Antisense RNA Enhanced Sensitivity of Methicillin-resistant Staphylococcus Aureus to Hydrogen Peroxide

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Research article

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Abstract

Background: *Staphylococcus aureus* (*S. aureus*) is the leading cause of various infective diseases including topical soft tissue infections. Due to an increasing prevalence of the methicillin-resistant *Staphylococcus aureus* (MRSA), *S. aureus* infection become more challenging to being treated over recent years. The goals of this study were to investigate the roles of antisense *yycF/G* RNAs in the regulation of the biofilm formation and pathogenicity. **Methods:** MRSA AS*yycF/G* overexpression mutants were constructed. The biofilm biomass was determined by crystal violet microtiter assay and scanning electron mi-ceroscopy (SEM). Quantitative RT-PCR and western blotting analysis were used to detect the transcripts and translations of biofilm-related genes. The effects of the antisense RNAs (AS*yycF/G*) strategy on the susceptibility of biofilm-producing *S. aureus* to hydrogen peroxide were also investigated. **Result**: The AS*yycF/G* transcript leaded to reduction in the biofilm formation. Overexpression of AS*yycF/G* inhibited the transcripts and translations of biofilm-related genes. The sensibility to vancomycin was improved in AS*yycF/G* overexpression MRSA. **Conclusions**: The biofilm biomass and the transcripts of the pathogenicity associated genes decreased in the AS*yycF/G* overexpression mutant. Thus, the current evidence may provide a supplementary strategy for managing MRSA infections.

Introduction

Staphylococcus aureus (*S. aureus*), as Gram-positive opportunistic pathogen, is the leading cause of various infective diseases including topical soft tissue infections, osteomyelitis, and even high mortality endocarditis [1]. Due to an increasing prevalence of multi-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), *S. aureus* infection become more challenging to being treated over recent years [2]. Biofilm formation of MRSA is responsible for persistent infections and difficult to being eradicated as biofilm conditions which is much more resistant to environmental stimulus. Hydrogen peroxide (H_2O_2), as a common biocide, is used for cleaning and debriding chronic wound infections. However, the activity of H_2O_2 is significant decreased in against biofilm with drug resistant bacteria. Thus, targeting biofilm has become an alternative strategy to attenuate the pathogenicity of bacterium.

In bacteria, two-component signal transduction systems (TCSs) play an essential activity on adaptation to environment changes [3]. In *S. aureus*, 16 TCSs including the only essential YycFG are encoded contribute to the pathogenicity and virulence [4–5]. Commonly YycFG the only essential TCS also known VicRK/WalRK TCS was consisting of a sensor histidine protein kinase YycG and its cognate response regulator YycF [6–7]. Our previous study showed YycFG could modulate extracellular polysaccharide (EPS) synthesis via *ica operon*, which is associated with biofilm construction [7]. Hence, YycFG TCS represents a promising target to modulate *S. aureus* biofilm.

An antisense oligonucleotide can hybridize to the target mRNA by base-pairing recognition [9]. It can inhibit translation of the corresponding gene by targeting sequences within pre-mRNA as a kind of antimicrobial oligonucleotides to co-administered with biocides [9–10]. The *yycF* gene, as the first gene of *yycFG* operon [11], encodes YycF response regulator. From this point we designed an antisense *yycF*

(AS*yycF*) according to *yycF* sequence to inhibit the expression of *yycF*, which has a positive association with *ica* expression [7]. We hypothesis this AS*yycF* could enhance H_2O_2 bactericide effect on methicillin-resistant *S. aureus* by depression the construction of *ica*-dependent biofilm as a novel antimicrobial agent for infection elimination.

Methods And Materials

Bacterial strains and biofilms growth conditions

Methicillin-resistant *S. aureus* strain ATCC43300 provided by the Department of Laboratory Medicine (West China Hospital, Sichuan University, Chengdu, China) was cultured in tryptic soy broth (TSB). After overnight incubation at (37°C, 5% CO_2). Five hundred microfilters of *S. aureus* overnight suspension was inoculated into 10 mL fresh TSB medium to mid-logarithmic phase with OD_{600} value at 0.5. For the formation of biofilm, the sterilized glass disks (diameter in 14 mm) were applied for 24 hours biofilms for further analyses according to our previous study [12].

Construction of S. aureus antisense yycF/yycG overexpression strains

Recombination of plasmid pDL278 containing antisense *yycF* or *yycG* was conducted by inserting antisense *yycF* (AS*yycF*) or *yycG* (AS*yycG)* sequence into restriction sites between BamHI and EcoRI by Sangon Biotech (Shanghai, China). According to our previous study, an AS*yycF* overexpression methicillin-resistant *S. aureus* (AS*yycG* mutants) or an AS*yycG* overexpression methicillin-resistant *S. aureus* (AS*yycF mutants*) was constructed by adding recombinant pDL278 AS*yycG* or AS*yycF* plasmid. The empty pDL278 plasmid did not exert any effects on the viability of *S. aureus* [12].

Analysis of gene expression using quantitative real-time PCR (qRT-PCR)

MRSA, AS*yycG* and AS*yycF* strains were cultured to the midlogarithmic phase. Then, we treated the *S. aureus* planktonic cultures with 100mM H_2O_2 for 60 min, washed and resuspended in PBS (pH 7.2). The total RNAs were extracted and purified with the MasterPureTM RNA Purification Kit (Epicentre Technologies, Epicentre, Madison, WI, USA) including MRSA + H_2O_2 group, AS*yycG* + H_2O_2 group, and AS*yycF* + H_2O_2 group. The purified RNA was reverse transcribed to cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The quantitative real-time PCR assays were applied with LightCycler 480 system (Roche, Basel, Switzerland) with the primers listed in Table 1 with the 16Sr RNA gene as an internal control. Each sample was analyzed in triplicate, and the threshold cycle values (CT) were quantified [7].

Table 1
Sequences of primers in this study

Primers	sequence 5'-3' (Forward/Reverse)
RT-qPCR	
icaA	5'- GATTATGTAATGTGCTTGGA – 3'/
	5'- ACTACTGCTGCGTTAATAAT – 3'
yycF	5' - TGGCGAAAGAAGACATCA – 3'/
	5' – AACCCGTTACAAATCCTG- 3'
yycG	5' - CGGGGCGTTCAAAAGACTTT – 3'/
	5' - TCTGAACCTTTGAACACACGT – 3'
icaD	5' - ATGGTCAAGCCCAGACAGAG-3'/
	5' –CGTGTTTTCAACATTTAATGCAA – 3'
16S rRNA	5' - GTAGGTGGCAAGCGTTATCC – 3'/
	5' –CGCACATCAGCGTCAACA-3'

Western Blotting

For protein extraction, the bacterial cells including MRSA + H_2O_2 _group, AS*yycG* + H_2O_2 group, and AS*yycF* + H_2O_2 group were mechanically disrupted and collected by centrifugation as previously described [12]. For western blot analysis, the collected protein was probed with purified YycG- and YycF-specific antibodies (1:1000, HuaBio Biotechnology, Hangzhou, China). A BioRad GS-700 Imaging Densitometer was used to determine the signal density of Western blot bands for comparation.

Biofilms exposure to hydrogen peroxide

To determine the susceptibility of *S. aureus* ATCC43300, AS*yycG* mutants and AS*yycF* mutants to oxidants in biofilms conditions, all groups were treated with 3% H_2O_2 (for clinical irrigation, H_2O_2 is usually 3%) for 30 min at 37°C. Residual H_2O_2 was removed by diluting biofilms samples in PBS buffer. The treated biofilms samples were cultured in TSB medium for another 24 hours for further investigation, including MRSA + H_2O_2 group, AS*yycG* + H_2O_2 group, and AS*yycF* + H_2O_2 group.

The microtiter dish assay and epifluorescence staining for biofilm biomass

The microtiter dish assay was applied to evaluate the biomass of treated biofilm after cultured with another 24 hours with crystal violet (CV) following previous protocol [12]. The dye bound to the biofilms was transferred into a new plate and the absorbance was measured with a microplate reader (ELX800, Gene) under OD_{600} nm.

For epifluorescence staining, the biofilms were labeled with SYTO9 (LIVE/DEAD Bacterial Viability Kit reagent; BacLight, Invitrogen, Grand Island, NY, USA); live cells were stained green, while dead cells appeared red. The cells were visualized using epifluorescence microscopy (Nikon Eclipse TE-2000S, Melville, NY) at 40×magnification. Notably, three random fields in each specimen were selected.

Characterizing biofilm morphologies

The 24 hours biofilms of MRSA + H_2O_2 group, AS*yycG* + H_2O_2 group, and AS*yycF* + H_2O_2 group were labeled with SYTO9 (Invitrogen; Thermo Fisher Scientific, Inc.). The observation was implemented using epifluorescence microscopy (Nikon Eclipse TE-2000S, Melville, NY) at 40 ×magnification. Notably, five random fields in each specimen were selected. To assess the structure of biofilms, the scanning electron microscopy (Inspect, Hillsboro, OR, USA; SEM) was conducted. Briefly, the treated biofilm samples were diluted twice using PBS and fixed with 2.5% glutaraldehyde for 4 hours. Then the fixed samples were serially dehydrated and dried using a critical point dryer. After coated with gold powder, the micrographs of biofilm samples were evaluated.

Data analysis

All statistical data were analyzed in SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The Shapiro–Wilk test was used to analyze the distribution of data, and the Bartlett test was used to determine the homogeneity of variances. For parametric testing, we adopted a one-way ANOVA analysis to assess the statistical significance of variables followed by the Tukey test. The significant differences of data were set at $P < 0.05$.

Results

Antisense yycF **regulated the biofilm associated genes and protein in MRSA**

Quantitative RT-PCR analyses demonstrated that the expression levels of the *yycG* and *yycF* genes in were significantly reduced, which may be attributable to the overexpression of AS*yycF* in the *S. aureus* treated with H_2O_2. Furthermore, the expression levels of *icaA* related to the oxide stress reaction and biofilm-associated *ica* genes were more significantly reduced by AS*yycF* overexpression in the AS*yycG* strains treated with H_2O_2 than in the MRSA parent strains ($P < 0.05$; Fig. 1A). Consistently, the YycF protein expression was significantly downregulated in the AS*yycF* group treated with H_2O_2 compared with the MRSA parent strains (Fig. 1B). These results indicated that antisense *yycF* inhibited the production of the YycF, which may have contributed to reductions in downstream *icaA* gene expression.

Antisense yycF **sensitized MRSA to H_2O_2 intervention and vancomycin antibiotic**

The SEM results similarly showed the biofilm construction in ASyycF + H_2O_2 group most sparse than other groups (Fig. 2A). Vancomycin is the primary option for MRSA infections. By E-test, the sensitivity of MRSA to vancomycin decreased from 5 to 3 mg/L after AS*yycG* overexpression and 5 to 1 mg/L after

AS*yycF* overexpression (Fig. 2B). These results also showed that antisense *yycF* was much more effectively than antisense *yycG* on enhance the susceptibility of MRSA to H_2O_2.

The antibacterial effect of H_2O_2 was significantly enhanced by AS*yycF* and AS*yycG* compared with MRSA group even after 24hrs intervention with H_2O_2 and culture in TSB (Fig. 3A). Quantitively, we evaluated the ability of the MRSA strains to form biofilms after 24hrs intervention with H_2O_2 and culture in TSB. Immunofluorescence density for the dead/live ratios exhibited AS*yycF* + H_2O_2 group was at 0.3 around (Fig. 3B). The biomass was quantified via a microtiter dish assay, and AS*yycF* + H_2O_2 group exhibited more reduced biofilm formation compared with MRSA group from 1.4 to 0.8. Similarly, As*yycG* + H_2O_2 group reduced biofilm formation compared with MRSA group, but less effective than AS*yycF* + H_2O_2 group (Fig. 3C).

Discussion

Staphylococcus aureus is a major human pathogen, which is responsible for a wide range of infective diseases. With the development of antibiotic resistant strains such as MRSA, *S. aureus*-based infections are becoming more difficult to being treated [13]. The propensity of bacteria to produce biofilms was one of most essential factors, which contributed to the pathogenesis and resistance [14]. In this study, antisense *yycF* strategy was applied to MRSA strains to significantly down-regulate biofilm production associated YycFG pathway, which could as a promising strategy to overcome this biofilm developed infections.

Two-component systems (TCSs) were ubiquitously found in bacteria [15]. The highly conserved YycFG (also known as WalRK) TCSs reveal a major role in controlling biofilm formation in low-G + C gram-positive bacteria including *S. aureus* [7]. Antisense is a kind of complementary sequences hybridized to their target mRNA and inhibits the transcriptions of the corresponding genes [16]. In our study, the expression of YycFG TCS and biofilm production associated *ica* genes were accordingly down-regulated by antisense *yycF* or *yycG*. In *S. epidermidis*, a classical staphylococci bacterium, biofilm formation could also be inhibited via the blockade of extracellular domain of the YycG protein [17–18]. From this point, YycFG TCS may be a potential candidate for the eradication of staphylococci infections. YycG proteins act as a sensor sensing environmental signals and YycF could directly regulate different sets of vital functional genes by binding to the promoter regions [19]. Our results also showed antisense *yycF* not only depresses the sensitive of MRSA strains to environmental clues but also disrupted cytoplasmic adaptive activities by downregulation both YycF and YycG expressions.

In *S. aureus*, the only polysaccharide intercellular adhesin (PIA) is associated with *ica* locus [20]. PIA strongly contributes to biofilm formation leading resistance to antimicrobial agent. According to our previous study, *ica* expression was controlled by YycFG [7]. In our study, the antisense could cause a significant biofilm reduction by inhibiting YycFG as well as *ica* expression. Two-component signal transduction systems were essential in response to environmental stimuli, involving adaptation to

oxidative stress [21]. Therefore, there will be an adaptive regulation when occurred with H_2O_2 treatment. In present study, YycFG expression can be significantly decreased by AS*yycF*, even under oxidative stress intervention with H_2O_2. From this point, AS*yycF* has a potential as a kind of enhancer to improve the antibacterial efficiency of H_2O_2 by suppression resistance and adaption to oxidative stress. *S. aureus* is the leading cause of osteomyelitis with biofilm formation on the destructed bone matrix or necrotic bone, 1,000 times more tolerant to antibiotic and greatly contributions to cause of treatment recalcitrance were identified [22]. Without the shelter of biofilm, the susceptibility of pathogen was reversible [23]. The vancomycin was a gold treatment for clinical MRSA infections [24]. With the interruption of antisense, MRSA biofilms were significantly reduced without the shelter of biofilm, which enhanced the sensitivity of vancomycin. In addition, the resistance of hydrogen peroxide, which was a common biocide used for cleaning and debriding infections was destructed synergically by the antisense strategies [25].

Antisense oligonucleotides provide a therapeutic potential for treating infection disease [26]. However, nucleases are capable to degrading the antisense oligonucleotides rapidly, which limit their efficacy [27]. Hydrogel scaffolds such as alginate-based hydrogel has been designed for infection inducing bone defects [28]. They could be employed as gene carrier's protection from the inflammation-mediated degradation and control release [29]. Our previous study showed nano sized platform such as graphene oxide as a kind of nano knife could significantly improving the transformation effectiveness of antisense oligonucleotides [30]. Therefore, further investigations on composition nano material functioned hydrogel will widen the possibility of antisense strategy for clinical infection control.

Conclusions

In the current study, overexpression of AS*yycF/G* significantly downregulate biofilm formation and the transcripts of the pathogenicity associated genes. Also, the sensibility to vancomycin was improved in AS*yycF/G* overexpression MRSA. Furthermore, the antisense RNAs, as post-transcriptional regulators, reveal a potential supplementary strategy for managing MRSA infections.

Abbreviations

S. aureus
Staphylococcus aureus;
ASyycG
antisense yycG RNA;
PIA
Polysaccharide intercellular adhesion;
S. epidermidis
Staphylococcus epidermidis;
TCSs
two-component signal transduction systems;
RR

response regulator;
ASRNA
Antisense RNA;
EMSA
electrophoretic mobility shift assay;
EPS
Extracellular polysaccharide substance;
PIA
Polysaccharide intercellular adhesion;
TSB
Tryptic soy broth; CSP:Competence stimulating peptide;
KEGG
Kyoto Encyclopedia of Genes and Genomes;
CV
Crystal violet;
CFU
Colony-forming units;
SEM
Scanning electron microscopy;
qRT-PCR
Quantitative real-time polymerase chain reaction;
CT
Cycle values;
PVDF
Polyvinylidene fluoride;
APS
Ammonium persulfate;
TEMED
tetramethylethylenediamine;
sarA
Staphylococcal accessory regulator;
yycG
membrane-bound sensor histidine kinase associated with cell wall metabolism

Declarations

Ethics approval and consent to participate:Not applicable.

Consent for publication: All authors understand that the text and any pictures or videos published in the article, and give our consent for information to be published in JOSR.

Availability of data and materials: All data generated or analyzed during this study are included in this published article. The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare no conflict of interest.

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Figures

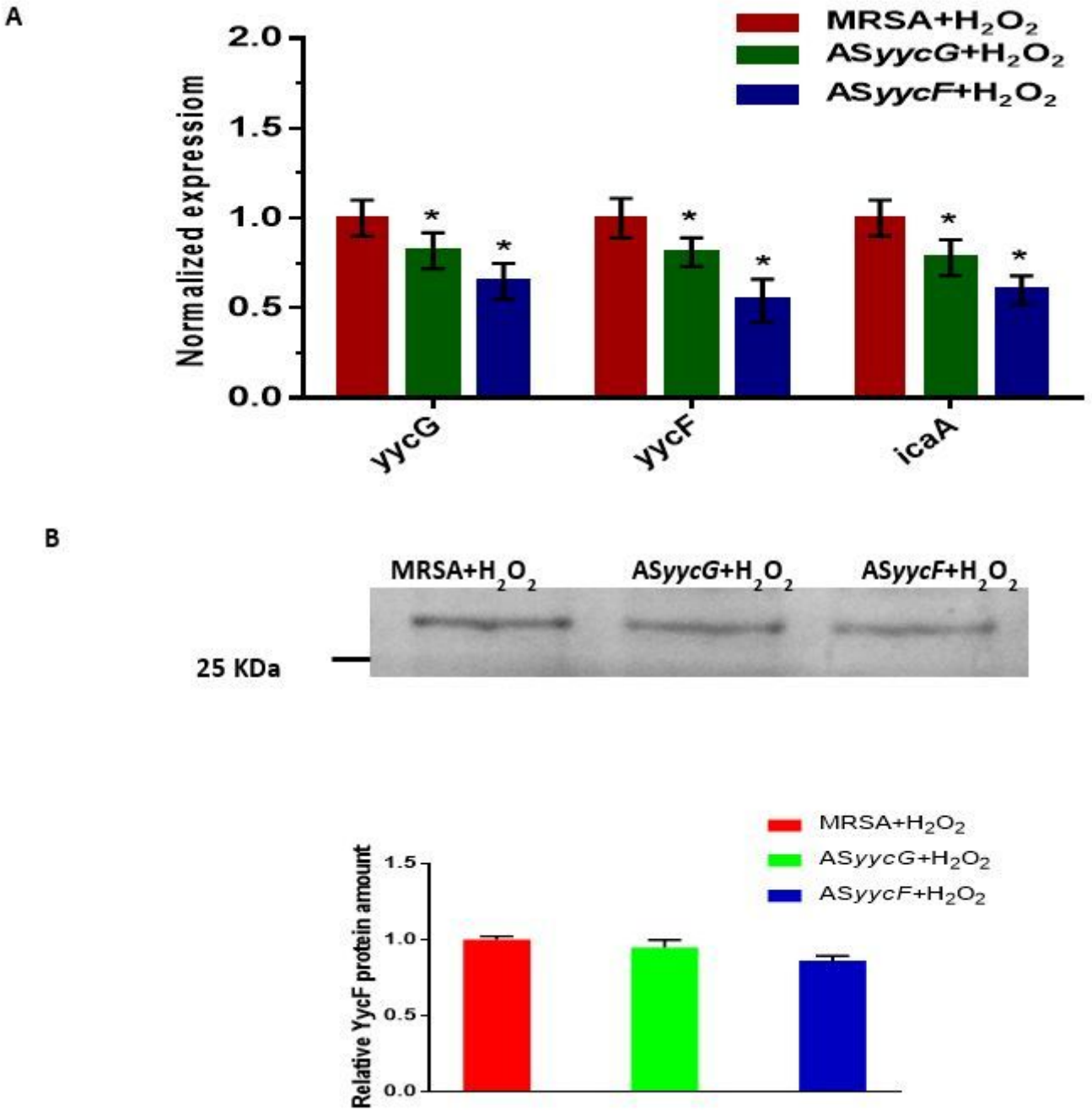

Figure 1

ASyycF/G overexpression inhibited the transcription of biofilm-related genes when treated with H2O2. A. Quantitative RT-PCR analysis showed the gene transcription in S. aureus, using 16S as an internal control (n=5, *P<0.05); B. The productions of YycF were quantified in the cells of S. aureus for Western blotting (upper lane); the lower panel showed Quanti-tative analysis for the relative YycF protein amounts.

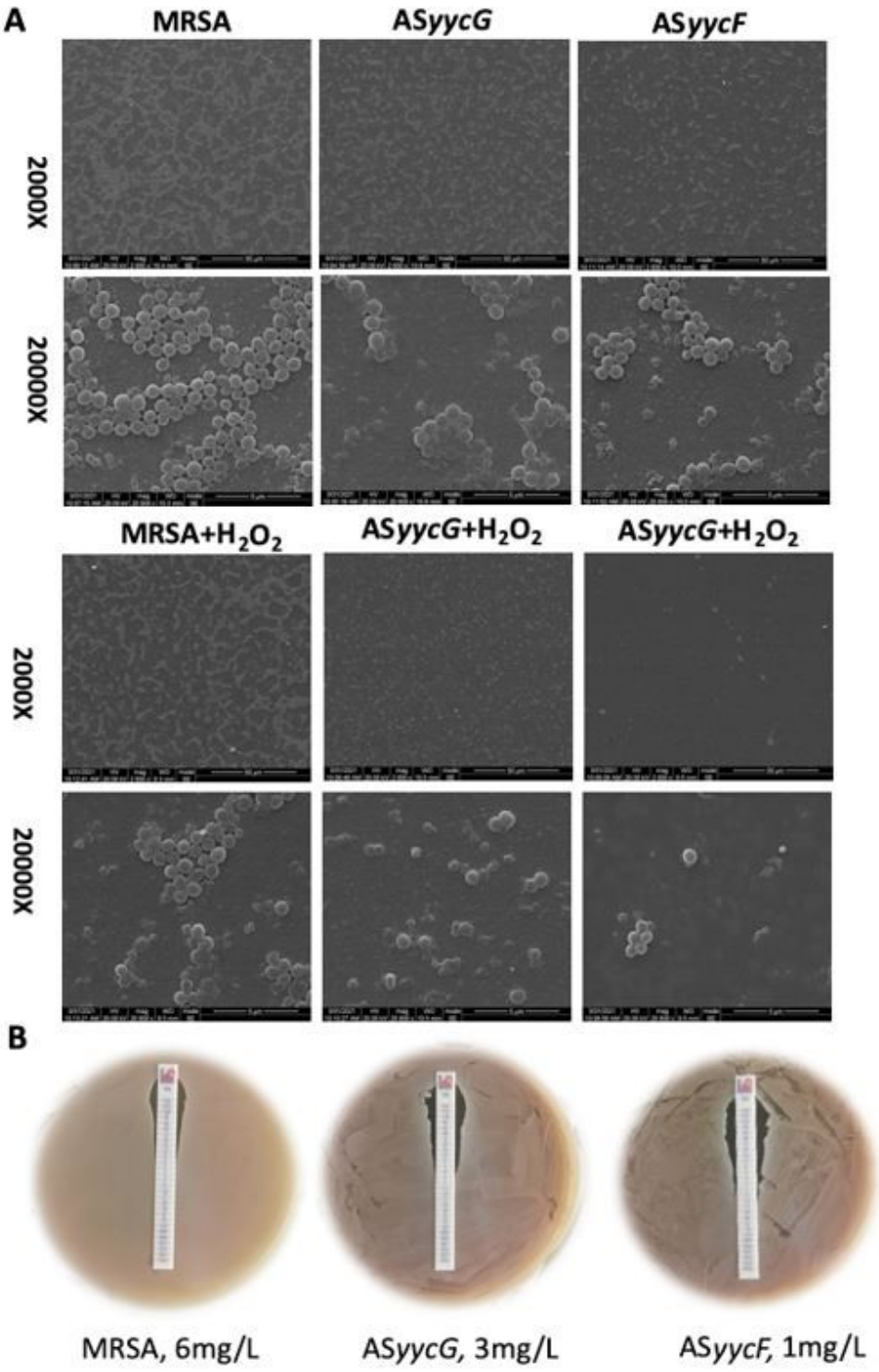

Figure 2

ASyycF/G modulated the biofilm organization and antibiotics sensitivity A. SEM images of MRSA strains after ASyycF or ASyycG overexpression; B. E-test for the sensitivity of MRSA to vancomycin.

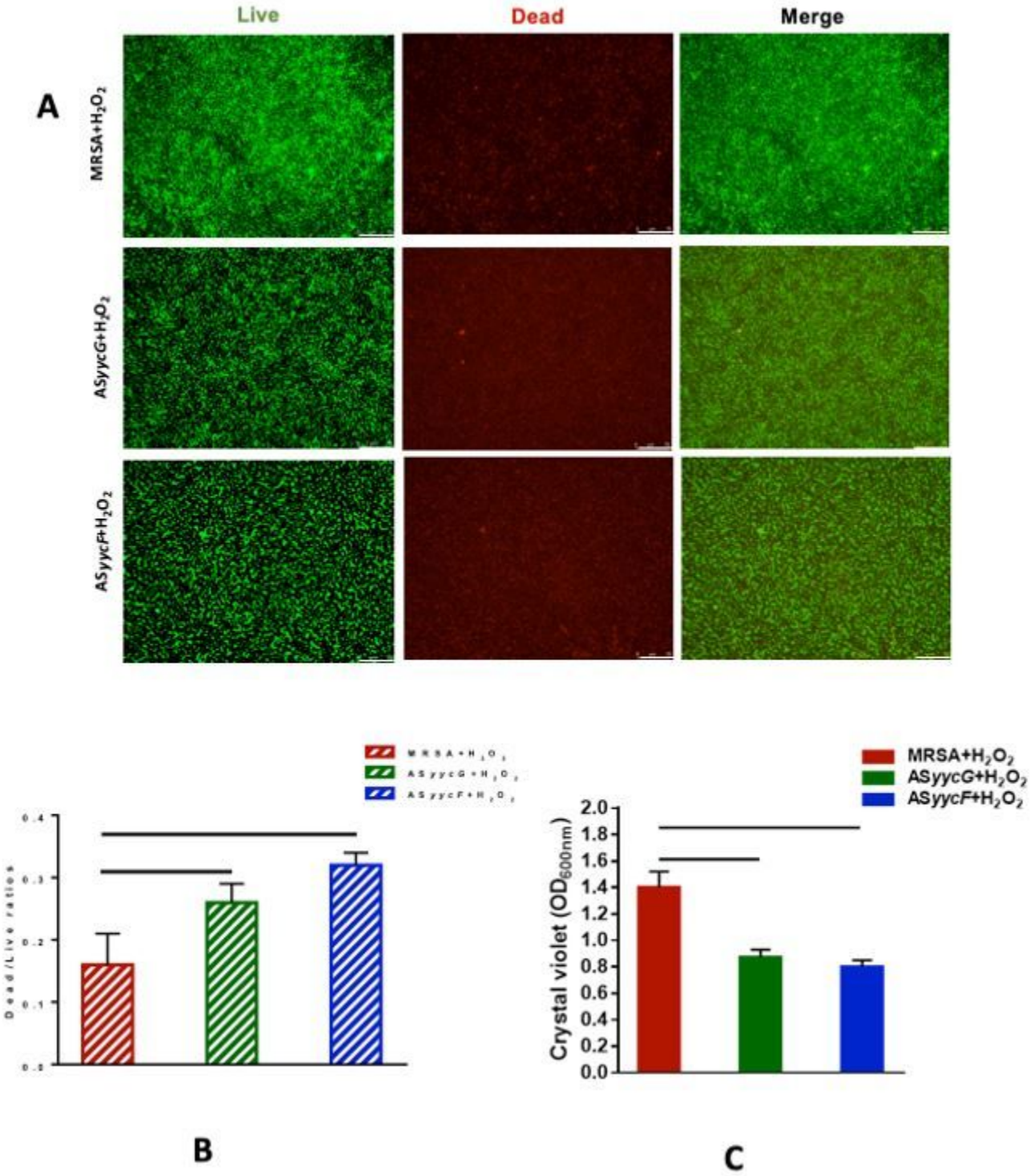

Figure 3

ASyycF/G overexpression inhibited the bacterial activities when treated with H2O2. A. Intensity of fluorescence for MRSA strains after ASyycF/G overexpression (Scale bar= 100μm). B. Immunofluorescence density for the dead/live ratios (n=5, *P<0.05). C. Biomass was quantified by crystal violet staining. Optical densities at 600 nm were measured (n=5, *P<0.05).