Identification of *Staphylococcus aureus* Cellular Pathways Affected by the Stilbenoid Lead Drug SK-03-92 Using a Microarray

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**Abstract:** The mechanism of action for a new lead stilbene compound coded SK-03-92 with bactericidal activity against methicillin-resistant *Staphylococcus aureus* (MRSA) is unknown. To gain insight into the killing process, transcriptional profiling was performed on SK-03-92 treated vs. untreated *S. aureus*. Fourteen genes were upregulated and 38 genes downregulated by SK-03-92 treatment. Genes involved in sortase A production, protein metabolism, and transcriptional regulation were upregulated, whereas genes encoding transporters, purine synthesis proteins, and a putative two-component system (SACOL2360 (MW2284) and SACOL2361 (MW2285)) were downregulated by SK-03-92 treatment. Quantitative real-time polymerase chain reaction analyses validated upregulation of *srtA* and *tdk* as well as downregulation of the MW2284/MW2285 and purine biosynthesis genes in the drug-treated population. A quantitative real-time polymerase chain reaction analysis of MW2284 and MW2285 mutants compared to wild-type cells demonstrated that the *srtA* gene was upregulated by both putative two-component regulatory gene mutants compared to the wild-type strain. Using a transcription profiling technique, we have identified several cellular pathways regulated by SK-03-92 treatment, including a putative two-component system that may regulate *srtA* and other genes that could be tied to the SK-03-92 mechanism of action, biofilm formation, and drug persisters.

**Keywords:** stilbene; microarray; *Staphylococcus aureus*; gene regulation; drug mechanism of action; sortase; biofilm
1. Introduction

*Staphylococcus aureus* is a common inhabitant of the human body that also causes numerous infections, including skin and soft tissue infections as well as more serious infections, such as pneumonia and bacteremia [1]. Presently, around 60% of *S. aureus* clinical isolates are methicillin-resistant *S. aureus* (MRSA) [2], and this bacterium is a leading cause of nosocomial infections in the United States [3,4]. In 1997, community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains emerged in the United States, causing infections in younger people, including necrotizing pneumonia [5–7]. Although skin infections caused by CA-MRSA are still prevalent, invasive MRSA infections have decreased [3,8]. In addition to methicillin resistance, CA-MRSA strains are becoming multidrug resistant at an alarming rate [9–11]. Heterogeneous vancomycin-intermediate *S. aureus* and vancomycin-resistant strains of *S. aureus* have led to vancomycin being less effective against some *S. aureus* infections [12–15]. Tolerance to vancomycin now has been reported to be as low as 3% and as high as 47% [16,17]. New drugs are needed to treat MRSA infections; however, most drugs currently in development are derivatives of drugs already being marketed [18,19]. *S. aureus* is one of the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) targeted by the 10 × 20 initiative to develop 10 new, safe and effective antibiotics approved by 2020 [20].

In support of the 10 × 20 initiative, a new antibiotic identified as (E)-3-hydroxy-5-methoxystilbene with promising activity against *S. aureus* was identified from *Comptonia peregrina* (L.) Coulter (“sweet fern”) [21]. A structure–activity relationship analysis identified our lead compound, (E)-3-(2-(benzo[b]thiophen-2-yl)vinyl)-5-methoxyphenol; for simplicity, SK-03-92. SK-03-92 was rapidly bactericidal (killing 90% of the population within an hour) against every Gram-positive species that was tested, including MRSA strains [22]. Importantly, a combined safety and pharmacokinetic study demonstrated that the SK-03-92 lead drug was safe in mice [23]. As with all antimicrobials, therapeutic treatment can result in residual bacteria not being killed by that antimicrobial, a phenomenon known as persistence [24–26]. Drug persisters are phenotypically different than the parent strain, but are not true drug resistant variants because the MICs of the drug persisters are the same as their parent strains [27,28]. Persisters are thought to be a major component of bacterial biofilms, allowing significant drug tolerance [29,30]. Many drugs used to treat *S. aureus* infections have drug persister population emerge that are recalcitrant to treatment. To gain insight into the mechanism of action of SK-03-92 and the mechanism of *S. aureus* persistence to SK-03-92 treatment, the effect of SK-03-92 on *S. aureus* cells was assessed by transcriptional profiling in the *S. aureus* strain MW2.

2. Results and Discussion

2.1. General Transcriptome Response of SK-03-92 Treatment

New drugs to treat *S. aureus* infections are urgently needed, and SK-03-92 holds considerable promise. SK-03-92 has a stilbenoid backbone [22] and is bactericidal within an hour; however, 10% of the population survives as drug persisters that can grow in media containing up to 32 µg/mL of SK-03-92 but with an MIC equivalent to untreated *S. aureus* cells. The mechanism of action for SK-03-92 is unknown. To ascertain the effects of SK-03-92 treatment on the transcriptome of *S. aureus*, total RNA was isolated from *S. aureus* strain MW2 cultures (Table 1) treated for 30 min with 8 × the MIC of SK-03-92 and untreated MW2 cultures and an RNA microarray was performed. A total of 52 genes were dysregulated by the SK-03-92 drug treatment (Table 2), representing 2% of the total *S. aureus* transcriptome. This is remarkable because transcriptional profiling of other bactericidal compounds has shown a larger effect on the *S. aureus* transcriptome, including ortho-phenylphenol (24%) [31], amicoumacin A (20%) [32] and daptomycin (5% to 32%) [33,34]. Interestingly, the number of downregulated genes (73.1%) greatly surpassed the number of upregulated genes (26.9%).

An examination of genes affected by treatment with other stilbene type compounds demonstrates the disparity in their transcript profile compared to treatment with SK-03-92. Pterostilbene, another
stilbenoid compound, in *Saccharomyces cerevisiae* showed 1189 genes that were dysregulated: 1007 upregulated (85%) and 182 downregulated (15%) [35]. Microarray analysis with resveratrol treated *Schizosaccharomyces pombe* showed 480 genes dysregulated, 377 genes that were upregulated and 103 that were downregulated [36]. RNA sequence analysis of resveratrol treated *S. aureus* cells demonstrated 444 dysregulated genes, 201 upregulated and 243 downregulated [37]. The majority of the genes in our study had a two- to four-fold difference in transcript abundance when comparing SK-03-92 treated vs. untreated *S. aureus* cultures. Very few genes dysregulated by SK-03-92 were previously shown to be dysregulated by resveratrol (e.g., downregulation of the purD, purH, purL, lrgA, and sdhC genes). Only three genes had a 10-fold or higher change in transcript levels, which included two genes annotated to be part of a putative two-component system (TCS) (*SACOL2360* (annotated as *MW2284* in MW2 strain) = 14.1-fold lower and *SACOL2361* (annotated as *MW2285* in MW2 strain) = 26.9-fold lower) as well as the *glpD* gene encoding glycerol-3-phosphate dehydrogenase (10-fold higher).

**Table 1.** Bacterial strains used in this study.

| Bacterial Strain | Genotype | Reference |
|------------------|----------|-----------|
| *S. aureus*      |          |           |
| MW2              | USA400 wild-type | [7] |
| JE2              | USA300 wild-type | [38] |
| NE272            | JE2 MW2284 mutant | [38] |
| NE671            | JE2 MW2285 mutant | [38] |
| NE1363           | JE2 srtB mutant | [38] |
| NE1787           | JE2 srtA mutant | [38] |
| *L. monocytogenes* |          |           |
| EGD              | Wild-type | [39] |
| EGD sRTA         | EGD sRTA mutant | [39] |
| EGD sRTB         | EGD sRTB mutant | [39] |

Dysregulated genes tied to a potential mechanism of action for SK-03-92 included *glpD, adhE* (*SACOL0135*), *adhP* (*SACOL0660*), and *sdhC* (*SACOL1158*). GlpD funnels electrons into the respiratory chain via quinone or menaquinone reduction coupled to the oxidation of glycerol-3-phosphate to glycerone phosphate (dihydroxyacetone phosphate) [40], which can be enzymatically or non-enzymatically transformed into methylglyoxal (MG) [41]. Higher concentrations of MG are thought to halt bacterial growth by damaging proteins by acting as a protein glycating agent that mainly affects arginine residues [42,43]. In *Candida albicans*, ADH1 catalyzes the NAD+ linked oxidation of MG to pyruvate and disruption of the *adh1* gene in *C. albicans* caused accumulation of MG followed by inhibition of growth [44]. The dysregulation of *glpD* and *adh* genes suggests that MG was accumulating and glycation was occurring in SK-03-92-treated *S. aureus*. MG glycation of proteins, lipids, and DNA generate advanced glycation end products (AGEs) [43]. Importantly, GlpD has been implicated in drug persistence in *Escherichia coli* [45] and *S. aureus* [34].

A number of genes involved in metabolism were also dysregulated by SK-03-92 treatment, including the *gcvH* gene that encodes GcvH, which shuttles the methylamine group of glycine from the P-protein to the T-protein via a lipoyl group [46]. Genes associated with protein degradation and repair had altered transcript abundance in SK-03-92-treated *S. aureus*. Transcripts encoding a putative repair system for deglycation of Amadori protein adducts derived from ribose-5-P (*ptpA*) [47] showed altered abundance in SK-03-92-treated *S. aureus*, as did the transcript encoding the enzyme that produces ribose-5-P (*SACOL2605*). The formation of Amadori protein adducts occurs spontaneously via a dehydrogenation mechanism when ribose-5-P interacts with an amine, such as the lysine residues of proteins. Amadori glycated proteins undergo further spontaneous reactions to become AGEs. AGEs promote protein aggregation [47,48]. Since *ptpA* transcript abundance was increased 2.3-fold and the
kinase transcript SACOL2605 was decreased 9.6-fold, ribulosamine substrates produced were likely not being deglycated, and protein repair was not occurring. Phase-dark and phase-bright inclusions were observed microscopically in SK-03-92-treated B. subtilis, consistent with perturbation of proteostasis resulting in visible accumulation of protein aggregates [49]. Uncontrolled protein aggregation is toxic to cells [48].

Table 2. Microarray analysis of genes dysregulated in S. aureus MW2 cells treated with 8× the SK-03-92 MIC vs. untreated cells.

| Locus    | Fold-Difference | Description                                                                 |
|----------|-----------------|-----------------------------------------------------------------------------|
| SACOL1759| −2.3            | universal stress protein family                                             |
| SACOL0086| −2.0            | drug transporter, putative                                                  |
| SACOL0155| −5.7            | cation efflux family protein                                               |
| SACOL0178| −2.9            | PTS system, IIBC components (scrBC)                                        |
| SACOL0400| −2.6            | ascorbate-specific PTS system subunit IIC (ulaA)                           |
| SACOL0454| −2.3            | sodium:dicarboxylate symporter family protein                              |
| SACOL1018| −2.3            | sodium:alanine symporter family protein                                     |
| SACOL1872| −3.0            | epidermin immunity protein F (epfE)                                        |
| SACOL2146| −2.7            | PTS system, mannitol-specific IIBC components (mtlA)                       |
| SACOL2333| −2.8            | YnaF family protein                                                        |
| SACOL2573| −3.2            | copper ion binding protein (copZ)                                          |
| SACOL2664| −2.3            | mannose-6-phosphate isomerase (manA)                                       |
| SACOL2718| −4.6            | 2-oxoglutarate/malate translocator, sodium sulfate symporter               |
| SACOL2360| −14.1           | LytTR family regulator protein                                             |
| SACOL2361| −26.9           | histidine kinase sensor membrane protein                                   |
| SACOL2340| 2.2             | transcriptional regulator TetR-family                                      |
| SACOL0151| −2.7            | UDP-N-acetylglucosamine 2-epimerase Cap5P (cap5P)                          |
| SACOL0247| −3.2            | holin-like protein LrgA (lrgA)                                             |
| SACOL0612| −2.1            | glycosyl transferase, group 1 family protein                               |
| SACOL1071| −2.2            | chitinase-related protein (iraE)                                           |
| SACOL2554| −2.0            | holin-like protein CidB (cidB)                                             |
| SACOL2539| 4.2             | sortase A (srtA)                                                           |
| SACOL013 | −2.1            | 5’ nucleotidase family protein                                             |
| SACOL1078| −3.2            | phosphoribosylformylglycinamidine synthase II (purL)                       |
| SACOL1082| −2.5            | bifunctional purine biosynthesis protein (purH)                            |
| SACOL1083| −2.6            | phosphoribosylamine-glycine ligase (purD)                                  |
| SACOL2329| −3.5            | ribose 5-phosphate isomerase (ripA)                                        |
| SACOL2111| 2.2             | thymidine kinase (tdk)                                                     |
| SACOL2377| 2.3             | conserved hypothetical protein                                              |
| SACOL0855| −2.5            | peptidase, M20.M25/M40 family                                              |
| SACOL2605| −9.6            | ribulosamine 3-kinase                                                      |
| SACOL0457| 2.6             | conserved hypothetical protein, heat induced stress                        |
| SACOL0590| 2.4             | 30S ribosomal protein L7 Ae                                                 |
| SACOL0877| 2.5             | glycine cleavage system H protein (gcvH)                                   |
| SACOL1907| 2.4             | ribosomal large subunit pseudouridine synthase (rlaD)                      |
| SACOL1939| 2.3             | phosphotyrosine protein phosphatase (pptA)                                |
| SACOL2596| 2.6             | metallo-dependent amidohydrolase                                           |
Three genes associated with purine synthesis were downregulated: purD, purH, and purL. Purine metabolism is a necessary part of DNA synthesis and energy production in *S. aureus* [50]. Genes involved in purine metabolism are often downregulated after treatment with a drug or plant extract [51–53]. In addition, one gene associated with pyrimidine synthesis, *tdk*, was upregulated. Thymidine kinase transfers the terminal phosphate from ATP to thymidine or deoxyuridine [54]. A decrease in the synthesis of purines coupled with an increase in phosphorylation of pyrimidines could result in a dramatic reorganization of the intracellular nucleotide pool. Moreover, less purine metabolism is often tied to drug persister populations [55,56]. Disruption of nucleotide metabolism in a library of *S. aureus* transposon insertion mutants caused a decrease in persister formation frequency when treated with rifampicin [57].

Consistent with the formation of persister strains, mRNA levels of genes linked to programmed cell death (PCD) were decreased in *S. aureus* cultures treated with SK-03-92. Specifically, the Cid/Lrg (holin/antiholin) system, which controls autolysis and affects the distribution of extracellular DNA in *S. aureus* during biofilm development [58–60]. This prokaryotic PCD is analogous to the bcl-2 pro-apoptotic effector and anti-apoptotic mediated apoptosis in eukaryotes [61,62].

Twelve putative transport genes were dysregulated encoding for proteins involved in anion transport, a cation efflux family protein, two phosphotransferase system (PTS) transporters, a sodium:alanine symporter, sodium:dicarboxylate symporter family protein, and a copper ion binding protein. The only true virulence factor genes affected by SK-03-92 treatment were the *SACOL0151 cap5P*, *epiE*, *SACOL2333* gene encoding a YnfA family protein putative transport small multidrug resistance family-3 protein [63], and the *srtA* gene encoding sortase A that will be described in more detail below [64]. Five genes identified by the microarray were annotated as hypothetical proteins with no known function (three downregulated and two upregulated).

### 2.2. Genes of a Putative TCS Are Significantly Downregulated by SK-03-92 Treatment

A surprising microarray result that was no known *S. aureus* global regulatory genes were shown to be affected by the drug treatment. Microarray analysis of daptomycin treated *S. aureus* demonstrated that the the *icaR* gene was dysregulated compared to untreated cells [34]. Our microarray showed that a tetR-family transcriptional regulator, *SACOL2340*, and two genes that comprise a putative TCS in *S. aureus* annotated as MW2284 (14.1-fold downregulated) and MW2285 in strain MW2 (26.3-fold downregulated) were downregulated. A bioinformatic analysis of the putative MW2284 and MW2285
proteins suggest that they comprise a putative two-component regulatory system where MW2284 (LytTR superfamily regulator protein) is the response regulator protein and MW2285 (membrane protein) is the sensor kinase protein. MW2284 was identified as a 440-bp ORF encoding a putative 14.7-kDa transcriptional regulator protein and MW2285 was identified as a 455-bp ORF encoding a putative 15.1-kDa histidine kinase sensor protein. The MW2285 ORF has a 3-bp overlap with the MW2284 ORF. BLASTP, PSI-BLAST, and BLASTN bioinformatics analyses [65] showed that MW2284 aligned with other two-component regulatory system regulator proteins and MW2285 aligned with other two-component regulatory system sensor proteins. Both proteins have homology with LytTR superfamily proteins involved in the regulation of bacterial genes [66]. LytTR proteins regulate virulence gene expression in a variety of bacterial species including S. aureus. The AgrA transcriptional regulator is one of these LytTR-type proteins [67]. Moreover, the MW2284 and MW2285 ORFs appeared to be conserved across a wide number of Gram-positive species, including all Staphylococcus and Streptococcus species, as well as Bacillus, Clostridium, Lactobacillus, Listeria, and Leuconostoc.

The same LytTR TCS dysregulated in SK-03-92-treated S. aureus was upregulated in purine synthesis deficient mutants in S. aureus [68]. The putative sensor kinase (MW2285) was upregulated in purH mutants and the response regulator (MW2284) was upregulated in purA mutants (adenylosuccinate synthetase involved in purine biosynthesis). The response regulator component transcript was also upregulated during anaerobic growth in another study [69]. A transposon mutant of the sensor kinase component has been previously shown to be viable, capable of producing a more robust biofilm, and had a lower LD50 than the parent strain [70,71]. The mechanistic link between defects in purine synthesis, persister formation, and the LytTR regulatory system remains unclear. Furthermore, RNAseq analysis of resveratrol treated S. aureus cells showed an almost 8-fold downregulation of the MW2284 gene, but no effect on the MW2285 gene [37].

2.3. Validation of Microarray Data by qRT-PCR

The microarray results were confirmed using qRT-PCR analyses on RNAs from 8× the MIC SK-03-92 treated MW2 cells vs. untreated MW2 cells. Transcription of the srtA gene was significantly upregulated almost 6-fold (p < 0.006, Figure 1) and the tdk gene was also upregulated 2.1-fold (p < 0.03) in SK-03-92 treated cells vs. untreated cells. On the other hand, several genes involved in purine biosynthesis (purD, purH, and purL) were shown to be significantly downregulated 2.2- to 2.4-fold (p < 0.01 to 0.04), whereas the MW2284 and MW2285 genes were downregulated 4- (p < 0.01) and 3-fold (p < 0.003), respectively, in the SK-03-92 treated samples. These results confirmed that treatment with the SK-03-92 lead compound caused dysregulation of the srtA, tdk, purD, purH, purL, MW2284, and MW2285 genes.

![Figure 1](image-url)  
**Figure 1.** Quantitative reverse transcribed-polymerase chain reaction results of S. aureus MW2 cells treated with 8× the SK-03-92 MIC vs. untreated cells. The data represents the mean ± standard deviation from at least three separate runs.
2.4. SK-03-92 Treatment Causes Alteration of Nucleotide Pool

Because three pur genes involved in purine synthesis and the tdk gene were dysregulated by SK-03-92 treatment, the rapid accumulation of the bacterial alarmone (p)ppGpp and the state of the intracellular nucleotide pool were examined using high-performance liquid chromatography (HPLC, Waters, Milford, MA, USA). Inhibition of isoleucyl tRNA synthetase by mupirocin has been shown to induce production of (p)ppGpp in S. aureus [72,73]. A highly phosphorylated ribonucleotide, (p)ppGpp, can be identified via rapid separation of the S. aureus nucleotide pool using anion-exchange HPLC, where (p)ppGpp elutes as a late peak, which can be detected by absorbance at 254 and 280 nm [74]. In control experiments, this late peak was not detected in untreated cells (Figure 2A), but was detected following treatment with mupirocin (Figure 2B). No (p)ppGpp was detected following treatment with SK-03-92 (Figure 2C). However, the composition and quantity (area under curve) of the nucleotide pool was altered in SK-03-92 treated S. aureus as compared to untreated cells (Figure 2A vs. Figure 2C), suggesting that dysregulation of tdk and the three purine biosynthesis genes by SK-03-92 treatment depleted the nucleotide pool.

![Figure 2](image)

**Figure 2.** Absorbance (254 and 280 nm) of the formic acid extracted nucleotide pool of log-phase S. aureus ATCC 29213 after 20 min with either (A) no treatment, (B) 60 µg/mL mupirocin, or (C) 16 µg/mL SK-03-92. Arrow denotes the (p)ppGpp peak.

2.5. Biofilm Formation Increases as the Concentration of SK-03-92 Increases

With an increase in srtA transcript abundance shown by the qRT-PCR results, an increase in biofilm formation would be expected following SK-03-92 treatment. To further analyze the effects of the increase in srtA transcription, a biofilm assay in microtiter plates was performed after SK-03-92 drug treatment (Figure 3). Wild-type JE2 and MW2 cultures were tested following SK-03-92 drug treatment (range 0.5–0.64 µg/mL). The JE2 culture grown without drug showed an OD570 of 2.41, whereas the MW2 culture had an OD570 of 2.50.

The SK-03-92 drug had a biphasic effect on the wild-type strains. At low concentrations, the drug reduced biofilm formation as exhibited by the 0.5 and 1 µg/mL data points that were significant for both strains that were tested (p < 0.05). As concentrations of SK-03-92 increased, the OD570 readings increased, plateauing at 32 µg/mL for both strains. Strain MW2 showed significant increases in biofilm formation going from 0 µg/mL to 8–64 µg/mL SK-03-92 concentration (p < 0.05). A similar finding was observed when Candida species grown as a biofilm were exposed to varying concentrations
of echinocandin [75]. *Candida* treated with low drug concentrations killed the fungal cells but at concentrations higher than the MIC showed there was an increase in the cell density of the biofilms. Echinocandin acting on the *Candida* species has the same effect as our SK-03-92 drug, triggering upregulation of a specific gene that increases biofilm formation.

![Figure 3](image-url)

**Figure 3.** The effects of SK-03-92 drug concentration on 24 h biofilm formation (OD$_{570}$) for *S. aureus* strains JE2 (black column) and MW2 (white column). All experiments represent the mean + standard deviation of at least 10 runs done in triplicate.

Under normal growth conditions, biofilm formation is not necessary for a cell, but under stressful environmental conditions, such as exposure to the SK-03-92 drug, biofilm formation would greatly benefit the *S. aureus* population. Formation of a biofilm would benefit cells by allowing for the formation of persister cell populations [76]. When biofilms form, the cells at the base of the biofilm slow or stop most cell metabolism and go into a dormant state, allowing the organisms to survive in the presence of a drug, for example SK-03-92. In addition, cells in a biofilm often undergo quorum sensing, which also can lead to the emergence of persister cells [77].

### 2.6. A Sortase A Mutant Has a Lower MIC against SK-03-92 Than Wild-Type

Since the putative MW2284/MW2285 TCS appears to repress transcription of the *srtA* gene, this regulatory effect could be tied to the mechanism of action of the SK-03-92 drug. Sortase A was first described in *S. aureus* in 1999 [64]. The protein covalently anchors surface proteins (e.g., fibronectin-binding protein, fibrinogen-binding protein, protein A, clumping factors, collagen adhesion protein) to the cell wall of *S. aureus* and other Gram-positive bacteria [77]. An LPXTG motif [78–80] is common among these anchored proteins and many are important for phase I of biofilm formation that allows attachment to biotic or abiotic surfaces [81]. A mutation of the *srtA* gene caused less expression of several cell wall anchored surface proteins [82,83]. Moreover, *srtA* mutants are attenuated compared to the wild-type strain in a variety of murine models of infection [82,84,85].

Because *srtA* and MW2284/MW2285 transcription were affected by SK-03-92 treatment, MICs were performed using the SK-03-92 lead compound on an *srtA* mutant (NE1787), *srtB* mutant (control, NE1363), MW2284 mutant (NE671), and MW2285 mutant (NE272) compared to the wild-type strain JE2 [38]. The *srtB*, MW2284, and MW2285 mutants had MICs that were equal to the wild-type strain (Table 3). However, the *srtA* mutant had an MIC that was 2-fold lower than the wild-type strain. When a *Listeria monocytogenes srtA* mutant was tested [39], the MIC for the *srtA* strain was 8-fold lower than the wild-type strain. A *L. monocytogenes srtB* mutant had the same MIC as the wild-type bacteria.
we obtained transposon mutant strains from the Nebraska Transposon Mutant Library [38] with the assumption that such mutations would be similar to a mutation in the MW2285 gene. The regulatory effect could be derepression of transcription, respectively, suggesting that this putative two-component regulatory system may be involved in repressing the srtA gene.

Table 3. MIC results for S. aureus and L. monocytogenes mutants and wild-type strains against SK-03-92.

| Strain | Genotype | MIC |
|--------|----------|-----|
| JE2    | Wild-type| 1 a |
| NE272  | MW2285   | 1   |
| NE671  | MW2284   | 1   |
| NE1363 | srtB     | 1   |
| NE1787 | srtA     | 0.5 |

L. monocytogenes

|        |           |     |
|--------|-----------|-----|
| EGD    | Wild-type | 1   |
| EGD srtA| srtA      | 0.125|
| EGD srtB| srtB      | 1   |

*Mean + standard deviation from three separate runs.

Presumably, SK-03-92 treatment causes downregulation of the MW2285 gene with an effect that would be similar to a mutation in the MW2285 gene. The regulatory effect could be derepression of srtA transcription. Either event would create more SrtA protein that in turn would allow greater extracellular presentation of proteins on the surface of S. aureus cells. This result may suggest that something tethered to the cell walls by sortase A that is conserved in both species may be tied to the mechanism of action of the SK-03-92 drug, and we are exploring this possibility.

2.7. Mutations in the MW2284/MW2285 Two-Component Regulatory Genes Cause an Upregulation of the srtA Gene

Since the microarray results showed significant upregulation of the srtA gene and downregulation of the MW2284 and MW2285 genes, we hypothesized that the MW2284 gene product, a putative transcriptional regulator protein, may be repressing the srtA gene. To confirm that the putative two-component regulatory system (MW2284/MW2285) may be involved in repressing the srtA gene, we obtained transposon mutant strains from the Nebraska Transposon Mutant Library [38] with insertion mutations in the MW2284 and MW2285 genes. A qRT-PCR analysis was then undertaken on RNA isolated from the NE272 (MW2285 mutation) and NE671 (MW2284 mutation) strains compared to the wild-type strain JE2, targeting the srtA gene. The results showed that mutations in both the MW2284 and MW2285 genes led to a 9.2-fold (p < 0.0008) and 8.1-fold (p < 0.0008) upregulation of srtA transcription, respectively, suggesting that this putative two-component regulatory system may be repressing transcription of the srtA gene (Figure 4).

![Figure 4](image-url)  
**Figure 4.** Quantitative reverse transcribed-polymerase chain reaction results of S. aureus srtA transcription in wild-type bacteria compared to MW2284 and MW2285 mutants. The data represents the mean + standard deviation from three separate runs.
3. Experimental Section

3.1. SK-03-92 Synthesis

SK-03-92 was synthesized as described previously [22].

3.2. Bacterial Strains and Growth Conditions

The S. aureus MW2 strain [7] used for the initial microarray and confirmatory qRT-PCRs (Table 1) was obtained from Jean Lee (Brigham and Young Hospital, Boston, MA, USA). S. aureus strains JE2 (wild-type), NE671 (MW2284), and NE272 (MW2285) were obtained from the Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA) strain repository (Table 1), representing part of the Nebraska Transposon Mutant Library [38]. Strain JE2 is a plasmid-cured derivative of a USA300 CA-MRSA [86]. Phillip Klebba (Kansas State University, Manhattan, KS, USA) [39] provided the Listeria monocytogenes wild-type strain EGD as well as the isogenic srtA and srtB mutant strains. All strains were grown in brain heart infusion broth (Becton Dickinson, Franklin Lakes, NJ, USA) or trypticase soy broth (Becton Dickinson) shaken 250 rpm at 37 °C. The transposon mutant strains had 5 µg/mL of erythromycin (Sigma-Aldritch, St. Louis, MO, USA) added to the media.

3.3. RNA Extractions

Total RNA was isolated from S. aureus MW2 cells grown to exponential growth phase (OD600 approximately 0.5) either treated with dimethyl sulfoxide (DMSO) or 8× the MIC of SK-03-92 dissolved in DMSO using TRIzol extraction (Life Technologies, Carlsbad, CA, USA) according to manufacturer’s instructions with an additional lysostaphin treatment step to help lyse the S. aureus cell walls. The RNA samples were digested with DNase I (New England Biolabs, Ipswich, MA, USA) followed by phenol and chloroform extractions to remove the protein. RNAs were run on 0.8% agarose gels to confirm concentration and integrities of the RNAs. To assess DNA contamination of the samples, PCRs were performed on the RNA samples using SaFtsZ1 and SaFtsZ2 primers (see Table 4). The PCR conditions for amplification with the SaFtsZ1/SaFtsZ2 primers was as follows: 94 °C, 1 min; 55 °C, 1 min; and 72 °C, 1 min for 35 cycles.

Table 4. Oligonucleotide primers used in this study.

| Primer   | Gene | Sequence                  |
|----------|------|---------------------------|
| SaFtsZ1  | ftsZ  | 5’-GTGGTGAAGTTGCTGCGGTAA-3’ |
| SaFtsZ2  |      | 5’-TCATTGGCCTAGATATTGTC-3’ |
| GuaBF1   | guaB | 5’-GCTCGTCAAGGTGGTTAGGTG-3’ |
| GuaBR1   |      | 5’-TAAGACATGCACACCTGCTTCG-3’ |
| SrtA1    | srtA | 5’-TCGCTGGTGCTGATACATC-3’  |
| SrtA2    |      | 5’-CAGGTTGCTGCTGCTTGG-3’   |
| MW2284A  | MW2284| 5’-CAATTGCAAATGACACGGAATCT-3’ |
| MW2284B  |      | 5’-GAATAATGGTATCGTGTACG-3’  |
| MW2285A  | MW2285| 5’-GTGATATTGCGACAGCGCAA-3’  |
| MW2285B  |      | 5’-AACCGACAGATCCTGCGATG-3’  |
| SA2043A  | tdk  | 5’-CATTGGTCACTGACAGCGCTCA-3’ |
| SA2043B  |      | 5’-AGCGCGATGCTGACTAATGTTG-3’ |
| SaPurD1  | purD | 5’-CAGCGCCTATTAGCTGATGATT-3’ |
| SaPurD2  |      | 5’-AGCACAATCTGCTTCGCAAAT-3’ |
| SaPurH1  | purH | 5’-CCAGAAATAATGGATGCGCG-3’  |
| SaPurH2  |      | 5’-TGCCGAGTCAAATTTGTTG-3’  |
| SaPurL1  | purL | 5’-TTATGGTGGAAGCATTTG-3’   |
| SaPurL2  |      | 5’-AGCCCGATAGCAGATATGTC-3’  |
3.4. Microarray

Total RNAs from cells treated with DMSO or 8× the MIC of SK-03-92 were converted to cDNAs, biotinylated, and hybridized to S. aureus GeneChips following the manufacturer’s recommendations (Affymetrix, Santa Clara, CA, USA). Agilent GeneSpring G×7.3 software (Santa Clara, CA, USA) was used to gauge transcript differences and a two-fold or higher difference in the transcript level for one population over the other was considered significant. Nucleic acid sequences with a ≥2-fold change in transcriptional abundance were mapped to the S. aureus COL genome (taxid: 93062) via BLASTN, BLASTX, or PSI-BLAST analysis [65] through the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) website and their putative products were annotated.

3.5. cDNA Synthesis

The cDNAs were synthesized from 5 µg of total RNA from SK-03-92 treated or untreated S. aureus MW2 using a First-Strand Synthesis kit (Life Technologies) according to manufacturer’s instructions.

3.6. Real Time-Quantitative Polymerase Chain Reaction (qRT-PCR)

All of the qRT-PCRs were performed using the LightCycler FastStart DNA MasterPLUS SYBR Green kit according to manufacturer’s instructions (Roche, Indianapolis, IN, USA). Primers used in this study were based off of the MW2 sequenced genome [87] and synthesized by Integrated DNA Technologies (Coralville, IA, USA) that are shown in Table 4. A LightCycler 1.5 machine (Roche) or a CFX96 machine (BioRad, Hercules, CA, USA) were used throughout the study. The guaB and ftsZ housekeeping genes were used as standardization controls. Each RT-qPCR run followed the minimum information for publication of quantitative real-time PCR experiments guidelines [88]. The qRT-PCRs were done at least three times under the following conditions: 94 °C, 20 s; 55 °C, 30 s; and 72 °C, 1 min for 35 cycles. The level of target gene transcripts in MW2 cells was compared to the guaB and ftsZ genes. Crossover points for all genes were standardized to the crossover points for ftsZ and guaB in each sample using the \( 2^{\Delta\Delta CT} \) formula [89].

3.7. HPLC

High-performance liquid chromatography was used to detect the presence of (p)ppGpp in the intracellular nucleotide pools of mid-log phase S. aureus cells following treatment with SK-03-92, mupirocin (positive control), or dimethyl sulfoxide (negative control) [90–92]. SK-03-92 was added at 16 µg/mL to 100 mL mid-log (OD\(_{600}\) = 0.4–0.6) culture in cation-adjusted Mueller Hinton broth and incubated for 20 min with shaking at 37 °C. Mupirocin was added at 60 µg/mL. Cells were collected after a 20 min incubation by centrifugation at 10,000 × g for 10 min at 4 °C. The supernatant was discarded and the cell pellet suspended in 12 mL of ice cold 0.4 M formic acid (pH 3.5). After 30 min on ice, the cell extract was centrifuged at 10,000 × g for 10 min at 4 °C to remove cell debris. The supernatant was evaporated under vacuum and filtered (0.2 µm pore size). Filtered cell extract was stored at −20 °C until use. Fifty microliters of filtered cell extract were loaded on a Hypersil SAX column (Thermo Fisher Scientific, Waltham, MA, USA) (5 µm, 4.6 × 250 mm) at a flow rate of 1.0 mL/min in 0.45 M potassium phosphate 0.05 M magnesium sulfate buffer (pH 3.5) using a Waters 600E pump and 996 photodiode array detector (Waters, Milford, MA, USA). Absorbance of the separated nucleotide pool was monitored at 254 and 280 nm.

3.8. Biofilm Assay

To determine the effect of SK-03-92 treatment on the ability of S. aureus to form a biofilm, a biofilm assay was performed [93]. The S. aureus parent strains MW2 and JE2 were treated with SK-03-92 at concentrations of 0.5–64 µg/mL and those plates were compared to wells with bacteria not treated with the drug. After drying, the remaining dried crystal violet dye stained biofilm material was extracted
with 160 µL 33% glacial acetic acid per well and the OD₅₇₀ was measured for each well. The total biofilm assay was performed a minimum of 10 times for each strain to achieve statistical significance.

3.9. MICs

In vitro minimum inhibitory concentration (MIC) determinations were performed on the S. aureus strains using SK-03-92 according to the Clinical and Laboratory Standards Institute guidelines [94]. All MICs were done a minimum of three times.

3.10. Statistical Analysis

A two-tailed Student’s t-test was run for the qRT-PCR comparisons and an ANOVA analysis was used for the biofilm assays to assess probabilities. *p*-values < 0.05 were considered significant.

4. Conclusions

Drug treatment with the stilbenoid compound SK-03-92 caused more genes to be transcriptionally downregulated than upregulated compared to other bactericidal and stilbenoid compounds (e.g., pterostilbene and resveratrol). The methoxy substitution on the main benzene ring at position 5 is likely to be responsible for this effect. A putative TCS, MW2284/MW2285, is clearly downregulated by SK-03-92 treatment. Is the TCS the prime target of the SK-03-92 lead compound and could targeting this TCS be the mechanism of action for SK-03-92 in Gram-positive bacteria? We hypothesize that one of the SK-03-92 targets is this putative TCS. Knockouts of both MW2284 and MW2285 showed substantial upregulation of the srtA gene that encodes sortase A. Sortase A may present something on the exterior of the S. aureus cell that causes rapid cell lysis. Furthermore, the MW2284 and MW2285 ORFs lie just upstream of the MW2286 ORF, which is thought to encode a malate:quinone oxidoreductase gene important in the electron transport chain. If the MW2284/MW2285 TCS positively regulates this gene, then a mutation in either gene or treatment of S. aureus with a SK-03-92 drug may, in turn, cause downregulation of this gene as well as *sdhC* and *glpD* that would disrupt the electron transport chain in S. aureus. Evidence presented in this study also suggests the existence of a conserved bacterial pathway, involving PCD and persister formation, which is triggered by protein glycation and aggregation that may be responsible for the killing mechanism of SK-03-92. Could this putative TCS be tied to these phenomena? Further study may help us determine if the SK-03-92-induced S. aureus cell lysis is caused by a disruption of the electron transport chain, regulation of a conserved prokaryotic PCD pathway, or a combination of both of these events.

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Author Contributions: W.R.S. conceived the experiments, wrote the paper, designed some of the primers, and ran data analysis: P.M.D. ran the microarray analysis and initial microarray annotation; A.M., J.M.C., V.V.N.P.B.T., C.W., and M.T.R. synthesized the SK-03-92 lead compound used in the study; S.M.B., M.L., L.L., and A.B. isolated the RNA samples, designed primers, and ran qRT-PCR; A.W. performed the biofilm assays, D.B. ran the biofilm statistical analysis; and R.P. and M.R. completed the HPLC and some bioinformatic analysis of the microarray results.

Conflicts of Interest: W.R.S., M.R., A.M., and J.M.C. hold a composition of matter and use a patent covering the SK-03-92 lead compound.

References

1. Suaya, J.A.; Mera, R.M.; Cassidy, A.; O’Hara, P.; Amrine-Madsen, H.; Burstin, S.; Miller, L.G. Incidence and cost of hospitalizations associated with *Staphylococcus aureus* skin and soft tissue infections in the United States from 2001 to 2009. *BMC Infect. Dis.* 2014, 14, 296. [CrossRef] [PubMed]
2. Klein, E.Y.; Sun, L.; Smith, D.L.; Laxminarayan, R. The changing epidemiology of methicillin-resistant \textit{Staphylococcus aureus} in the United States: A national observational study. \textit{Am. J. Epidemiol.} \textbf{2013}, \textit{177}, 666–674. [CrossRef] [PubMed]

3. Hidron, A.I.; Edwards, J.R.; Patel, J.; Horan, T.C.; Sievert, D.M.; Pollock, D.A.; Fridkin, S.K.; National Healthcare Safety Network Team; Participating National Healthcare Safety Network Facilities. NHSN annual update: Antimicrobial-resistant pathogens associated with healthcare-associated infections: Annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. \textit{Infec. Control Hosp. Epidemiol.} \textbf{2008}, \textit{29}, 996–1011. [PubMed]

4. Maree, C.L.; Daum, R.; Boyle-Vavra, S.; Matayoshi, K.; Miller, L. Community associated methicillin-resistant \textit{Staphylococcus aureus} isolates causing healthcare-associated infections. \textit{Emerg. Infect. Dis.} \textbf{2007}, \textit{13}, 236–242. [CrossRef] [PubMed]

5. Herold, B.C.; Immergluck, L.C.; Maranan, M.C.; Lauderdale, D.S.; Gaskin, R.E.; Boyle-Vavra, S.; Leitch, C.D.; Daum, R.S. Community-acquired methicillin-resistant \textit{Staphylococcus aureus} in children with no identified predisposing risk. \textit{JAMA} \textbf{1998}, \textit{279}, 593–598. [CrossRef] [PubMed]

6. Lina, G.; Piémont, Y.; Godail-Gamot, F.; Bes, M.; Peter, M.O.; Gauduchon, V.; Vandenesch, F.; Etienne, J. Involvement of Panton-Valentine leukocidin-producing \textit{Staphylococcus aureus} in primary skin infections and pneumonia. \textit{Clin. Infect. Dis.} \textbf{1999}, \textit{29}, 1128–1132. [CrossRef] [PubMed]

7. Center for Disease Control and Prevention. Four pediatric deaths from community-acquired methicillin-resistant \textit{Staphylococcus aureus}—Minnesota and North Dakota, 1997–1999. \textit{Morbid. Mortal. Wkly. Rep.} \textbf{1999}, \textit{52}, 88.

8. Dantes, R.; Mu, Y.; Belflower, R.; Aragon, D.; Dumyati, G.; Harrison, L.H.; Lessa, F.C.; Lynfield, R.; Nadle, J.; Petit, S.; et al. National burden of invasive methicillin-resistant \textit{Staphylococcus aureus} infections, United States, 2011. \textit{JAMA Intern. Med.} \textbf{2013}, \textit{173}, 1970–1978. [PubMed]

9. Patel, A.J.; Terribilini, R.G.; Ghobadi, F.; Azhir, A.; Barber, A.; Pearson, J.M.; Kalantari, H.; Hassen, G.W. Antibiotics for methicillin-resistant \textit{Staphylococcus aureus} skin and soft tissue infections: The challenge of outpatient therapy. \textit{Am. J. Emerg. Med.} \textbf{2014}, \textit{32}, 135–138. [CrossRef] [PubMed]

10. Pendleton, J.N.; Gorman, S.P.; Gilmore, B.F. Clinical relevance of the ESKAPE pathogens. \textit{Expert Rev. Anti-Infect. Ther.} \textbf{2013}, \textit{11}, 297–308. [CrossRef] [PubMed]

11. Stryjewski, M.E.; Corey, G.R. Methicillin-resistant \textit{Staphylococcus aureus}: An evolving pathogen. \textit{Clin. Infect. Dis.} \textbf{2014}, \textit{58}, S10–S19. [CrossRef] [PubMed]

12. Bae, I.G.; Federspiel, J.J.; Miró, J.M.; Woods, C.W.; Park, L.; Rybak, M.J.; Rude, T.H.; Bradley, S.; Bukovski, S.; de la Maria, C.G.; et al. Heterogeneous vancomycin-intermediate susceptibility phenotype in bloodstream methicillin-resistant \textit{Staphylococcus aureus} isolates from an international cohort of patients with infective endocarditis: prevalence, genotype, and clinical significance. \textit{J. Infect. Dis.} \textbf{2009}, \textit{200}, 1355–1366. [CrossRef] [PubMed]

13. Gomes, D.M.; Ward, K.E.; LaPlante, K.L. Clinical implications of vancomycin heteroresistant and intermediately susceptible \textit{Staphylococcus aureus}. \textit{Pharmacotherapy} \textbf{2015}, \textit{35}, 424–432. [CrossRef] [PubMed]

14. Moise, P.A.; North, D.; Steenbergen, J.N.; Sakoulas, G. Susceptibility relationship between vancomycin and daptomycin in \textit{Staphylococcus aureus}: Facts and assumptions. \textit{Lancet Infect. Dis.} \textbf{2009}, \textit{9}, 617–624. [CrossRef]

15. Sader, H.S.; Jones, R.N.; Rossi, K.L.; Rybak, M.J. Occurrence of vancomycin-tolerant and heterogeneous vancomycin-intermediate strains (hVISA) among \textit{Staphylococcus aureus} causing bloodstream infections in nine USA hospitals. \textit{J. Antimicrob. Chemother.} \textbf{2009}, \textit{64}, 1024–1028. [CrossRef] [PubMed]

16. Jones, R.N. Microbiological features of vancomycin in the 21st century: Minimum inhibitory concentration creep, bactericidal/static activity, and approved breakpoints to predict clinical outcomes or detect resistant strains. \textit{Clin. Infect. Dis.} \textbf{2006}, \textit{42}, S13–S24. [CrossRef] [PubMed]

17. Traczewski, M.M.; Katz, B.D.; Steenbergen, J.N.; Brown, S.D. Inhibitory and bactericidal activities of daptomycin, vancomycin, and teicoplanin against methicillin-resistant \textit{Staphylococcus aureus} isolates collected from 1985–2007. \textit{Antimicrob. Agents Chemother.} \textbf{2009}, \textit{53}, 1735–1738. [CrossRef] [PubMed]

18. Bassetti, M.; Righi, E. Development of novel antibacterial drugs to combat multiple resistant organisms. \textit{Langenbecks Arch. Surg.} \textbf{2015}, \textit{400}, 153–165. [CrossRef] [PubMed]

19. Coates, A.R.M.; Halls, G.; Hu, Y. Novel classes of antibiotics or more of the same? \textit{Br. J. Pharmacol.} \textbf{2011}, \textit{163}, 184–194. [CrossRef] [PubMed]
20. Infectious Diseases Society of America. The 10 × 20 initiative: Pursuing a global commitment to develop 10 new antibacterial drugs by 2020. Clin. Infect. Dis. 2010, 50, 1081–1083.

21. Kabir, M.S.; Engelbrecht, K.; Polanowski, R.; Krueger, S.M.; Ignasiak, R.; Rott, M.; Schwan, W.R.; Stemper, M.E.; Reed, K.D.; Sherman, D.; et al. New classes of Gram-positive selective antibacterials: Inhibitors of MRSA and surrogates of the causative agents of anthrax and tuberculosis. Bioorg. Med. Chem. Lett. 2010, 18, 5745–5749. [CrossRef] [PubMed]

22. Schwan, W.R.; Kabir, M.S.; Kallas, M.; Krueger, S.; Monte, A.; Cook, J.M. Synthesis and minimum inhibitory concentrations of SK-03-92 against Staphylococcus aureus and other gram-positive bacteria. J. Infect. Chemother. 2012, 18, 124–126. [CrossRef] [PubMed]

23. Schwan, W.R.; Kolesar, J.M.; Kabor, M.S.; Elder, E.J., Jr.; Williams, J.B.; Minerath, R.; Cook, J.M.; Witzgmann, C.M.; Monte, A.; Flaherty, T. Pharmacokinetic/toxicity properties of the new anti-staphylococcal lead compound SK-03-92. Antibiotics 2015, 4, 617–626. [CrossRef] [PubMed]

24. Cohen, N.R.; Lobritz, M.A.; Collins, J.J. Microbial persistence and the road to drug resistance. Cell Host Microbe 2013, 13, 632–642. [CrossRef] [PubMed]

25. Conlon, B.P. Staphylococcus aureus chronic and relapsing infections: Evidence of a role for persister cells: An investigation of persister cells, their formation and their role in S. aureus disease. Bioessays 2014, 36, 991–996. [CrossRef] [PubMed]

26. Lechner, S.; Lewis, K.; Bertram, R. Staphylococcus aureus persisters tolerant to bactericidal antibiotics. J. Mol. Microbiol. Biotechnol. 2012, 22, 235–244. [CrossRef] [PubMed]

27. Lewis, K. Persister cells. Annu. Rev. Microbiol. 2010, 64, 357–372. [CrossRef] [PubMed]

28. Keren, I.; Shah, D.; Spoering, A.; Kaldalu, N.; Lewis, K. Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli. J. Bacteriol. 2004, 186, 8172–8180. [CrossRef] [PubMed]

29. Spoering, A.L.; Lewis, K. Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials. J. Bacteriol. 2001, 183, 6746–6751. [CrossRef] [PubMed]

30. Stewart, P.S.; Costerton, J.W. Antibiotic resistance of bacteria in biofilms. Lancet 2001, 358, 135–138. [CrossRef]

31. Jang, H.; Nde, C.; Toghrol, F.; Bentley, W.E. Microarray analysis of toxicogenic effects of ortho-phenylphenol in Staphylococcus aureus. BMC Genomics 2008, 9, 411. [CrossRef] [PubMed]

32. Lama, A.; Pané-Farré, J.; Chon, T.; Wiersma, A.M.; Sit, C.S.; Vederas, J.C.; Hecker, M.; Nakano, M.M. Response of methicillin-resistant Staphylococcus aureus to amicoumacin A. PLoS ONE 2012, 7, e34037. [CrossRef] [PubMed]

33. Muthaiyan, A.; Silverman, J.A.; Jayaswal, R.K.; Wilinson, B.J. Transcriptional profiling reveals that daptomycin induces the Staphylococcus aureus cell wall stress stimulus and gene responsive to membrane depolarization. Antimicrob. Agents Chemother. 2008, 52, 980–990. [CrossRef] [PubMed]

34. Lechner, S.; Prax, M.; Lange, B.; Huber, C.; Eisenreich, W.; Herbig, A.; Nieselt, K.; Bertram, R. Metabolic and transcriptional activities of Staphylococcus aureus challenged with high-doses of daptomycin. Int. J. Med. Microbiol. 2014, 304, 931–940. [CrossRef] [PubMed]

35. Pan, Z.; Agarwal, A.K.; Xu, T.; Feng, Q.; Baerson, S.R.; Duke, S.O.; Rimando, A.M. Identification of molecular pathways affected by pterostilbene, a natural dimethylether analog of resveratrol. BMC Med. Genomics 2008, 20, 1–7. [CrossRef] [PubMed]

36. Wang, Z.; Gu, Z.; Shen, Y.; Wang, Y.; Li, J.; Lv, H.; Huo, K. The natural product resveratrol inhibits yeast cell separation by extensively modulating the transcriptional landscape and reprogramming the intracellular metabolome. PLoS ONE 2016, 11, e0150156. [CrossRef]

37. Qin, N.; Tan, X.; Jiao, Y.; Liu, L.; Zhao, W.; Yang, S.; Jia, A. RNA-Seq-based transcriptome analysis of methicillin-resistant Staphylococcus aureus biofilm inhibition by ursolic acid and resveratrol. Sci. Rep. 2014, 4, 5467. [CrossRef] [PubMed]

38. Fey, P.D.; Endres, J.L.; Yajala, V.K.; Widhelm, T.J.; Boissy, R.J.; Bose, J.L.; Bayles, K.W. A genetic resource for rapid and comprehensive phenotype screening of nonessential Staphylococcus aureus genes. mBio 2013, 4, e00537012. [CrossRef] [PubMed]

39. Xiao, Q.; Jiang, X.; Moore, K.J.; Shao, Y.; Pi, H.; Dubail, I.; Charbit, A.; Newton, S.M.; Klebba, P.E. Sortase independent and dependent systems for acquisition of haem and haemoglobin in Listeria monocytogenes. Mol. Microbiol. 2011, 80, 1581–1597. [CrossRef] [PubMed]
40. Yeh, J.I.; Chinte, U.; Du, S. Structure of glycerol-3-phosphate dehydrogenase, an essential monomeric enzyme involved in respiration and metabolism. Proc. Nat. Acad. Sci. USA 2008, 105, 3280–3285. [CrossRef] [PubMed]

41. Ramasamy, R.; Yan, S.F.; Schmidt, A.M. Methylglyoxal comes of AGE. Cell 2006, 124, 258–260. [CrossRef] [PubMed]

42. Ackerman, R.S.; Cozzarelli, N.R.; Epstein, E.W. Accumulation of toxic concentrations of methylglyoxal by wild-type Escherichia coli K-12. J. Bacteriol. 1974, 119, 357–362. [PubMed]

43. Rabban, N.; Thornalley, P.J. Methylglyoxal, glyoxalase 1 and the dicarbonyl proteome. Amino Acids 2012, 42, 1133–1142. [CrossRef] [PubMed]

44. Kwak, M.; Ku, M.; Kang, S. NAD+-linked alcohol dehydrogenase 1 regulates methylglyoxal concentration in Candida albicans. FEMS Lett. 2014, 588, 1144–1153. [CrossRef] [PubMed]

45. Spoering, A.L.; Vulić, M.; Lewis, K. GlpD and PIsB participate in persister cell formation in Escherichia coli. J. Bacteriol. 2006, 188, 5136–5144. [CrossRef] [PubMed]

46. Stauffer, L.T.; Steiert, P.S.; Steiert, J.G.; Stauffer, G.V. An NAD+-linked alcohol dehydrogenase 1 regulates methylglyoxal concentration in Escherichia coli. J. Bacteriol. 2006, 188, 5136–5144. [CrossRef] [PubMed]

47. Gemayel, R.; Fortpied, J.; Rzem, R.; Vertommen, D.; Veiga-da-Cunha, M.; van Schaftingen, E. Many fructosamine 3-kinase homologues in bacteria are ribulosamine/erythrulosamine 3-kinases potentially involved in protein deglycation. FEBS J. 2007, 274, 4360–4374. [CrossRef] [PubMed]

48. Polanowski, R.; Rott, M.; University of Wisconsin-La Crosse, La Crosse, WI, USA. unpublished data. 2016.

49. Performance Standards for Antimicrobial Susceptibility Testing, 16th Informational Supplement; NCCLS document M100-S16; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2006.

50. Bednarska, N.G.; Schymkowitz, J.; Rousseau, F.; van Eldere, J. Protein aggregation in bacteria: The thin boundary between functionality and toxicity. Microbiology 2013, 159, 1795–1806. [CrossRef] [PubMed]

51. Wood, R.C.; Steers, E. Study of the purine metabolism of Staphylococcus aureus. J. Bacteriol. 1959, 77, 760–765. [PubMed]

52. Subramanian, D.; Natarajan, J. Network analysis of S. aureus response to ramoplanin reveals modules for virulence factors and resistance mechanisms and characteristic novel genes. Gene 2015, 574, 149–162. [PubMed]

53. Cuaron, J.A.; Dulal, S.; Song, Y.; Singh, A.K.; Montelongo, C.E.; Yu, W.; Nagarajan, V.; Jayaswal, R.K.; Wilkinson, B.J.; Gustafson, J.E. Tea tree oil-induced transcriptional alterations in Staphylococcus aureus. Phytother. Res. 2013, 27, 390–396. [CrossRef] [PubMed]

54. Cuacon, J.A.; Dulal, S.; Song, Y.; Singh, A.K.; Montelongo, C.E.; Yu, W.; Nagarajan, V.; Jayaswal, R.K.; Wilkinson, B.J.; Gustafson, J.E. Tea tree oil-induced transcriptional alterations in Staphylococcus aureus. Phytother. Res. 2013, 27, 390–396. [CrossRef] [PubMed]

55. Shen, F.; Tang, X.; Wang, Y.; Yang, Z.; Shi, X.; Wang, C.; Zhang, Q.; An, Y.; Cheng, W.; Jin, K.; et al. Phenotype and expression profile analysis of Staphylococcus aureus biofilms and planktonic cells in response to licochalcone A. Appl. Microbiol. Biotechnol. 2015, 99, 359–373. [CrossRef] [PubMed]

56. Blakely, R.L.; Vitiol, E. The control of nucleotide biosynthesis. Annu. Rev. Biochem. 1968, 37, 201–224. [CrossRef] [PubMed]

57. Fung, D.K.; Chan, E.W.; Chin, M.L.; Chan, R.C. Delineation of a bacterial starvation stress response network which can mediate antibiotic tolerance development. Antimicrob. Agents Chemother. 2010, 54, 1082–1093. [CrossRef] [PubMed]

58. Maisonneuve, E.; Cerdes, K. Molecular mechanisms underlying bacterial persisters. Cell 2014, 157, 539–548. [CrossRef] [PubMed]

59. Yee, R.; Cui, P.; Shi, W.; Feng, J.; Zhang, Y. Genetic screen reveals the role of purine metabolism in Staphylococcus aureus persistence to rifampicin. Antibiotics 2015, 4, 627–642. [CrossRef] [PubMed]

60. Ranjit, D.K.; Endres, J.L.; Bayles, K.W. Staphylococcus aureus CidA and LrgA proteins exhibit holin-like properties. J. Bacteriol. 2011, 193, 2468–2476. [CrossRef] [PubMed]

61. Sadykov, M.R.; Bayles, K. The control of death and lysis in staphylococcal biofilms: A coordination of physiological signals. Curr. Opin. Microbiol. 2012, 15, 211–215. [CrossRef] [PubMed]

62. Yang, S.J.; Rice, K.C.; Brown, R.J.; Patton, T.G.; Liou, L.E.; Park, Y.H.; Bayles, K.W. A LysR-type regulator, CidR, is required for induction of the Staphylococcus aureus cidABC operon. J. Bacteriol. 2005, 187, 5893–5900. [CrossRef] [PubMed]

63. Bayles, K.W. Bacterial programmed cell death: Making sense of a paradox. Nat. Rev. Microbiol. 2014, 12, 63–69. [CrossRef] [PubMed]
63. Tanouchi, Y.; Lee, A.J.; Meredith, H.; You, L. Programmed cell death in bacteria and implications for antibiotic therapy. *Trends Microbiol.* 2013, 21, 265–270. [CrossRef] [PubMed]

64. Sarkar, S.K.; Bhattacharyya, A.; Mandal, S.S. YnfA, a SMP family efflux pump is abundant in *Escherichia coli* isolates from urinary infection. *Indian J. Med. Microbiol.* 2015, 33, 139–142. [PubMed]

65. Mazmanian, S.K.; Liu, G.; Ton-That, H.; Schneewind, O. *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. *Science* 1999, 285, 760–763. [CrossRef] [PubMed]

66. Altschul, S.F.; Madden, T.L.; Schäffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 1997, 25, 3389–3402. [CrossRef] [PubMed]

67. Nikolskaya, A.N.; Galperin, M.Y. A novel type of conserved DNA-binding domain in the transcriptional regulators of the AlgR/AgrA/LytR family. *Nucleic Acids Res.* 2002, 30, 2453–2459. [CrossRef] [PubMed]

68. Nicod, S.S.; Weinzierl, R.O.; Burchell, L.; Escalera-Maurer, A.; James, E.H.; Wigneshweraraj, S. Systematic mutational analysis of the LysTR DNA binding domain of *Staphylococcus aureus* virulence gene transcription factor AgrA. *Nucleic Acids Res.* 2014, 42, 12523–12536. [CrossRef] [PubMed]

69. Lan, L.; Cheng, A.; Dunman, P.M.; Missiakas, D.; He, C. Golden pigment production and virulence gene expression are affected by metabolisms in *Staphylococcus aureus*. *J. Bacteriol.* 2010, 192, 3068–3077. [CrossRef] [PubMed]

70. Fuchs, S.; Pané-Farré, J.; Kohler, C.; Hecker, M.; Engemann, S. Anaerobic gene expression in *Staphylococcus aureus*. *J. Bacteriol.* 2007, 189, 4275–4289. [CrossRef] [PubMed]

71. Kadurugamuwa, J.L.; Sin, L.; Albert, E.; Yu, J.; Francis, K.; DeBoer, M.; Rubin, M.; Bellinger-Kawahara, C.; Parr, T.R., Jr.; Contag, P.R. Direct continuous method for monitoring biofilm infection in a mouse model. *Infect. Immun.* 2003, 71, 882–890. [CrossRef] [PubMed]

72. Xiong, Y.Q.; Willard, J.; Kadurugamuwa, J.L.; Yu, J.; Francis, K.P.; Bayer, A.S. Real-time in vivo bioluminescent imaging for evaluating the efficacy of antibiotics in a rat *Staphylococcus aureus* endocarditis model. *Antimicrob. Agents Chemother.* 2005, 49, 380–387. [CrossRef] [PubMed]

73. Reiß, S.; Pané-Farré, J.; Fuchs, S.; François, P.; Liebeke, M.; Schrenzel, J.; Lidequist, U.; Lalk, M.; Wolz, C.; Hecker, M.; Engemann, S. Global analysis of the *Staphylococcus aureus* response to mupirocin. *Antimicrob. Agents Chemother.* 2012, 56, 787–804. [CrossRef] [PubMed]

74. Anderson, K.L.; Roberts, C.; Disz, T.; Vonstein, V.; Hwang, K.; Overbeck, R.; Olson, P.D.; Projan, S.J.; Dunman, P.M. Characterization of the *Staphylococcus aureus* heat shock, cold shock, stringent, and SOS responses and their effects on log-phase mRNA turnover. *J. Bacteriol.* 2006, 188, 6739–6756. [CrossRef] [PubMed]

75. Fischer, M.; Zimmerman, T.P.; Short, S.A. A rapid method for the determination of guanosine 5′-diphosphate-3′-diphosphate and guanosine 5′triphosphate-3′-diphosphate by high performance liquid chromatography. *Anal. Biochem.* 1982, 121, 135–139. [CrossRef]

76. Melo, A.; Colombo, A.; Arthington-Skaggs, B. Paradoxical growth effect of caspofungin observed on biofilms and planktonic cells of five different *Candida* species. *Antimicrob. Agents Chemother.* 2007, 51, 3081–3088. [CrossRef] [PubMed]

77. Wood, T.; Knabels, S.; Kwan, B. Bacterial persister cell formation and dormancy. *Appl. Environ. Microbiol.* 2013, 79, 7116–7121. [CrossRef] [PubMed]

78. Marraffini, L.A.; DeDent, A.C.; Schneewind, O. Sortases and the art of anchoring proteins to the envelopes of Gram-positive bacteria. *Microbiol. Mol. Biol. Rev.* 2006, 70, 192–221. [CrossRef] [PubMed]

79. Fischetti, V.A.; Pancholi, V.; Schneewind, O. Conservation of a hexapeptide sequence in the anchor region of surface proteins from gram-positive bacteria. *Mol. Microbiol.* 1990, 4, 1603–1605. [CrossRef] [PubMed]

80. Boekhorst, J.; de Been, M.W.; Kleerebezem, M.; Siezen, R.J. Genome-wide detection and analysis of cell wall-bound proteins with LPXTG-like sorting motifs. *J. Bacteriol.* 2005, 187, 4928–4934. [CrossRef] [PubMed]

81. Ton-That, H.; Liu, G.; Mazmanian, S.K.; Faull, K.F.; Schneewind, O. Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of *Staphylococcus aureus* at the LPXTG motif. *Proc. Natl. Acad. Sci. USA* 1999, 96, 12424–12429. [CrossRef] [PubMed]

82. Foster, T.J.; Hook, M. Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol.* 1998, 6, 484–488. [CrossRef]
83. Mazmanian, S.K.; Liu, G.; Jensen, E.R.; Lenoy, E.; Schneewind, O. Staphylococcus aureus mutants defective in the display of surface proteins and in the pathogenesis of animal infections. Proc. Natl. Acad. Sci. USA 2000, 97, 5510–5515. [CrossRef] [PubMed]

84. Sibbald, M.J.; Yang, X.-M.; Tsompanidou, E.; Qu, D.; Hecker, M.; Becher, D.; Buist, G.; Maarten van Dijl, J. Partially overlapping substrate specificities of staphylococcal group—A sortases. Proteomics 2012, 12, 3049–3062. [CrossRef] [PubMed]

85. Jonsson, I.M.; Mazmanian, S.K.; Schneewind, O.; Bremell, T.; Tarkowski, A. The role of Staphylococcus aureus sortase A and sortase B in murine arthritis. Microbes Infect. 2003, 5, 775–780. [CrossRef]

86. Weiss, W.J.; Lenoy, E.; Murphy, T.; Tardio, L.; Burgio, P.; Projan, S.J.; Schneewind, O.; Alksne, L. Effect of srtA and srtB gene expression on the virulence of Staphylococcus aureus in animal infection. J. Antimicrob. Chemother. 2004, 53, 480–486. [CrossRef] [PubMed]

87. Voyich, J.M.; Braughton, K.R.; Sturdevant, D.E.; Whitney, A.R.; Said-Salim, B.; Porcella, S.F.; Long, R.D.; Dorward, D.W.; Gardner, D.J.; Kreiswirth, B.N.; et al. Insights into mechanisms used by Staphylococcus aureus to avoid destruction by human neutrophils. J. Immunol. 2005, 175, 3907–3919. [CrossRef] [PubMed]

88. Baba, T.; Takeuchi, F.; Kuroda, M.; Yuzawa, H.; Aoki, K.; Oguchi, A.; Nagai, Y.; Iwama, N.; Asano, K.; Naimi, T.; et al. Genome and virulence determinants of high virulence community-acquired MRSA. Lancet 2002, 359, 1819–1827. [CrossRef]

89. Bustin, S.S.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; et al. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 2009, 55, 611–622. [CrossRef] [PubMed]

90. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2^−ΔΔCT Method. Methods 2001, 25, 402–408. [CrossRef] [PubMed]

91. Ochi, K. Occurrence of the stringent response in Streptomyces sp. and its significance for the initiation of morphological and physiological differentiation. J. Gen. Microbiol. 1986, 132, 2621–2631. [PubMed]

92. Ochi, K. Metabolic initiation of differentiation and secondary metabolism by Streptomyces griseus: Significance of the stringent response (ppGpp) and GTP content in relation to A factor. J. Bacteriol. 1987, 169, 3608–3616. [CrossRef] [PubMed]

93. Wilson, J.M.; Oliva, B.; Cassels, R.; O’Hanlon, P.J.; Chopra, I. SB 205952, a novel semisynthetic monic acid analog with at least two modes of action. Antimicrob. Agents Chemother. 1995, 39, 1925–1933. [CrossRef] [PubMed]

94. Stepanovic, S.; Vukovic, D.; Pavlovic, M.; Svabic-Vlahovic, M. Influence of dynamic conditions on biofilm formation by staphylococci. Eur. J. Clin. Microbiol. Infect. Dis. 2001, 20, 502–504. [CrossRef] [PubMed]