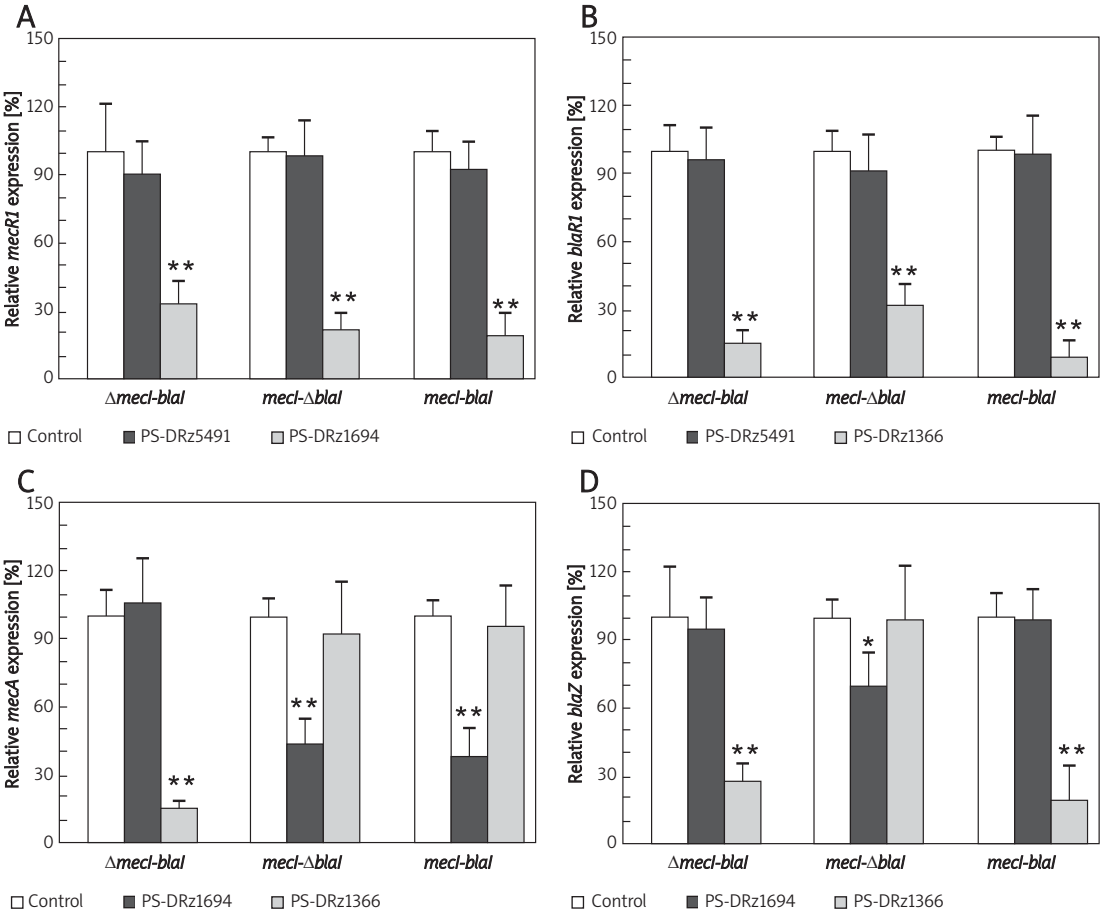


Figure 2. Comparison of expression of antibiotic resistant genes in three clinical MRSA isolates, MRSA080302 (Δ*mecI-blaI*), MRSA080305 (*mecI-*Δ*blaI*) and MRSA080309 (*mecI-blaI*). Relative mRNA expression of *mecR1* (**A**), *blaR1* (**B**), *mecA* (**C**), and *blaZ* (**D**)

p < 0.05,p < 0.01 vs. control, Δ indicates that the repressor is absent*

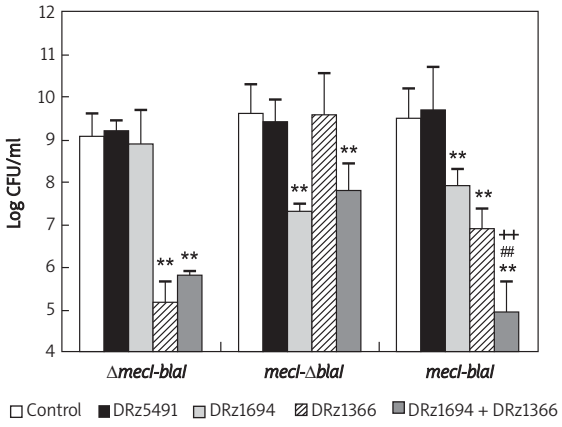


Figure 3. Effects of PS-DRz1694 or PS-DRz1366 on the growth of three clinical MRSA isolates – MRSA080302 (Δ*mecI-blaI*), MRSA080305 (*mecI-*Δ*blaI*) and MRSA080309 (*mecI-blaI*). DRz1366 group, DRz1694 group and DRz1694 + DRz1366 group vs. control **p < 0.05, **p < 0.01; DRz1694 + DRz1366 group vs. DRz1694 group, ⁺p < 0.05, ⁺⁺p < 0.01; DRz1694 + DRz1366 group vs. DRz1366 group, #p < 0.05, ##p < 0.01. The following are different treated groups: control, 10 mg/l PBS; DRz5491, 10 mg/l DRz5491; DRz1694, 10 mg/l DRz1694; DRz1366, 10 mg/l DRz1366; DRz1694 + DRz1366, 10 mg/l DRz1694 + 10 mg/l DRz1366. The data are shown as mean ± SD for 8 samples*

PS-DRz1694 and anti-*blaR1* PS-DRz1366 correlated with the restoration of susceptibility of MRSA clinical isolates to β-lactam antibiotics. The numbers of MRSA080302 colonies in PS-DRz1366 alone or PS-DRz1366 and PS-DRz1694 combination-treated cultures on Mueller-Hinton agar containing oxacillin (6 μg/ml) were reduced by 10^3- to 10^4-fold, respectively (Figure 3). The 10^2- to 10^3-fold reduction of MRSA080305 colonies was achieved by treatment of either PS-DRz1694 alone or PS-DRz1694 and PS-DRz1366 combination (Figure 3). In MRSA080309, oxacillin gave rise to a 10^4- or 10^5-fold striking reduction in CFU after PS-DRz1694 or PS-DRz1366 treatment, respectively (Figure 3). The combination of these two DNAzymes caused stronger effects on CFU reduction and led to a synergistic effect on the reversal of antibiotic resistance of MRSA080309 (Figure 3).

In liquid medium containing oxacillin (6 mg/l), the growth of PS-DRz1366-treated MRSA080302 and PS-DRz1694-treated MRSA080305 cells was inhibited, respectively (Figures 4 A, 4 B). However, the growth of MRSA080302 was not influenced by treatment with either PS-DRz1694 alone or mismatched PS-DRz5491 (Figures 3, 4 A). Similarly, the growth of MRSA080305 was not affected by treatment with either PS-DRz1366 alone or mismatched PS-DRz5491 (Figures 3, 4 B). The growth of MRSA080309 was inhibited by treatment with either PS-DRz1694 or PS-DRz1366 alone. Combination treatment of PS-DRz1694 and

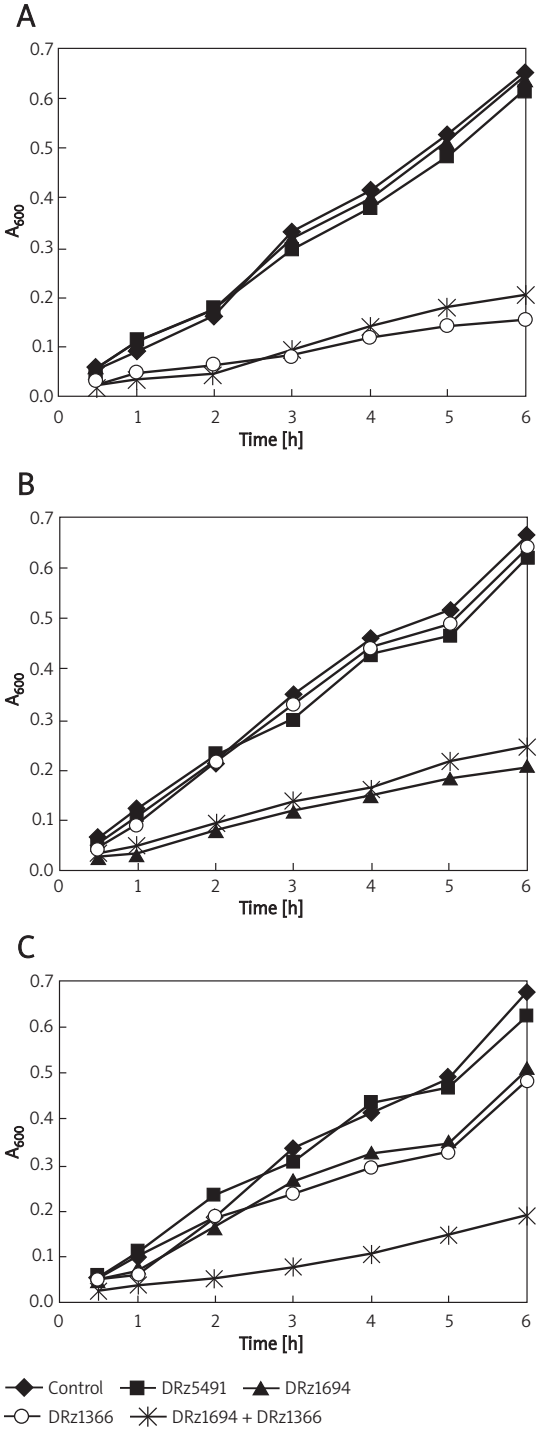


Figure 4. Effects of PS-DRz1694 or PS-DRz1366 on the growth of three clinical MRSA isolates in liquid medium (6 mg/l of oxacillin). The growth of different groups was monitored by using OD_{600} measurements. (**A**) The OD_{600} value of MRSA080302 in different treated groups. (**B**) The OD_{600} value of MRSA080305 in different treated groups. (**C**) The OD_{600} value of MRSA080309 in different treated groups. The following are different treated groups: control, 10 mg/l PBS; DRz5491, 10 mg/l DRz5491; DRz1694, 10 mg/l DRz1694; DRz1366, 10 mg/l DRz1366; DRz1694 + DRz1366, 10 mg/l DRz1694 + 10 mg/l DRz1366. The data are shown as mean ± SD for 5 samples

PS-DRz1366 resulted in the more significant restoration of susceptibility of MRSA080309 to oxacillin (Figures 3, 4C).

Meanwhile, PS-DRz1366 reduced the MIC of oxacillin for MRSA080302 (Δ*mecI-blaI*) from 1,024 mg/l to 1 mg/l and PS-DRz1694 reduced the MIC of oxacillin on MRSA080305 (*mecI*-Δ*blaI*) from 512 mg/l to 2 mg/l (Table III), both of which are within the oxacillin sensitivity range for the MRSA strains on the basis of the interpretive criteria recommended by the CLSI, representing the full restoration of MRSA susceptibility to oxacillin. The PS-DRz1366 or PS-DRz1694 alone did not alter the MIC of oxacillin for MRSA080305 or MRSA080302 respectively (Table III). On MRSA080309 strain harbouring wild type *mecI/blaI*, PS-DRz1694 or PS-DRz1366 only reduced the MIC of oxacillin from 1024 mg/l to 512 mg/l or from 1024 mg/l to 256 mg/l, respectively, which indicated a partial restoration of susceptibility of MRSA080309 to oxacillin. However, the combined administration of PS-DRz1694 and PS-DRz1366 to MRSA080309 caused a dramatic reduction on MIC of oxacillin from 1024 mg/l to 1 mg/l, which represented a full restoration of MRSA susceptibility to oxacillin. In addition, the MICs of oxacillin on MRSA080302 and MRSA080305 were decreased from 1024 mg/l to 2 mg/l and from 512 mg/l to 4 mg/l respectively under DRz1694 and DRz1366 combination treatment (Table III).

Discussion

Methicillin-resistant *Staphylococcus aureus* is resistant to all commercially available β-lactam antibiotics and also represents a therapeutic challenge, because effective therapeutic options are becoming limited. The expression of antibiotic-resistant genes *mecA* and *blaZ* is involved in antibiotic resistance of MRSA. Inhibition of bacterial gene expression by the antisense approach has been proposed as a promising strategy for bacterial infection therapy through targeting specific genes in bacteria [22-25]. Especially, catalytic oligodeoxynucleotides are valuable tools to downregulate the expression of resistant genes in a sequence-specific manner and have been widely applied *in vitro* and *in vivo* [26, 27].

In this study, we designed two deoxyribozymes (PS-DRz1694 and PS-DRz1366) specifically targeting *mecR1* and *blaR1* to restore susceptibility of clinical MRSA isolates. First, the consistency of catalytic activities of deoxyribozyme were demonstrated *in vitro* and *in vivo*. With the increase of the reaction time, the substrate *mecR1* or *blaR1* was cleaved into two cleavage products by the two DNAzymes *in vitro*, respectively. Then DNAzymes were electroporated into bacteria and the transcription levels of *mecR1* and *blaR1* were tested. The repression of *mecR1* or *blaR1* was observed in all MRSA strains treated by anti-*mecR1* or anti-*blaR1* DNAzyme respectively and showed specific catalytic activity of deoxyribozyme *in vivo*.

However, the inhibitory efficiency on the *mec* regulated *mecA* or *bla* regulated *blaZ* expression was different in all MRSA strains. In the *blaI* deleted strain MRSA080305 (*mecI*-Δ*blaI*), expression of both *mecA* and *blaZ* was decreased significantly by anti-*mecR1* DNAzyme PS-DRz1694, but not by anti-*blaR1* DNAzyme PS-DRz1366. Meanwhile in *mecI* deleted strain MRSA080302 (Δ*mecI-blaI*), anti-*mecR1* DNAzyme PS-DRz1366 caused significant reduction of *mecA* and *blaZ* expression. In *mecI-blaI* non-mutation strain MRSA080309, PS-DRz1366 or PS-DRz1694 only cleaved its target gene, *blaR1* or *mecR1*, and thereafter led to *blaZ* or *mecA* repression respectively. The outcomes indicated identically that *mecR1* cleaved by PS-DRz1694 could prevent the cleavage of *MecI*. Similarly, *blaR1* cleaved by PS-DRz1366 could merely prohibit the cutting of *BlaI*. So, we demonstrated that *mecR1* and *blaR1* are specific for their own homologous repressor and are not interchangeable in repressor

Table II. Oligonucleotide primers used for PCR

Genes	Primers	Primer sequence (5'-3')	Location [bp]	Size [bp]	Annealing temperature [°C]
mecR1	Forward	acacgacttcttcggttag	218-236	336	58
	Reverse	gtacaatttgggatttcact	534-553		
mecA	Forward	gcaatcgctaaagaactaag	553-572	225	58
	Reverse	aatgggaccaacataaccta	758-777		
blaR1	Forward	acaatgaagtagaagccgatagat	719-742	489	55
	Reverse	gtcggtcaagtccaaaca	1207-1190		
blaZ	Forward	agagatttgcctatgcttca	311-330	461	56
	Reverse	agtatctccgcttttattattt	771-750		
16SrRNA	Forward	gttattagggaagaacatatgtg	446-468	750	55
	Reverse	ccaccttcctccggtttgtcacc	1195-1173		

Table III. MICs of oxacillin in the presence/absense of PS-DRz1694/1366 in MRSA080302, MRSA080305 or MRSA080309 broth culture

Strain	Competent	Electroporation	PS-DRz1694 [mg/l]	PS-DRz1366 [mg/l]	MIC [mg/l]
ATCC29213	–	–	–	–	0.5
MRSA080302	–	–	–	–	1024
MRSA080302	+	+	–	–	1024
MRSA080302	+	+	10	0	1024
MRSA080302	+	+	0	10	1
MRSA080302	+	+	10	10	2
MRSA080305	–	–	–	–	512
MRSA080305	+	+	–	–	512
MRSA080305	+	+	10	0	2
MRSA080305	+	+	0	10	512
MRSA080305	+	+	10	10	4
MRSA080309	–	–	–	–	1024
MRSA080309	+	+	–	–	1024
MRSA080309	+	+	10	0	512
MRSA080309	+	+	0	10	256
MRSA080309	+	+	10	10	1

"+" Represents competent cells or electroporation processing; "–" refers to non-competent cells or no electroporation processing. The MIC of oxacillin to Staphylococcus aureus: sensitive (S): MIC ≤ 2 mg/l; resistant (R): MIC ≥ 4 mg/l

cleavage. Furthermore, PS-DRz1366-mediated uncleavage of MecI could effectively inhibit the expression of not only *mecA* but also *blaZ* in MRSA080302. It is the same for PS-DRz1694 in MRSA080305. Therefore, we demonstrated that *mecA* and *blaZ* can be mutually regulated by either MecI or BlaI, which might be the result of the fact that MecI and BlaI are almost identical and are interchangeable in repression of target gene transcription.

Further investigations on bacteria growth and susceptibility in the presence of oxacillin demonstrated a high correlation between targeted gene repression and MRSA growth suppression. The anti-*mecR1* PS-DRz1694 caused repression of *mecA* and *blaZ* in MRSA080305. Also, reduction of CFU and inhibition of the growth curve were observed in MRSA080305, but not in *mecI* deleted strain MRSA080302. In contrast, anti-*blaR1* PS-DRz1366 effectively inhibited the expression of *mecA* and *blaZ* in MRSA080302, and only inhibited MRSA080302 growth. A high correlation between the down-regulation of *blaR1*/*mecR1* and suppression of MRSA growth was also observed in non-*mecI-blaI* mutation strain MRSA080309 after treatment with PS-DRz1366 or PS-DRz1694 respectively. As both *mecR1-mecI-mecA* and *blaR1-blaI-blaZ* systems play roles in induction of antibiotic resistance in MRSA [13, 15, 28], blockade of each signal pathway only partially restored the susceptibility. The combination treatment of PS-DRz1694 and PS-DRz1366 on MRSA080309 resulted in synergic effects on susceptibility restoration to oxacillin.

It is particularly encouraging that MICs of oxacillin to MRSA080302, MRSA080305 and MRSA080309 were fully restored to values within the sensitivity defining range by PS-DRz1366, PS-DRz1694 and combined administration of these two DNAzymes, respectively (Table III). Although PS-DRz1366 and PS-DRz1694 only worked in certain MRSA strains, more importantly, we demonstrated that simultaneous administration of the two DNAzymes to knockdown *mecR1* and *blaR1* resulted in increased susceptibility of all MRSA strains tested in this study. This is thought to be accomplished by reducing the level of *mecA* and *blaZ*. When the levels of *mecA* and *blaZ* are lowered, the MRSA is not protected against oxacillin and the MRSA is killed.

The results of the present study indicate that co-blockade of *blaR1-blaZ* and *mecR1-mecA* signal pathways without detecting *mecI* or *blaI* mutation is a feasible strategy to restore the susceptibility of MRSA clinical strains to existing β-lactam antibiotics.

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