RNA-Seq-based transcriptome analysis of methicillin-resistant Staphylococcus aureus biofilm inhibition by ursolic acid and resveratrol

Nan Qin1*, Xiaojuan Tan2*, Yinning Jiao3*, Lin Liu1,4, Wangsheng Zhao5, Shuang Yang4 & Aiqun Jia2

1State Key Laboratory for Diagnosis and Treatment of Infectious Disease, the First Affiliated Hospital, Zhejiang University, Hangzhou 310003, China, 2School of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing 210094, China, 3Department of Bioinformatics, State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou 310058, China, 4NGS Sequencing Department, Beijing Genomics Institute (BGI), Shenzhen 518083, China, 5Clinical Laboratory Department of the First Affiliated Hospital, Nanjing Medical University, Nanjing 210029, China.

Bacterial biofilms are particularly problematic since they become resistant to most available antibiotics. Hence, novel potential antagonists to inhibit biofilm formation are urgent. Here the influences of two natural products, ursolic acid and resveratrol, on biofilm of the clinical methicillin-resistant Staphylococcus aureus (MRSA) isolate were investigated using RNA-seq, and the differentially expressed genes were analyzed using Cuffdiff. The results showed that ursolic acid inhibition of biofilm formation may reduce amino acids metabolism and adhesins expression and resveratrol may disturb quorum sensing (QS) and the synthesis of surface proteins and capsular polysaccharides. In addition, the transcriptome analysis of resveratrol and the combination of resveratrol with vancomycin inhibition of established biofilm revealed that resveratrol would disturb the expression of genes related to QS, surface and secreted proteins, and capsular polysaccharides. These findings suggest that ursolic acid and resveratrol could be useful to be adjunct therapies for the treatment of MRSA biofilm-involved infections.

Methicillin-resistant Staphylococcus aureus (MRSA) is an important human pathogen that causes abscesses in many organ tissues, septicemia, and even life-threatening infections1. Many reports have shown that staphylococcal infections were associated with biofilm formation2,3. Bacterial biofilms are particularly problematic because sessile bacteria can often withstand a host’s immune response and the bacteria within biofilms become resistant to most available antibiotics4. Hence, the research and development of novel potential antagonists to inhibit biofilm formation or remove established biofilm is both urgent and necessary.

Increasing evidence suggests that quorum sensing (QS) is important for the construction and/or dissolution of biofilm communities5. As such, QS inhibitors (QSIs) have the potential to be used to be adjuvants in antimicrobial therapy. In addition, some QSIs could be used to suppress the emergence and spread of bacteria-forming biofilm6.

To develop less or non-toxic natural products (such as QSIs) that inhibit MRSA biofilm formation or remove established biofilm, hundreds of natural compounds isolated from traditional Chinese medicines were tested by our group. Ursolic acid inhibited MRSA biofilm formation but has no impact on established biofilm (Supplementary Fig. S1a online, Supplementary Table S1 online), whereas resveratrol inhibited MRSA biofilm formation and removed partially established biofilm (Supplementary Fig. S1b online, Supplementary Table S2 online).

Studies on global S. aureus biofilm transcriptional profiles suggested that planktonic and biofilm cultures showed distinct patterns of gene expression using microarray7-9. However, few papers have reported on MRSA biofilm treated with drugs at the genetic level using microarray5,6. In the current study, we quantitatively compared the abundance of each gene under different conditions and pooled and normalized fragments of each sample data size. The resulting number, fragments per kilobase of transcript per million mapped fragments (FPKM), allows for comparisons between differently expressed genes and growth conditions10. Here, to further investigate the mechanisms of inhibiting biofilm formation or removing
Results

MICs and MBCs determination. The susceptibilities of the MRSA planktonic cells to ursolic acid and resveratrol were determined in vitro by methods recommended by the CLSI. The MICs and MBCs are given in Supplementary Table S3. The MIC of ursolic acid against the MRSA was lower (37 μg/mL) than resveratrol (350 μg/mL). In addition, the MBC of resveratrol against the MRSA was higher (>800 μg/mL) than ursolic acid (175 μg/mL).

Resveratrol and ursolic acid inhibit MRSA biofilm formation. The in vitro effects of resveratrol and ursolic acid on MRSA biofilm formation were investigated using crystal violet staining semi-quantitative assays and scanning electron microscopy (SEM) images. MRSA was able to form biofilm on 96-well plates after 18 h incubation. The SEM images also showed that MRSA formed thick, heterogenous clumps on the coverslip (Fig. 1a). Crystal violet staining assays revealed that the inhibiting effect of ursolic acid on MRSA biofilm formation was stronger (66.3%) than that of resveratrol (39.85%) (Table 1, Supplementary Fig. S2 online). Moreover, the SEM images further qualitatively showed that resveratrol and ursolic acid could inhibit MRSA biofilm formation (Fig. 1b,c).

Quantitative analysis of the global genes expression after treatment MRSA biofilm. In this study, the expression level distribution of all genes revealed that samples in the inhibitory biofilm formation conditions (MR100 & MU30) showed more differences than those samples in the removed established biofilm conditions (MR150 & MVR) (Supplementary Figs. S4–S5 online). The global expression level distribution profiles for mRNA isolated from all the test conditions were shown in Fig. 2 (Supplementary Fig. S3 online). The number of mapped cDNA reads, 12.1–30.0 million (100 bp each) per sample, totaled 17,693,687,500 bases of sequenced cDNA (Table 2). Using the Staphylococcus aureus COL genome as a reference, all of the MRSA transcripts were assignable to the genome. Each sample yielded a high number of mRNA reads (up to 90.8%). A total of 15,773,232,900 bp sequences of cDNA from mRNA were mapped along the relative position through the genome without bias (Supplementary Fig. S3 online). The distribution of the reads in each sample could be randomly mapped along the relative position through the genome without bias (Supplementary Fig. S3 online). The number of mapped cDNA reads, 12.1–30.0 million (100 bp each) per sample, totaled 17,693,687,500 bases of sequenced cDNA (Table 2). Using the Staphylococcus aureus COL genome as a reference, all of the MRSA transcripts were assignable to the genome. Each sample yielded a high number of mRNA reads (up to 90.8%). A total of 15,773,232,900 bp sequences of cDNA from mRNA were mapped along the relative position through the genome without bias (Supplementary Fig. S3 online). The distribution of the reads in each sample could be randomly mapped along the relative position through the genome without bias (Supplementary Fig. S3 online). The number of mapped cDNA reads, 12.1–30.0 million (100 bp each) per sample, totaled 17,693,687,500 bases of sequenced cDNA (Table 2). Using the Staphylococcus aureus COL genome as a reference, all of the MRSA transcripts were assignable to the genome. Each sample yielded a high number of mRNA reads (up to 90.8%). A total of 15,773,232,900 bp sequences of cDNA from mRNA were mapped along the relative position through the genome without bias (Supplementary Fig. S3 online). The distribution of the reads in each sample could be randomly mapped along the relative position through the genome without bias (Supplementary Fig. S3 online). The number of mapped cDNA reads, 12.1–30.0 million (100 bp each) per sample, totaled 17,693,687,500 bases of sequenced cDNA (Table 2). Using the Staphylococcus aureus COL genome as a reference, all of the MRSA transcripts were assignable to the genome. Each sample yielded a high number of mRNA reads (up to 90.8%). A total of 15,773,232,900 bp sequences of cDNA from mRNA were mapped along the relative position through the genome without bias (Supplementary Fig. S3 online). The distribution of the reads in each sample could be randomly mapped along the relative position through the genome without bias (Supplementary Fig. S3 online). The number of mapped cDNA reads, 12.1–30.0 million (100 bp each) per sample, totaled 17,693,687,500 bases of sequenced cDNA (Table 2). Using the Staphylococcus aureus COL genome as a reference, all of the MRSA transcripts were assignable to the genome. Each sample yielded a high number of mRNA reads (up to 90.8%). A total of 15,773,232,900 bp sequences of cDNA from mRNA were mapped along the relative position through the genome without bias (Supplementary Fig. S3 online). The distribution of the reads in each sample could be randomly mapped along the relative position through the genome without bias (Supplementary Fig. S3 online).

| Table 1 | Quantification of the treatment of methicillin-resistant Staphylococcus aureus (MRSA) biofilm with different compounds |
|---|---|---|
| Condition | Compound | Inhibitory rate |
| Inhibiting biofilm formation | Ursolic acid 30 μg/mL (MU30) | 66.30% ± 0.18 |
| | Resveratrol 100 μg/mL (MR100) | 39.85% ± 0.15 |
| | The combination of resveratrol and vancomycin (MVR) | 55.43% ± 0.17 |
| Removing established biofilm | Ursolic acid 30 μg/mL (MU30) | 66.30% ± 0.18 |
| | Resveratrol 100 μg/mL (MR100) | 39.85% ± 0.15 |
| | Vancomycin 8 μg/mL (MV) | 27.71% ± 0.13 |
| | The combination of resveratrol and vancomycin (MVR) | 55.43% ± 0.17 |
regulated in the removed conditions (Fig. 2c, fifth and sixth circles) but there were a few differences in the inhibitory conditions (Fig. 2a,b, third circles). The chemical structure of resveratrol had no correlation with that of ursolic acid, so we performed the differential expression analyses independently between MR100 and MU30 (Fig. 2a,b).

The variance analysis package Cuffdiff was used to systematically search the transcriptome data for the subset of genes with statistical significance \((P < 0.05\), false discovery rate \((FDR) < 0.001; >2\)-fold differential expression change in treatment conditions versus controls)\(^{33}\). Although the expression levels of most genes did not differ markedly when the cells were obtained after treatment (Supplementary Fig. S6 online), the significant differences for the expression levels of some genes were established by the use of Cuffdiff on FPKM values in both conditions. In the inhibiting MRSA biofilm formation conditions, 193 genes were up-regulated and 48 genes were down-regulated in both samples. We also found that most differentially

### Table 2 | Summary of methicillin-resistant Staphylococcus aureus (MRSA) cDNA samples

| Sequenced sample | Total mapped reads | Total mapped mRNA reads | mRNA reads (% of all mapped reads) | Notes | Condition |
|------------------|--------------------|-------------------------|------------------------------------|-------|-----------|
| M18              | 19,937,650         | 17,232,901              | 86.4                               | Free-compound sample | In the inhibiting biofilm formation conditions |
| M18C06           | 13,205,414         | 11,715,293              | 88.7                               | Sample containing 0.6% ethanol resveratrol | |
| MR100            | 19,350,636         | 17,571,197              | 90.8                               | Sample containing 100 µg/mL vancomycin | |
| MU30             | 12,147,718         | 10,780,072              | 88.7                               | Sample containing 30 µg/mL ursolic acid | |
| M36              | 26,298,604         | 23,114,110              | 89.1                               | Free-compound sample | In the removing established biofilm conditions |
| MV               | 21,020,644         | 18,719,520              | 90.5                               | Sample containing 8 µg/mL vancomycin | |
| M36C05           | 20,477,274         | 18,531,582              | 90.6                               | Sample containing 0.5% ethanol | |
| MR150            | 14,487,371         | 13,127,118              | 90.0                               | Sample containing 150 µg/mL resveratrol | |
| MVR              | 30,011,564         | 26,940,536              | 90.0                               | Sample containing 150 µg/mL vancomycin | |
| Sum              | 176,936,875        | 157,732,329             | 89.1                               | | |

Key genes associated with MRSA biofilm formation. Notably, the sets of genes that were highly up-regulated and down-regulated in both conditions included some key known genes encoding virulence factors, surface proteins, capsular polysaccharides, and others related to biofilm formation of \(S. aureus\) (Fig. 5, Supplementary Tables S13–S14 online).

In the inhibiting biofilm formation conditions, \(hld\) encoding \(\delta\)-hemolysin was up-regulated in two samples, while \(rsbU\) encoding sigma factor B regulator protein was only up-regulated in MR100. Both genes could secrete virulence factors in \(S. aureus\). Staphylococcal protein A (\(spa\)), which encoded the protein A, was up-regulated in MR100. Genes (\(cap5ABC\)) mediating the synthesis of capsular polysaccharides were down-regulated in MR100. However, genes encoding adhesins (\(issB, srtB, ebh, \) and \(sdrC\)) were down-regulated in MU30. In addition, genes related to metabolism (\(arcA, arcB, arcD,\) and \(aur\)), which are important to biofilm survival, were also down-regulated in the MU30 except for genes associated with purine metabolism (\(purN, purH,\) and \(purD\)) (Fig. 5, Supplementary Table S13 online).

In the removing established biofilm conditions, genes associated with the QS systems of \(S. aureus\) (\(agrA, agrB, agrC, hld,\) and \(sra\)) were down-regulated in MR150 and MVR (Fig. 5c, 6, Supplementary Table S14 online). Moreover, genes encoding surface and secreted proteins (\(spa, sek, sdrD,\) and \(efb\)) were up-regulated in both samples. All \(cap\) genes were highly down-regulated in both samples, but \(cap5M\) and \(cap5N\) were not differentially expressed in MVR. Meanwhile, genes associated with biofilm survival (\(arcA, carA, carB, pyrB, pyrC, pyrE, pyrF, pyrG,\) and \(pyrR\)) were up-regulated in both samples. We also found that most differentially
to M36C05 sample, the level of expression of
fold. The levels of expression of
expression of
hld
SCIENTIFIC
and
spa
sample compared to M36C05 sample, the levels of expression of
seq experiment were determined by real-time RT-PCR. In MR150
tions, the expression of six genes associated with
expressed in MR150 and MVR (Fig. 5c, Fig. 6, Supplementary
related to virulence and biofilm formation, were differentially
expressed genes related to virulence and biofilm in MR150 were
differentially expressed genes in the resveratrol inhibiting methicillin-
medium, and low expression, respectively.

Figure 2 | Distribution of differentially expressed genes in inhibitory and
removed conditions Red, yellow, and blue correspond to genes with high,
medium, and low expression, respectively. (a) Distribution of
differentially expressed genes in the resveratrol inhibiting methicillin-
resistant Staphylococcus aureus (MRSA) biofilm formation condition. From outside to inside, the three circles in each plot correspond to the follow:
(1 and 2) log2 of the fragments per kilobase of transcript per
million mapped fragments (FPKM) of each gene in MR100 and M18C06;
(3) genes differentially expressed in MR100 compared with M18C06.
(b) Distribution of the differentially expressed genes in ursolic acid
inhibitory MRSA biofilm formation condition. From outside to inside, the
tree circles in each plot correspond to the follow: (1 and 2) log2 of
FPKM each gene in MU30 and M18C06, respectively; (3) genes
differentially expressed in MU30 compared with M18C06. (c) Distribution
of the differentially expressed genes in the resveratrol and its combination
with vancomycin removed established MRSA biofilm conditions. From
outside to inside, the six circles in each plot correspond to the follow:
(1, 2, and 3) log2 of FPKM of each gene in MVR, MR150, and M36C05,
respectively; (4) genes differentially expressed in MVR compared with
MR150; (5 and 6) genes differentially expressed in MVR and MR150
compared with M36C05, respectively.

genes were partially down-regulated. As
above key genes, other key genes, such as clfA and spVG genes
related to virulence and biofilm formation, were differentially
expressed in MR150 and MVR (Fig. 5c, Fig. 6, Supplementary
Table S14 online).

In addition, in the removing established MRSA biofilm condi-
tions, the expression of six genes associated with S. aureus biofilm
that were up- or down-regulated by more than twofold in the RNA-
seq experiment were determined by real-time RT-PCR. In MR150
sample compared to M36C05 sample, the levels of expression of sdrD
and spa increased by 2.55- and 1.45-fold, respectively. The levels of
expression of hld, agrA, and cap5C changed slightly. The level of
expression of cap5B decreased by 10-fold. In MVR sample compared
to M36C05 sample, the level of expression of sdrD increased by 7.13-
fold. The levels of expression of hld, cap5B, and cap5C decreased by
17.87-, 16.24-, and 5.67-fold, respectively (Supplementary Fig. S9
online). In general, the trends in regulating gene expression by
RNA-seq technique are consistent with the results of real-time RT-
PCR.

Discussion

Staphylococcus aureus subsp. aureus COL (S. aureus COL), a MRSA
strain, is also resistant to several antibiotics including penicillin and
tetracycline13 and shares the same phenotype as the MRSA strain. In
addition, all of the MRSA transcripts were assignable to the S. aureus
COL genome, which illustrated the suitability of using the S. aureus
COL genome as a reference here. In addition, the next generation
sequencing approach resulted in a massive amount of mRNA
informative reads to a level at which all genes could be covered.
The distribution of reads could be randomly mapped along the rela-
tive position through the genome in each sample (Supplementary
Fig. S3 online), which illustrated the suitability of using RNA-seq for
bacterial transcriptomic studies without bias and for the following
comparative transcriptome analysis.

It has become clear that there are at least two mechanisms of
biofilm formation in S. aureus14: one requires the production of
the polysaccharide intercellular adhesion proteins, which is encoded by
the ica locus15, and the other is an ica-independent mechanism16.
Alternatively, we did not find any icaADBC genes that showed sig-
nificant changes in inhibitory or removed conditions. As such, the
biofilm formation of the clinical MRSA isolate studied here may
belong to an ica-independent mechanism. Meanwhile, some of the
best studied factors involved in the ica-independent biofilm pro-
cesses are accessory gene regulator (agr) and staphylococcal access-
ory regulator A (sara) global regulatory systems17. The agr locus of S.
aureus is one QS cluster of five genes (hld, agrB, agrD, agrC, and
agrA) that up-regulates the production of secreted virulence factors,
including the α-, β-, and δ-hemolysins18. Of these virulence factors,
δ-hemolysin, which is encoded by hld and derived from translation
of RNA III, is uniquely regulated by agr operon19. Moreover,
Sakoulas et al. indicated that δ-hemolysin production could be used as
a maker of agr function20.

In the inhibiting biofilm formation conditions, we only found that
hld of the QS systems was up-regulated, which indicates that resver-
arl and ursolic acid may enhance MRSA agr function at the RNA
level to inhibit biofilm formation. Pratten et al. discovered that hld
expression was the highest at the base of the biofilm, where the high-
est numbers of bacteria were also observed21. However, Resch et al.
reported that toxins were up-regulated in planktonic growth condi-
tions. In addition, Vuong et al. proposed that δ-hemolysin might
also serve as a surfactant in vivo that prevents the adherence of
staphylococcal cells to surfaces22. Surfactants may also decrease the
adherence of staphylococci to biomaterials. Based on the results in
this study, the conclusion that hld expression was up-regulated
in MR100 and MU30 is appropriate. Moreover, rsbU encoding viru-
ence factor was up-regulated in MR100, which also corresponds to
the discovery of Resch et al. The expression level of spa was up-
regulated in MR100, which was consistent with the microarray and
real-time polymerase chain reaction comparison results23. In addi-
tion, cap genes, which mediate the synthesis of capsular polysacchar-
ides inducing human infections24, are important to S. aureus biofilm
formation. In MR100, cap genes were partially down-regulated. As
such, the above analysis suggested that resveratrol inhibited MRSA
biofilm formation by disturbing QS systems and the synthesis of
surface proteins and capsular polysaccharides, while ursolic acid
inhibited MRSA biofilm formation by reducing the metabolism of
some amino acids as well as the expression of adhesins. We also
speculated that the mechanism by which ursolic acid inhibited
MRSA biofilm formation could differ from that by which it inhibited
Escherichia coli biofilm formation, which may affect sulfur metabol-
ism (through cysB)25,26.
To date, two staphylococcal QS (SQS) systems have been described. Here we provide a relationship graph of SQS in *S. aureus* according to some studies (Supplementary Fig. S10 online). In the removing established MRSA biofilm conditions, the genes associated with QS were differentially expressed. Therefore, the use of resveratrol or its combination with vancomycin could reduce MRSA virulence and biofilm by impacting on the QS systems. Korem *et al.* demonstrated that in the absence of target of RNAIII-activating protein expression or phosphorylation, the gene expression levels for biofilm survival were reduced in *arcABC, pyrR, pyrP, pyrB, pyrC, carA,* and *carB* using microarray. In this study, however, *arcA, carA, carB, pyrB, pyrC, pyrE, pyrF, pyrG,* and *pyrR* were up-regulated in both MR150 and MVR (Supplementary Table S14 online), a finding that was contradictor to the microarray results, which may result from different phenotype between *S. aureus*. The expression levels of genes encoding surface and secreted proteins were up-regulated, a finding that is consistent with the microarray results. Reduced capsule gene expression may render an organism more sensitive to the host’s immune response, namely phagocytosis, which may result in more rapid clearance of infection. In MR150 and MVR, the reduction expression levels of genes encoding the synthesis of capsular polysaccharides is consistent with the microarray results. Therefore, the mechanisms of resveratrol and its combination with vancomycin inhibiting the development of established MRSA biofilm may impact on the expression levels of genes related to QS, surface proteins, secreted proteins as well as the synthesis of capsular polysaccharides. Moreover, there are some genes associated with virulence and biofilm, such as *clfA* and *spoVG*. In

![Volcano plots of the distribution of gene expression for methicillin-resistant *Staphylococcus aureus* (MRSA) in the inhibitory and removed conditions.](image-url)

Figure 3 | Volcano plots of the distribution of gene expression for methicillin-resistant *Staphylococcus aureus* (MRSA) in the inhibitory and removed conditions. Cuffdiff analysis was performed to show the differentially expressed genes. Green, red, and gray correspond to genes with >2 log₂-fold differential expression, >1 log₂-fold differential expression, and <1 log₂-fold differential expression, respectively. (a) Distribution of differentially expressed genes in MU30 compared with M18C06, (b) distribution of differentially expressed genes in MR100 compared with M18C06, (c) distribution of differentially expressed genes in MVR compared with M36C05, and (d) distribution of differentially expressed genes in MR150 compared with M36C05.
Cillin resistance and biofilm formation. Inactivation of vancomycin-resistant \textit{Staphylococcus epidermidis} SCIENTIFIC may reduce bacterial virulence. Inactivation illustrated that resveratrol and its combination with vancomycin this study, Figure 4 | Functional classification of differentially expressed genes in each condition. a) Inhibitory condition, and (b) removed condition. Clusters of orthologous gene designations are described in Supplementary Table S12 online.

We found that the expression level of \textit{spoVG} was down-regulated in \textit{S. aureus} after treatment with resveratrol or its combination with vancomycin. Therefore, the mechanisms of using resveratrol and its combination with vancomycin to inhibit the development of established biofilm may be similar. However, the effect of the combination on established biofilm was more obvious than the effects of resveratrol and vancomycin alone. The transcriptome analyses indicated that (i) the expression levels of the genes associated with purine metabolism (\textit{purADHN}) were induced in MVR and vancomycin (MV) but not in MR150; and (ii) vancomycin inhibited the development of established MRSA biofilm by disturbing the expression of genes related to metabolic instead of those associated with QS, surface proteins, secreted proteins as well as the synthesis of capsular polysaccharide (Supplementary Table S15 online). As such, we speculated that these induced genes related to purine metabolism (\textit{purADHN}) may be one reason why the use of combination resveratrol and vancomycin more effectively eliminates established biofilm.

In conclusion, the transcriptome analysis in this study suggested that ursolic acid and resveratrol may have different mechanisms of inhibiting MRSA biofilm formation. Resveratrol inhibiting biofilm formation may disturb QS, the synthesis of surface protein and capsular polysaccharides, whereas ursolic acid-induced inhibition of biofilm formation may reduce the metabolism of some amino acids and the expression of adhesins. These differences may explain why resveratrol inhibited the further development of established biofilm whereas ursolic acid only inhibited biofilm formation. In addition, the MRSA isolate is sensitive to vancomycin (See Methods section), but once biofilm was established, the effect of vancomycin against bacteria was sharply reduced. In contrast, the combination of resveratrol and vancomycin markedly increased efficacy against established biofilm (Table 1). Meanwhile, the transcriptome analysis showed that the tendency toward differential genes expression in MR150 was consistent with those of MVR (Fig. 4b).

In addition to affecting proteins synthesis in biofilm-associated cells, the presence of resveratrol and ursolic acid altered the expression of a number of genes encoding key virulence factors involved in the pathogenesis of \textit{S. aureus}. Studies on the effects of two natural compounds on MRSA biofilm using RNA-seq suggested that ursolic acid could be used to prevent \textit{S. aureus} biofilm formation but that amounts of the corresponding toxin (β-hemolysin) may be increased, which is obviously counterproductive. Therefore, the combination of ursolic acid and QS blockers may be used to inhibit \textit{S. aureus} biofilm formation. The combination of resveratrol with vancomycin may be used to inhibit the development of established biofilm. Further investigation with a large panel of MRSA isolates would be required to confirm these findings and determine whether ursolic acid and resveratrol may prove to be therapeutically useful in reducing the morbidity and mortality linked with \textit{S. aureus} biofilm-mediated infections.

Methods

\textbf{Strain and growth conditions.} The clinical MRSA isolate used in this study was provided by the Clinical Laboratory Department of the First Affiliated Hospital, Nanjing Medical University, Nanjing, China. The strain was isolated from blood and was resistant to many antibiotics except for linezolid, teicoplanin, and vancomycin (Supplementary Table S16 online). Moreover, the isolate was identified using a MicroFlex LT instrument (Bruker Daltonics) according to the manufacturer’s recommendations. Spectra were analyzed by Flexcontrol 3.0 software and Biotyper 2.0 database (Bruker Daltonics). Blood agar medium was used to culture the colonies. Nutrient broth (NB) medium was used for routine culturing of the strain, while trypticase soy broth (TSB) medium was used to study the effects of natural compounds on MRSA biofilm in 96-well flat-bottom polystyrene plates (Costar 3599; Corning, USA). The resveratrol and ursolic acid used in this study were isolated from natural products in our group and their purities were confirmed to be >98% using high performance liquid chromatography methods. They were dissolved in ethanol at 30 mg/mL and 5 mg/mL, respectively. The vancomycin used in this study was purchased from Sigma and dissolved in water at 5 mg/mL. All compounds were filtered with a 0.22-μm filter in sterile conditions and then stored at 4°C.

\textbf{Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays.} MICs and MBCs were determined by a microtitre broth dilution methods as recommended by the Clinical and Laboratory Standards Institute (CLSI) with a few modifications. Briefly, the test medium was TSB and the density of bacteria was \(5 \times 10^7\) colony forming units (CFU)/mL. Cell suspensions (200 μL) were inoculated into the wells with ursolic acid or resveratrol at different final concentrations (25, 30, 33, 35, 37, and 40 μg/mL for ursolic acid, 37.5, 75, 150, 300, 350, and 400 μg/mL for resveratrol). These
concentrations were selected owing to the solubility reducing of ursolic acid and resveratrol in TSB medium as their concentrations increasing. The inoculated microplates were incubated at 37°C for 18 h before being read. The MIC was defined as the lowest concentration of the drug that inhibited the growth of the test microorganism by ≥ 90%.

The MBC was obtained by subculturing 100 μL from each well from the MIC assay onto TSA plates. The plates were incubated at 37°C for 24 h and the MBC was defined as the lowest concentration of substance that produced subcultures growing no more than five colonies on each plate. Experiments were carried out on three separate occasions.

Resveratrol and ursolic acid inhibiting MRSA biofilm formation assays. This assay was performed as previously described with a few modifications. Briefly, one MRSA colony was used to inoculate 5 mL of NB medium (pH 7.2) and was grown in an orbital shaker for 24 h at 37°C. The culture was then diluted (1:100) in fresh TSB supplement with resveratrol or ursolic acid of which final concentrations were 100 μg/mL and 30 μg/mL, respectively, according to supplementary table S1 and table S2. Next, 200 μL samples were added to the wells of 96-well plates. The plates were incubated at 37°C for 18 h with shaking. We also prepared controls with or without ethanol to observe the effects of solvent (each well contained 0.6% [v/v] ethanol) on biofilm formation. For quantification of the total biofilm mass, the suspension cultures were decanted and the plates were washed three times with phosphate buffered saline (PBS) (pH 7.2). The remaining biofilm was fixed in 200 μL of methanol for 15 min and then dried at 60°C. The biofilm was stained with 50 μL of 0.1% crystal violet (w/v) for 15 min. The wells were also washed three times with PBS to remove unbound crystal violet dye and dried for 2 h at 60°C. After the addition of 200 μL of 95% ethanol (v/v) to each well, the plates were shaken for 1 h to release the stain from the biofilm, and the absorbance at OD570 nm was measured with a microplate reader (BioTek, USA). All assays were performed in triplicate at least and repeated three times starting from new cultures. The inhibitory rates were then calculated using the following formula:

Figure 5 | Heatmap of differentially expressed genes associated with methicillin-resistant Staphylococcus aureus (MRSA) biofilm formation and virulence. (a) Resveratrol inhibiting MRSA biofilm formation condition, (b) ursolic acid inhibiting MRSA biofilm condition, and (c) resveratrol and its combination with vancomycin removing established MRSA biofilm condition. Chemically, resveratrol had no correlation with ursolic acid, so we performed separate differential expression analyses.
Figure 6 | Coverage changes of differentially expressed genes associated with methicillin-resistant Staphylococcus aureus (MRSA) biofilm and virulence in removing established MRSA biofilm conditions. Red, blue, and green lines represent M36C05, MVR, and MR150, respectively. For the x-axis, the position of the genome increases from left to right, and the length of each gene is proportional to the width of the position in the x-axis. The use of ‘...’ indicates discontinuous gene segments.

Treatment of MRSA biofilm with resveratrol, vancomycin, or their combination. After 18 h of biofilm growth, the suspension cultures were removed from each well. The plates were then washed twice with sterile PBS. A total of 200 µL of fresh TSB with resveratrol (final concentration, 150 µg/mL according to supplementary table S2), vancomycin (final concentration, 8 µg/mL), or combination (resveratrol 150 µg/mL + vancomycin 8 µg/mL) was added to the wells. Controls were prepared to observe the effects of the solvent (each well contained 0.5% [v/v] ethanol) on the established biofilm. The plates were incubated at 37°C for 18 h with shaking. The quantitative methods of their effects on established biofilm were the same as the methods of inhibiting biofilm formation. All assays were performed in triplicate at least and repeated three times starting from new cultures.

SEM measurements assay. SEM was performed on biofilm formed on glass coverslips (0.2 mm thick and 6 mm in diameter) by dispensing 700 µL of the cell suspensions into the wells of 24-well flat bottomed polystyrene plates (Costar 3524; Corning, USA). The plates were incubated at 37°C with shaking. The coverslips were then washed three times with water and fixed using 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer for 15 min each, followed by three 50% ethanol (v/v) washes for 10 min each. The samples were freeze-dried for 5 h and ultimately coated with gold and palladium in an evaporator. The observations were performed with a scanning electron microscope (FEI Quanta 200; USA).

Enrichment and sequencing of mRNA. A total of 10 µg of each RNA sample was subjected to further purification to enrich the mRNA using a MICROBExpress Kit (Ambion) according to the manufacturer’s instructions. Each mRNA sample was suspended in 25 µL of RNA storage solution and the quality of mRNA obtained was determined using Agilent 2100 Bioanalyzer. Bacterial mRNA was fragmented using a RNA fragmentation kit (Ambion, Carlsbad, CA) according to the manufacturer’s instructions. An Illumina Paired End Sample Prep kit was used to prepare RNA-seq library according to the manufacturer’s instructions. All of the samples were sequenced using the HiSeq2000 (Illumina, CA) sequencer at Beijing Genomics Institute at Shenzhen.

Analysis pipeline. Reads were aligned to Staphylococcus aureus subsp. aureus COL (S. aureus COL) (RefSeq accession number NC_002951.2) using the Burrows-Wheeler Alignment tool (BWA)\(^1\). The RNA-seq data analysis included the following stages. (1) If the pair-end reads satisfied N > 2% and low quality (quality value < 20) >50%, the reads were removed. In addition, if the terminal 20 bp consisted of N or was of low quality, the reads were removed. Using the above quality control (QC) standards, clean data were produced. (2) The clean data were aligned to S. aureus COL using BWA. (3) When some reads were unmappable or had incorrect alignment, they were removed. Based on the above standard, the QC of alignment was produced. (4) The commonly used fragments per kilobase of transcript per million mapped fragments (FPKM) incorporate normalization steps to ensure that expression levels for different genes and transcripts can be compared across runs\(^2\). Based on FPKM normalization, we performed analyses of coverage, distribution, and differentially expressed genes (workflow shown in Supplementary Fig. S11 online).
Identification of differentially expressed genes. Differentially expressed genes were identified using Cuffdiff, a separate program in Cufflinks19, which calculated expression in two or more samples and tested the statistical significance of each observed expression changes between them11. Genes with an adjusted P value < 0.05, FDR < 0.001 and fold change > 2 were identified as being differentially expressed. Finally, CummeRbund11 was used to visualize and integrate all of the data produced by Cufflinks analysis.

Real-time RT-PCR. Six genes (sdrD, spa, hld, agra, cap5B, and cap5C) that were up- or down-regulated by more than twofold in the presence of resveratrol and the combination with vancomycin under the removing established biofilm condition were selected to validate the data generated from RNA-Seq study by real-time RT-PCR. These genes were selected because they encode a range of virulence factors, were selected to validate the data generated from RNA-Seq study by real-time RT-PCR. These genes were selected because they encoded a range of virulence factors, including those associated with biofilm formation, and capsule synthesis.

The primer pairs used in real-time RT-PCR are listed in Supplementary Table S17. Total RNA was reverse transcribed into cDNA using the Takara RNA PCR kit (AMV) ver. 3.0 (Takara, Japan), according to the manufacturer’s protocol. The resulting cDNAs were stored at -20°C until they were required. The real-time RT-PCR was carried out in a 20 µL volume using SYBR® Premix Ex Taq™ (Tli RNaseH Plus kit) (Takara, Japan) as recommended by the manufacturer. These reactions were performed using the Applied Biosystems 7300 Real-time PCR System by using the following cycle parameters: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 60°C for cap5C gene and 57°C for other genes for 30 sec, and 72°C for 30 sec; and one dissociation step of 95°C for 15 sec, 60°C for 30 sec, and 95°C for 15 sec. All measurements were independently conducted 3 times on 2 separate biological isolates. The specificity of primer sets used for real-time RT-PCR amplification was evaluated by melting curve analysis. The standard curve method was used for quantification against a known concentration of plasmid DNA, pMD18T. All primers used, and sequences are listed in Supplementary Table S17.

Statistical analysis. At least three independent replicates of each 96-well plate experiment were performed. The biofilm inhibition results and real-time RT-PCR results were statistically analyzed using SPSS software version 18.0 (SPSS, Chicago, IL, USA). P values ≤ 0.05 were considered significant.

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