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The anti-MRSA compound 3-O-alpha-L-(2'',3''-di-p-coumaroyl)rhamnoside (KCR) inhibits protein synthesis in *Staphylococcus aureus*.

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Abstract

Methicillin-resistant *S aureus* (MRSA) contributes to patient mortality and extended hospital stays. 3-O-alpha-L-(2'',3''-di-p-coumaroyl)rhamnoside (KCR) is a natural product antibiotic that is effective against MRSA but has no known mechanism of action (MOA). We used proteomics to identify the MOA for KCR.

Methicillin sensitive *S aureus* and a mixture of four KCR stereoisomers were tested. A time-kill assay was used to choose a 4 hour treatment using KCR at 5X its MIC for proteomic analysis. *S aureus* was treated in triplicate with KCR, oxacillin or vehicle and quantitative proteomic analysis was carried out using isobaric tags and mass spectrometry. 1190 proteins were identified and 552 were affected by KCR ($q < 0.01$). Ontology analysis identified 6 distinct translation-related categories that were affected by KCR (PIANO, 10% false-discovery rate) including structural constituent of ribosome, translation, rRNA binding, tRNA binding, tRNA processing and aminoacyl-tRNA ligase activity. Median fold changes (KCR vs Control) for small and large ribosomal components were 1.46 and 1.43 respectively. KCR inhibited the production of luciferase protein in an in vitro assay (IC₅₀ 39.6 µg/ml).

Upregulation of translation-related proteins in response to KCR indicates that KCR acts to disrupt *S aureus* protein synthesis. This was confirmed with an in vitro transcription/translation assay.

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⁶.Data Availability

Mass spectrometry data can be downloaded from: <ftp://massive.ucsd.edu/MSV000084282/>

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Graphical Abstract

Keywords

methicillin-resistant; *Staphylococcus aureus*; 3-O- α -L-(2'',3''-di-p-coumaroyl)rhamnoside; KCR; proteomics; mechanism of action

2. Introduction

Among the *Staphylococcus aureus* (*S. aureus*) bacteria, methicillin-resistant *S. aureus* (MRSA) causes the greatest patient mortality and extends hospital stays. MRSA alone is estimated to kill around 19,000 people every year in the U.S.[1, 2]. Recently, MRSA has emerged as a community-associated pathogen (CA-MRSA), infecting individuals who have not either been hospitalized or undergone a medical procedure[3]. Furthermore, MRSA is also known to be a common skin colonizer and causes the same spectrum of infections as methicillin-susceptible *S. aureus* (MSSA) infections[4]. Over 50% of *Staphylococcus* cases around the world are known to be caused by MRSA[5].

Currently, even though vancomycin is only successful in 35–57% of the infections[6], it is still the drug of choice for MRSA infections. Vancomycin has to be administered by *i.v.* injection/infusion, which may require prolonged hospitalization. Further, increased use of vancomycin in past decades has resulted in the emergence of vancomycin-resistant *S. aureus* (VISA) with reduced susceptibility to glycopeptides[7]. Oral antibiotic choices for MRSA include trimethoprim-sulfamethoxazole (TMP-SMX), clindamycin, doxycycline, minocycline, linezolid and rifampin. TMP-SMX is a sulfonamide antibiotic used as the initial antibiotic for CA-MRSA infection, but it is associated with several potentially serious adverse reactions and is not suitable for general use[8]. Clindamycin is another antibiotic frequently used as an initial therapeutic option, but resistance to clindamycin can develop rapidly[9, 10]. Doxycycline and minocycline also have been reported to be effective antibiotics for MRSA/CA-MRSA[11, 12]. Linezolid has been used in central nervous system (CNS) MRSA infections and has similar efficacy as vancomycin. Its oral bioavailability allows oral administration thus decreasing hospital stays and therapy costs[13]. Rifampin has been used in combination with other antibiotics, such as TMP-SMX, clindamycin, or doxycycline/minocycline since resistance may develop rapidly if it is

used as monotherapy[14]. However, the most effective anti-MRSA drug used currently is daptomycin, a cyclic lipopeptide derived from *Streptomyces roseosporus*. Daptomycin is useful in treatments of short duration and persistent MRSA unaffected by other drug treatments[15]. Its mechanism of action involves binding to the bacterial cell membrane, which causes depolarization of the membrane potential leading to inhibition of bacterial growth. Unfortunately, the FDA has reported serious side effects for daptomycin, including an increase in blood creatine phosphokinase, rhabdomyolysis, skin exfoliation and skin ulcers. Thus, there are great and urgent needs for novel antimicrobial agents that can effectively combat these new strains of drug-resistant “superbugs”[16]. One of the most promising sources of anti-MRSA agents is from natural products.

At the present time, about 60–75% of pharmaceutical sales for infectious diseases and cancer are from products derived from natural sources and approximately 60% of those compounds commercially available or in the late stages of clinical trials for the treatment of either infectious diseases or cancer are of natural product origin[17]. Rapid emergence of multi drug-resistant microbial pathogens is a serious problem to US human health.

American sycamore is a native US species already in culture for timber and pulp production. As a result, leaf material for drug production is highly scalable. Sycamore trees are characteristically known for their long-life spans, many living 400–500 years. Due to their long-life expectancy, they have uniquely evolved the ability to produce potent antimicrobials defending against pathogenic bacteria.[18] In a recent study, Ibrahim *et al.* isolated a group of glycosides from the leaf extract of American sycamore[6]. Fractionation of the extract produced an active kaempferol molecule, 3-*O*-alpha-L-(2'',3''-di-*p*-coumaroyl)rhamnoside (KCR) in four stereoisomeric forms [3-*O*-alpha-L-(2'',3''-di-*E*-*p*-coumaroyl)rhamnoside, 3-*O*-alpha-L-(2''-*E*-*p*-coumaroyl-3''-*Z*-*p*-coumaroyl)rhamnoside, 3-*O*-alpha-L-(2''-*Z*-*p*-coumaroyl-3''-*E*-*p*-coumaroyl)rhamnoside, and 3-*O*-alpha-L-(2'',3''-di-*Z*-*p*-coumaroyl)rhamnoside abbreviated as *EE*, *EZ*, *ZE*, and *ZZ*] (Figure 1). All four isomers exhibit potent anti-MRSA activity, both in cultures and in animal models with the *ZZ* isomer having the greatest potency[6, 19]. In 2004, the FDA established a Botanical Drug Guidance, instituting the requirements and special consideration of naturally derived drug mixtures in the drug approval process[20]. Only two botanical drug New Drug applications (NDAs) have been approved since 2004. The first botanical, Veregen™ (Polyphenon® E), uses green tea polyphenol extracts containing concentrated amounts of catechin to treat genital warts[21]. The second, Fulyzaq (Crofelemer) approved in 2012, provides a model representation of a botanical mixture consisting of heterogeneous proanthocyanidin oligomers to provide symptomatic relief for HIV/AIDS patients on anti-retroviral therapy[22]. Several studies explored a single chemical compound from active extracts of plants responsible for therapeutic effect and not developing resistance[23], however; it has been proven that the process of isolation of a single compound often leads to the loss or decrease in activity[24–26]. Mixture therapy can prevent the development of drug resistance through two routes. First, multiple mechanisms (each requiring different mutations) are required for resistance to occur to all drugs. Even if a small number of variants with the potential for resistance to individual agents exist before treatment, it is highly unlikely that some of these variants are able to resist all the drugs. Second, multiple drugs suppress replication more effectively than single agents[27, 28]. In addition, the use of multiple first-line therapies against malaria

yields a better clinical outcome than using a single therapy[29]. It is proposed that the administration of KCR as a botanical mixture of the four isomers exponentially reduces the ability of bacteria to evolve resistance. A common resistance mechanism introducing a potential threat to KCR is the enzymatic inactivation of the antibiotic using selective esterases to target and hydrolyze susceptible chemical bonds[30]. Fortunately, the development of a bacterial esterase able to selectively target and hydrolyze esters in each of the sterically different KCR isomers is greatly reduced. Active metabolites of these anti-MRSA extracts may possess a novel mechanism of action as they do not show cross-resistance or structural homology with established antibiotics. This represents the first plant-derived antibiotics to prevent growth of MRSA both on surfaces and systemically. Recent data (unpublished) have also shown that both the parent compound and individual isomers have potent anti-VRSA activity as well.

The mechanism of action (MOA) for KCR is not yet resolved. It inhibits NorA multidrug efflux transporter activity[31] and may inhibit topoisomerase activity[32] but no global analysis of the bacterial response to KCR has been conducted. Proteomic analysis has become a mainstay of antibiotic MOA studies[33–40]. Changes in the proteome following antibiotic treatment reflect the bacterial response to the antibiotic which in many cases is specific to the antibiotic MOA[41]. In this work, we set out to investigate the MOA for KCR using quantitative proteomic analysis.

3. Methods

Extraction and Isolation of KCR

The leaves of *P. occidentalis* were collected in March 2016 from Oxford, Mississippi. A specimen voucher (No. #03062016) is housed at the Department of Drug Discovery, School of Pharmacy, the Medical University of South Carolina. The air-dried and powered leaves (3.1 kg) were percolated with 200 proof ethanol (52 L) at room temperature and the drug purified as previously reported [6]. KCR purity and relative stereoisomer proportions were assessed using ¹H NMR and liquid chromatography and mass spectrometry (LCMS).

Cell Culture and Treatment Conditions.

A methicillin sensitive strain of *S. aureus* (ATCC 25923) was cultured in Mueller-Hinton broth. The minimum inhibitory concentration (MIC) was determined using a 2-fold dilution series of KCR. The time-kill assay was conducted using standard methods.

Proteomic Analysis.

S. aureus in exponential growth was treated for 4 hours with a mixture of KCR isoforms or with oxacillin at 5X MIC or with DMSO vehicle. Treatments were in triplicate. Bacteria were harvested by centrifugation, washed in phosphate buffered saline and immediately boiled in 1% sodium dodecyl sulfate for 5 minutes and then disrupted using a bead beater. The size of the bacteria pellet was similar for all treatments however a bicinchoninic acid protein assay indicated high protein concentrations in the KCR-treated samples. It was concluded that KCR interfered with the protein assay and therefore the samples were all precipitated twice in 80% acetone (overnight at –80 degrees C) to remove any residual KCR

and then were assumed to have the same amount of protein. Samples were reduced, alkylated, trypsinized overnight and labeled with respective Tandem Mass Tag TMT reagents (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Samples were pooled and analyzed by liquid chromatography-mass spectrometry with a Fusion mass spectrometer (Thermo Fisher Scientific) set for data dependent analysis with high resolution MS2 scans and equipped with an Easy nLC UPLC using a single 90 minutes analysis. Spectra were searched using Proteome discoverer v2.1 against a Uniprot database downloaded 12.18.2015 using the taxonomy id 46170 (11 658 entries). Cysteine carbamidomethylation was set as a fixed modification and methionine oxidation and n-terminal acetylation were variable modifications. Peptide spectrum matches were accepted at a 1% false discovery rate as determined by a reversed database search. Data are available in supplementary table S1. Subsequent data analysis and visualization was carried out in R v3.1.3. Proteomic analysis was carried out in duplicate. Both analysis had similar outcomes and the results from just one of them are reported here.

Statistical analysis.

Protein differential expression was assessed using a moderated t-test from the limma package[42]. Protein abundances were normalized so that each sample had the same median and log transformed prior to analysis. The resultant p-values were transformed to q-values to account for the false discovery rate (FDR)[43]. T-statistics were submitted to PIANO to find protein categories that were affected by KCR or oxacillin[44] using gene ontologies for taxonomy id 46170 that were downloaded from Uniprot. Categories were required to have between 10 and 100 proteins for analysis. 76 categories were tested. PIANO results have a built-in FDR correction.

Transcription/Translation Assay.

E coli lysate from Promega (product # L1020, Madison, WI) was used to test the ability of KCR to inhibit transcription and translation of luciferase which was quantified using a luminescence assay[45]. The cell free system was prepared according to the manufacturer's instructions except that the suggested reaction volumes were split in half to double the number of reactions, i.e. 25 μ l reactions instead of 50 μ l and the luciferase plasmid was omitted. KCR was added to a final concentration of 4–128 μ g/ml KCR in 5% dimethyl sulfoxide (DMSO) and incubated for 15 minutes prior to addition of the luciferase plasmid. The reaction was incubated at 37 °C for 60 minutes and diluted 10-fold prior to luciferase activity detection. Luminescence was measured using a Fluoroscan Ascent FL (Thermo Fisher Scientific). Curve fitting was done with the nplr package in R v3.1.3.

4. Results

KCR Isomers

Bacteria respond to antibiotic treatment by altering their protein complement in a way that reflects the systems that were disrupted[41]. Monitoring the changes in protein abundance that are implemented following treatment with an antibiotic can indicate its mechanism of action. Here we investigate the proteomic response of a methicillin sensitive strain of *S aureus* to KCR with the goal of inferring its mechanism of action. A mixture of KCR

isomers was used for this study. The mixture consisted of the four isomers in the ratio (*ZZ*: *EZ*: *ZE*: *EE*) is 1: 1.32: 1.71: 2.31 (fig. 2 and 3). Ratios were established by comparison to standards (fig. S1 and S2).

Treatment Optimization

Oxacillin treated *S aureus* samples were also analyzed. The oxacillin analysis provides a positive control to confirm that we can interpret results for a known mechanism and also allows us to test whether aspects of the KCR response are general to all stressors or potentially specific to KCR.

The choice of treatment time and dose are critical for the success of mechanism of action studies. A time kill assay was used as a guide in choosing treatment conditions (fig. 4). Serial aliquots of antibiotic treated bacteria were taken over 24 hours, diluted in media and allowed to grow. The number of colony-forming units per unit volume was then counted. The MIC for KCR in *S aureus* ATCC 25923 was previously determined to be 16 µg/ml (not shown). The time kill assay indicated that a 5X MIC dose of KCR had a no effect on bacterial clonogenicity after 2 hours of treatment and a small difference at 6 hours compared to the untreated control (fig. 4A). A preliminary experiment indicated very little change to the proteome in samples treated with 5X MIC KCR for 2 hours (not shown), so a 4 hour treatment was used here. Similar analysis were carried out for oxacillin and indicated that 5X MIC for 4 hours was a reasonable choice for oxacillin as well (fig. 4B).

Proteomic Analysis

A TMT 10-plex-based proteomic analysis was used for identification and quantification. Three replicate samples were prepared and TMT labeled for each treatment. The samples were pooled and analyzed in a single LC-MS/MS run using MS2 quantification. 1190 proteins were identified in this analysis and 1118 quantified. On average 1106 were quantified per sample with no substantial change in the number of proteins identified between treatment groups (fig. S3a). Prior to normalization the median and interquartile ranges were similar between treatment groups (fig. S3b). These data indicate that the amount of protein in each sample was similar between treatment groups. Principal component analysis was performed to reduce the number of dimensions required for visualization. The first two principal components, accounting for 70.9% of protein variance, separate the treatment groups into distinct clusters indicating that the treatments had substantial and distinct effects on the *S aureus* proteome (fig 5).

Moderated t-tests identified 552 proteins that changed abundance in response to KCR treatment and 178 in response to oxacillin ($q < 0.01$, table 1 and fig. 6). Proteins that responded to KCR treatment were evenly distributed with respect to protein abundance (fig. 6 A and C) and were not enriched in proteins with just 1 unique peptide (16.8% of affected proteins had just 1 unique peptide compared to 25.9% of all proteins) indicating that protein identification quality did not determine which proteins were affected by KCR. The effect of KCR was split approximately equally between increased and decreased protein abundance (fig. 6 A and B) while oxacillin primarily decreased protein abundances (fig. 6 C and D). It is notable that a single protein, named “membrane protein”, uniprot ID: A0A0D1JMH4 had

a 30-fold increase in KCR treated samples relative to controls. This protein is a predicted protein with no functional annotation in Uniprot. Therefore the functional significance of this strongly affected protein is unknown.

Utaida *et al*, 2003[46] identified a set of genes that were overexpressed in response to cell-wall active agents. Of 10 Utaida set members that were quantified in our dataset, 6 had increased abundance in oxacillin treated cells ($q < 0.05$). Further, the top 20 proteins most up-regulated in response to oxacillin contained a number of known responders to cell wall stress including Teichoic acid biosynthesis protein, Glycerol kinase and Penicillin-binding protein. These results indicate that the proteome response to oxacillin reflected the mechanism of action for that antibiotic.

Set-based analysis as implemented in the PIANO package[44] was used to identify protein categories that were affected by KCR. This analysis tests all members of a protein category collectively for a treatment effect rather than focusing on members that passed a statistical threshold. The goal was to identify systems that were affected by KCR and so may be targets of this compound. 15 non-redundant categories had overall increased abundance in KCR treated samples (table 2). 6 of them related directly to translation. Translation-related proteins appeared to fall into two distinct categories: Ribosome components and tRNA-related proteins (fig. 7 and S4). Most of the changes are less than 2-fold in magnitude. The median fold changes are 1.46, 1.43 and 1.19 for large ribosomal subunit components, small ribosomal components and tRNA associated proteins. However they are highly consistent within categories and 82, 80 and 50% of the proteins in those categories respectively were affected by KCR treatment ($q < 0.01$) suggesting that increased abundance of translational machinery is part of the response of *S aureus* to KCR treatment. 266 proteins were increased in abundance following KCR treatment ($q < 0.01$). 75 of them (28.2%) were involved in translation. Using a more stringent criteria, $q < 0.0001$, 41 proteins were affected and 24 (58.5%) were involved in translation. These data indicate that a substantial proportion of proteins with increased abundance after KCR treatment were related to translation, especially for the most strongly affected proteins. In contrast translation-related proteins have mixed response to oxacillin indicating that they are not a generalized stress response (fig. 7 and S4). These data suggest that KCR acts by disrupting the machinery of translation in *S aureus*.

Transcription/Translation assay

To confirm that KCR disrupts protein synthesis, it was tested in an *E coli in vitro* transcription/translation system. This system allows us to test the hypothesis that KCR acts on translation machinery without specifying the target protein or binding site. An *E. coli* lysate was incubated with a luciferase plasmid and KCR between 4 and 256 $\mu\text{g}/\text{ml}$ and the production of luciferase was measured. The experiment was conducted in 5% DMSO which reduced luciferase production by 70% (not shown). The IC₅₀ determined from that fit is 39.6 $\mu\text{g}/\text{ml}$ (fig. 8). The luciferase data support the hypothesis that KCR acts as an inhibitor of protein synthesis.

5. Discussion

This study used proteomics to get a global view of the response of *S aureus* to KCR and develop a hypothesis for its MOA. KCR elicited a clear response in *S aureus* to increase the abundance of proteins involved in translation, including ribosomal proteins and tRNA processing proteins. The up-regulation of small and large ribosomal subunits was uniform across almost all components identified (fig 7) and similarly uniform response was observed for tRNA synthesis proteins (supplementary figure S4). The dominance of translation-associated pathways in the PIANO results (table 2), the substantial fraction of proteins that were increased following KCR treatment (58.5% at $q < 0.0001$) and the consistent up-regulation of small and large ribosomal subunits and tRNA synthesis proteins indicate that *S aureus* increases its capacity for protein synthesis following KCR treatment. The bacterial response to antibiotic challenge is closely tied to the antibiotic target and increased abundance of ribosomal proteins is a hallmark of translation-targeting antibiotics [47, 48]. Taken together these indicate that KCR acts to disrupt protein synthesis. The increased abundance of translational machinery is a mechanism to compensate for the disruption of translation by KCR. This is supported by an *in vitro* assay where transcription/translation was inhibited by KCR. The increase in abundance in translational machinery is not a general response to stress because (1) no increase in translational machinery abundance was observed in oxacillin treated samples (fig 6 and 7) and (2) the literature on antibiotic MOA doesn't support increased translation as a general response to stress but rather the opposite: global translation is decreased in response to antibiotic stress [41, 47].

The *in vitro* transcription/translation assay has several limitations. First, it is not specific for translation but includes all steps required to get a functional protein from a DNA sequence. Second it uses an *E coli* cellular extract rather than one from *S aureus*. Protein synthesis inhibitors typically have a broad spectrum of activity in cell-free systems [49] suggesting that the action of KCR will be the same in both organisms. However a species-specific response to KCR can't be ruled out. In spite of these limitations we identified an inhibitory effect by KCR on transcription/translation of a luciferase construct which supports our proposal that KCR acts as a translation inhibitor. Previous studies have identified inhibition by KCR stereoisomers for the NorA efflux pump [31] and DNA topoisomerase [32]. This unbiased study indicates that the effect of KCR on translation is its most prominent and important effect in *S aureus*. Other activities of KCR are not ruled out by this study, but the proteomics evidence indicates that translation is the main target of KCR in this system.

KCR exists naturally as a mixture of 4 KCR stereoisomers that differ in the stereochemistry of a pair of coumaroyl groups. This study was conducted with a mixture of stereoisomers. It is possible that the 4 KCR stereoisomers *EE*, *EZ*, *ZE* and *ZZ* have distinct biological effects. All 4 inhibit growth of a panel of MSRA and VRSA strains with varying effectiveness (data not shown). No molecular-level studies have compared them: Holler et al [31] tested just the *EE* form. Further studies will be required to determine if there is stereoselectivity in the effect of KCR on translation in MRSA.

The strain used in this study, ATCC 25923 is sensitive to methicillin and vancomycin. This strain was selected as a test system as it doesn't require specialized procedures for culturing.

Both methicillin and vancomycin are cell wall active antibiotics. Methicillin resistance is due to expression of a penicillin-binding protein form that has low affinity for methicillin[50] and vancomycin resistance is due to a modified cell wall that is thicker and less cross-linked than the non-resistant form[51]. Protein translation, the proposed MOA for KCR, is distinct from the mechanisms for methicillin and vancomycin resistance so it is expected that KCR will have the same mechanism in MRSA and VRSA as in MSSA. Further experiments will be required to confirm this. KCR is known to be effective against MRSA and VRSA[6, 19].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

MRSA	methicillin-resistant <i>S aureus</i>
CA-MRSA	community-associated methicillin-resistant <i>S aureus</i>
VRSA	vancomycin-resistant <i>S aureus</i>
MSSA	methicillin-susceptible <i>S aureus</i>
TMP-SMX	trimethoprim-sulfamethoxazole
CNS	central nervous system
KCR	3-O-alpha-L-(2'',3'')-di- <i>p</i> -coumaroyl)rhamnoside
MOA	mechanism of action
MIC	minimum inhibitory concentration
TMT	tandem mass tag
UPLC	ultra performance liquid chromatography
LCMS	liquid chromatography-mass spectrometry
NMR	nuclear magnetic resonance
DMSO	dimethyl sulfoxide
IC50	concentration for 50% inhibition
FDR	false discovery rate

9. References

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Highlights

- A mixture of KCR isomers ZZ, EZ, ZE and EE (1: 1.32: 1.71: 2.31 ratio) was tested for antimicrobial activity against methicillin-sensitive *S aureus*.
- Proteomic analysis indicated that proteins involved in translation were more abundant in KCR treated bacteria than in controls suggesting that KCR acts to inhibit translation.
- An in vitro translation assay supported the proposed mechanism.

Significance

Methicillin-resistant *S aureus* (MRSA) contributes to patient mortality and extended hospital stays. 3-O-alpha-L-(2'',3''-di-p-coumaroyl)rhamnoside (KCR) is a natural product antibiotic that is effective against MRSA but has no known mechanism of action (MOA). Using proteomic analysis we determined that KCR acts by inhibiting protein synthesis. KCR is an exciting novel antibiotic and this work represents an important step in its development towards clinical use.

E-p-coumaroyl

Z-p-coumaroyl

EE stereoisomer: R1 = R2 = *E-p-coumaroyl*

EZ stereoisomer: R1 = *E-p-coumaroyl*, R2 = *Z-p-coumaroyl*

ZE stereoisomer: R1 = *Z-p-coumaroyl*, R2 = *E-p-coumaroyl*

ZZ stereoisomer: R1 = R2 = *Z-p-coumaroyl*

Figure 1:
Structures of the four stereoisomers of KCR

Fig. 2.

The ^1H NMR Spectrum of KCR. The ratio of four isomers (ZZ: EZ: ZE: EE) is 1: 1.32: 1.71: 2.31.

Fig. 3.
Representative chromatogram of KCR (10 μ L injection).

Figure 4. Time-kill assays for KCR (A) and oxacillin (B) treatment of *S. aureus* ATCC 25923 were used to determine optimal conditions for proteomic analysis. The number of colonies identified in diluted aliquots taken at the indicated times are shown for untreated (square), antibiotic at 5X MIC (triangle) and 10X MIC (circle). Data are the mean of 2 replicates.

Figure 5.

Principal component analysis of protein abundance in untreated and KCR and oxacillin treated samples. The treatment groups form distinct clusters indicating that antibiotic treatment is responsible for most of the protein variation in these samples.

Figure 6. Ratio-Intensity plots and Volcano plots for KCR vs Ctrl (A and B) and Oxacillin vs Ctrl (C and D). Proteins that were affected by treatment ($q < 0.01$) are colored.

Figure 7.

47 of the 54 ribosomal proteins identified in this study were up-regulated by KCR treatment relative to control ($q < 0.01$). Shown here are fold-changes for the large (A) and small (B) ribosomal subunit components in KCR (red) or oxacillin (blue) treated samples relative to control. $n=3$, error bars indicate standard deviation.

Figure 8.

Curve fitting for KCR inhibition of luciferase luminescence as a measure of translation. The IC₅₀ was 39.6 $\mu\text{g/ml}$. Points indicate luminescence normalized to untreated value. The line indicates the fit curve and the grey area indicates the 95% confidence interval.

Table 1.

Proteins that showed the greatest increase in abundance due to KCR treatment.

Accession	Description	KCR vs Ctrl		Oxacillin vs Ctrl		Translation Associated
		q-value	FC ^a	q-value	FC ^a	
A0A0D1JT97	Cro/C1 family transcriptional regulator	2.27E-06	4.87	0.30	0.98	
A0A0D1K004	Sodium ABC transporter ATP-binding protein	2.27E-06	4.41	0.08	1.15	
A0A0D1HHJ4	DNA topoisomerase I	2.27E-06	3.64	0.16	1.07	
A0A0D6DQ24	tRNA modification GTPase MnmE	2.27E-06	2.70	0.02	1.19	yes
A0A0D1FSM8	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase	2.27E-06	2.65	0.15	0.94	
A0A0D1JMH4	Membrane protein	2.27E-06	30.71	0.19	1.16	
A0A0D1I542	Mannose-6-phosphate isomerase	5.06E-06	4.87	0.06	1.23	
A0A0D1HFA7	Type II pantothenate kinase	1.30E-05	3.23	0.28	1.03	
A0A0D1ESJ8	Chromosome partitioning protein parB	4.54E-05	4.06	0.32	0.98	
A0A0D6DQ35	50S ribosomal protein L34	6.68E-05	3.36	0.03	0.69	yes
A0A0D1H2J5	50S ribosomal protein L32	8.13E-05	5.81	0.06	0.61	yes

^aFold-change treated vs untreated

Table 2.Protein categories with increased abundance in KCR treated samples (FDR adjusted $p < 0.05$).

Category	Number of Proteins	Adjusted p-value
RNA binding	74	8.44E-04
structural constituent of ribosome	54	8.44E-04
translation	92	8.44E-04
rRNA binding	37	8.44E-04
intracellular	59	8.44E-04
tRNA binding	16	8.44E-04
GTPase activity	12	8.44E-04
ligase activity	58	6.84E-03
aminoacyl-tRNA ligase activity	21	6.91E-03
purine nucleotide biosynthetic process	15	7.60E-03
methyltransferase activity	27	7.60E-03
GTP binding	19	7.60E-03
'de novo' IMP biosynthetic process	11	7.60E-03
tRNA processing	14	1.23E-02
cell wall organization	12	4.96E-02