Transcriptomic Analysis of the Activity of a Novel Polymyxin against Staphylococcus aureus

Jinxin Zhao,a,b Soon-Ee Cheah,b Kade D. Roberts,b,c Roger L. Nation,b Philip E. Thompson,c Tony Velkov,b Zongjun Du,a Matthew D. Johnson,b,d Jian Li,b,d
College of Marine Science, Shandong University at Weihai, Weihai, China; Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Victoria, Australia; Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, Monash University, Victoria, Australia; Monash Biomedicine Discovery Institute, Department of Microbiology, Monash University, Victoria, Australia

ABSTRACT
Polymyxin B and colistin are exclusively active against Gram-negative pathogens and have been used in the clinic as a last-line therapy. In this study, we investigated the antimicrobial activity of a novel polymyxin, FADDI-019, against Staphylococcus aureus. MIC and time-kill assays were employed to measure the activity of FADDI-019 against S. aureus ATCC 700699. Cell morphology was examined with scanning electron microscopy (SEM), and cell membrane polarity was measured using flow cytometry. Transcriptome changes caused by FADDI-019 treatment were investigated using transcriptome sequencing (RNA-Seq). Pathway analysis was conducted to examine the mechanism of the antibacterial activity of FADDI-019 and to rationally design a synergistic combination. Polymyxin B and colistin were not active against S. aureus strains with MICs of >128 mg/liter; however, FADDI-019 had a MIC of 16 mg/liter. Time-kill assays revealed that no S. aureus regrowth was observed after 24 h at 2× to 4× MIC of FADDI-019. Scanning electron microscopy (SEM) and flow cytometry results indicated that FADDI-019 treatment had no effect on cell morphology but caused membrane depolarization. The vancomycin resistance genes vraRS, as well as the VraRS regulon, were activated by FADDI-019. Virulence determinants controlled by SaeRS and the expression of enterotoxin genes yent2, sei, sem, and seo were significantly downregulated by FADDI-019. Pathway analysis of transcriptomic data was predictive of a synergistic combination comprising FADDI-019 and sulfamethoxazole. Our study is the first to examine the transcriptomics of S. aureus treated with a novel synthetic polymyxin analog. We also show the potential of transcriptomic and pathway analysis as tools to design synergistic antibiotic combinations.

IMPORTANCE
S. aureus is currently one of the most pervasive multidrug-resistant pathogens and commonly causes nosocomial infections. Clinicians are faced with a dwindling armamentarium to treat infections caused by S. aureus, as resistance develops to current antibiotics. This accentuates the urgent need for antimicrobial drug discovery. In the present study, we characterized the global gene expression profile of S. aureus treated with FADDI-019, a novel synthetic polymyxin analogue. In contrast to the concentration-dependent killing and rapid regrowth in Gram-negative bacteria treated with polymyxin B and colistin, FADDI-019 killed S. aureus progressively without regrowth at 24 h. Notably, FADDI-019 activated several vancomycin resistance genes and significantly downregulated the expression of a number of virulence determinants and enterotoxin genes. A synergistic combination with sulfamethoxazole was predicted by pathway analysis and demonstrated experimentally. This is the first study revealing the transcriptomics of S. aureus treated with a novel synthetic polymyxin analog.

KEYWORDS: Staphylococcus aureus, gene expression, polymyxins

Received 5 May 2016 Accepted 15 June 2016 Published 27 July 2016
Citation Zhao J, Cheah S-E, Roberts KD, Nation RL, Thompson PE, Velkov T, Du Z, Johnson MD, Li J. 2016. Transcriptomic analysis of the activity of a novel polymyxin against Staphylococcus aureus. mSphere 1(4):e00119-16. doi:10.1128/mSphere.00119-16.
Editor Paul D. Fey, University of Nebraska Medical Center
Copyright © 2016 Zhao et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.
Address correspondence to Jian Li, Colistin.Polymyxin@gmail.com.
J.Z. and S.-E.C. contributed equally to this article. M.D.J. and J.L. are joint senior authors.
Polymyxin B and colistin (i.e., polymyxin E) are naturally occurring cationic antimicrobial peptides (CAMPs) that have been used in the clinic for over 50 years to treat multidrug-resistant Gram-negative bacterial infections (1–3). Polymyxins primarily interact with lipid A, which is located in the outer leaflet of the Gram-negative outer membrane (OM) (1, 3, 4). It has been reported that CAMPs interact with cell membranes and form membrane-spanning pores, which results in cell death (5, 6). In addition to causing membrane perturbation, CAMPs can also inhibit intracellular metabolic processes, including biosynthesis of the cell wall, nucleic acids, and proteins (7, 8). In these cases, the cell death may be the result of multiple inhibitory effects. It has been shown that the interaction of polymyxins with lipid A is essential to their antimicrobial activity in Gram-negative bacteria (9). Gram-positive bacteria lack an OM or lipid A, rendering them inherently resistant to polymyxin B and colistin. Interestingly, we have shown that novel polymyxins can have very different activities against both Gram-negative and Gram-positive pathogens (3, 9). Specifically, FADDI-019 is a novel polymyxin B derivative with a D-octylglycine at position 6 and an octanoyl fatty acyl chain at the N terminus, as opposed to polymyxin B1 and colistin A, which have either a D-phenylalanine (polymyxin B1) or D-leucine (colistin A) at position 6 and a 6-S-methyloctanoyl fatty acyl chain (9). With increased hydrophobicity within its heptapeptide ring, FADDI-019 exhibits increased activity against Gram-positive bacteria, including *Staphylococcus aureus* (9). More recently, other polymyxin-based synthetic CAMPs have been designed that exhibit antimicrobial activities against Gram-positive bacteria (10, 11). Synthetic CAMPs containing tryptophan, arginine, and N-leucine alterations showed greater activities against Gram-positive bacteria; in addition, these CAMPs showed low cytotoxicities and activities against Gram-negative bacteria, equivalent to those of polymyxin B (10, 12). Gallardo-Godoy et al. generated a library of synthetic polymyxin B derivatives, four of which showed significantly reduced MICs against *S. aureus* (11). Despite the growing number of synthetic polymyxin-like CAMPs, there is currently no understanding of the transcriptional changes caused by their activity against Gram-positive bacteria.

Many Gram-negative species use well-characterized two-component systems to develop resistance to polymyxin (13, 14). In *Salmonella enterica* serotype Typhimurium, polymyxins activate the two-component system PhoPQ, causing the activation of the PhoPQ regulon (13, 15). The *pmr* (polymyxin resistance) locus is part of that regulon, and the activation of the *pmr* locus results in modifications of lipid A, which in turn serve to decrease the interaction with polymyxins. This mechanism has also been shown in *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae* (16–20). Due to the inactivity of polymyxin B and colistin against Gram-positive bacteria, the transcriptional response of Gram-positive bacteria to polymyxins has not been investigated. The transcriptional responses to CAMPs other than polymyxins have been reported in *S. aureus* and *Bacillus subtilis* (21, 22). Pietiäinen et al. characterized the transcriptional response of *S. aureus* to three CAMPs, ovisporin-1, temporin L, and dermaseptin K4-S4 (22). Significant activation of the *vraSR* and *vraDE* genes, which are involved in vancomycin resistance and cell wall homeostasis, suggested that these CAMPs perturb the cell wall. In *B. subtilis*, treatment with CAMPs resulted in the activation of the SigW and SigM cytoplasmic sigma factors and the YxdkJ and LiaRS two-component systems (21). In scenarios where antimicrobials target multiple components of the bacterial cell, transcriptome analysis is capable of identifying affected pathways (21–24). In the present study, we characterized the response of *S. aureus* ATCC 700699 treated with a novel synthetic polymyxin, FADDI-019, using phenotypic assays and transcriptomics. Furthermore, we successfully predicted a synergistic combination by targeting a choke point identified using pathway analysis of our transcriptomic data.

**RESULTS**

**Antimicrobial activity of FADDI-019 against *S. aureus***. In order to characterize the antimicrobial activities of FADDI-019, the MICs of FADDI-019 were measured against...
S. aureus ATCC 700699 (Mu50), S. aureus ATCC 700698 (Mu3), P. aeruginosa ATCC 27853, and Acinetobacter baumannii ATCC 19606 (Table 1). The MICs of FADDI-019 against S. aureus strains ATCC 700699 and ATCC 700698 were both 16 mg/liter, consistent with our previous report (9). We also tested polymyxin B and colistin against both S. aureus strains and observed no activity (MICs of >128 mg/liter) for both compounds, confirming that these S. aureus strains are intrinsically resistant to the naturally occurring, clinically available polymyxins. The results in Table 1 show that FADDI-019 had MICs between 1 and 2 mg/liter against Gram-negative bacteria. This level of activity is comparable to those of polymyxin B and colistin against the same strains (Table 1). In summary, FADDI-019 displayed antibacterial activity against S. aureus strains that were resistant to polymyxin B and colistin.

**FADDI-019 killing kinetics against S. aureus and SEM analysis of cell morphology.** Figure 1 shows the killing kinetics of FADDI-019 at three concentrations (i.e., 2×, 4×, and 8× MIC) against S. aureus strain ATCC 700699. Contrary to the typical rapid-killing kinetics of polymyxin B and colistin against Gram-negative bacteria (25–28), FADDI-019 caused slow killing against S. aureus ATCC 700699 at all concentrations tested; interestingly, regrowth was not observed even at 2× MIC. Scanning electron microscopy (SEM) was used to investigate the effects of FADDI-019 treatment on cell morphology and the bacterial cell surface of S. aureus ATCC 700699 (Fig. 2). Treatment with polymyxin B (99.5 mg/liter) was used for comparison, which showed no significant morphological alterations of S. aureus 700699 cells. Interestingly, compared to the results for the untreated control, no major perturbations of cell morphology were observed in the treated samples at 1× or 4× MIC of FADDI-019, even with significant bacterial killing (Fig. 1). Cell morphology changes were only evident at 8× MIC of FADDI-019 against S. aureus ATCC 700699.

**Flow cytometry analysis of the membrane polarity during FADDI-019 treatment.** S. aureus cells treated with polymyxin B at 99.5 mg/liter for up to 1 h

| Strain                  | MIC (mg/liter) of: | Polymyxin B | Colistin | FADDI-019 |
|-------------------------|--------------------|-------------|----------|-----------|
| A. baumannii ATCC 19606 | 0.5                | 1           | 1        |
| A. baumannii ATCC 17978 | 2                  | 0.5         | 2        |
| P. aeruginosa ATCC 27853| 2                  | 2           | 2        |
| S. aureus ATCC 700698   | >128               | >128        | 16       |
| S. aureus ATCC 700699   | >128               | >128        | 16       |

**FIG 1** Time-kill kinetics of FADDI-019 against S. aureus ATCC 700699. The error bars show the standard deviations of the results from three independent biological repeats.
showed no indication of depolarization relative to the results for the control at time zero (Fig. 3). Interestingly, there was a significant shift in the red fluorescence when cells were treated with FADDI-019 at 4×MIC within 15 min, indicating that treatment with FADDI-019 resulted in membrane depolarization (Fig. 3). In addition, there was no evidence of recovery from membrane depolarization after 60 min of treatment (Fig. 3), which suggests that the depolarization effect of FADDI-019 was permanent.

Transcriptome profiling and enrichment of *S. aureus* ATCC 700699 in response to FADDI-019 treatment. The transcriptome variances of all samples, treatments, and time points were analyzed by principal component analysis (PCA) (Fig. 4A). PCA analysis separated FADDI-019-treated from untreated samples across principal component 1, indicating that treatment with FADDI-019 was responsible for 51.5% of total variance across all samples (Fig. 4A). Samples taken at different time points were separated across principal component 2, which was responsible for 14.9% of the total variance between all samples (Fig. 4A). Individual repeats clustered together, showing the excellent reproducibility of our transcriptomic study (Fig. 4A). Voom and limma linear modeling and Degust (Fig. 4B) were used to enrich for genes that were differentially regulated at 15 min and sustained this response after 60 min of FADDI-019 treatment (false discovery rate [FDR] = 0.05, >2-fold change) (Fig. 4B). After this enrichment, a total of 208 differentially regulated genes were identified, of which 140 were significantly upregulated and 68 significantly downregulated (FDR = 0.05, >2-fold change) (Fig. 4B). The use of a time course allowed enrichment analysis, which limited the list of differentially regulated genes to those involved in a specific response to FADDI-019 (see Table S1 in the supplemental material).

Induction of the vancomycin resistance regulon in *S. aureus* ATCC 700699 by FADDI-019. Genes involved in similar cellular processes are often regulated in kind (29). We analyzed the enriched gene lists to identify the pathways affected by FADDI-019 treatment. Analysis of genes upregulated by FADDI-019 in *S. aureus* ATCC 700699 showed a significant increase in the expression of the vancomycin resistance genes. Constituents of the two-component signaling system comprising vraRS were upregulated by FADDI-019 5.04- and 4.90-fold, respectively (Fig. 5A). In order to confirm the activation of the remaining VraSR regulon, we analyzed the expression of the genes known to be under the control of the VraSR two-component system. These included hypothetical proteins SAV1424, SAV1423, SAV1422, SAV1421, and SAV2556, which were upregulated 3.50-, 3.90-, 3.73-, 3.50-, and 4.28-fold, respectively, hypothetical membrane protein SAV1650, which was upregulated 3.54-fold, and a peptidyl-prolyl isomerase, *prsA*, which was upregulated 3.41-fold (Fig. 5A). These results show that, in addition to the activation of VraSR, genes in the VraSR regulon were also upregulated during FADDI-019 treatment.

Activation of genes involved in the metabolism of energy, folate, and amino acids by FADDI-019. FADDI-019-treated *S. aureus* ATCC 700699 showed an increase in the expression of genes involved in basic metabolic processes, including peptide import, metabolism of folate and sugar, and amino acid biosynthesis. The phosphotransferase system genes *pstSACB*, which code for proteins involved in sugar import, were upregulated 2.08-, 2.88-, 2.88-, and 3.57-fold, respectively (Fig. 5B). In addition, the galactose metabolism genes *lacCDEF* and the *malA* gene were upregu-
lated 2.37-, 2.18-, 2.27-, 2.09-, and 2.79-fold, correspondingly (Fig. 5C). Genes involved in folate biosynthesis, fhs and folD, were also upregulated 2.14- and 2.47-fold, respectively (Fig. 5D). The most-upregulated genes during FADDI-019 treatment were those

![FIG 3 Scatter plots of flow cytometry analysis of S. aureus ATCC 70099 during treatment with polymyxin B and FADDI-019. DiOC₃(3) was used to stain the cells, and the fluorescence of each incident (indicated as a dot) in the red and green channels was measured and plotted onto a 2-D scatterplot. Shifting of the scatter in the red fluorescence channel is indicative of membrane depolarization.](image-url)
involved in amino acid biosynthesis, and the levels of gene expression ranged from 13.12- to 23.07-fold higher than in the control (Fig. 5E). In addition to amino acid biosynthesis, peptide import genes were also significantly upregulated. The oppABCDF locus encodes a peptide import system, and genes in this operon were 3.11- to 3.31-fold upregulated in FADDI-019-treated cells relative to their expression in the control (Fig. 5F).

Repression of key virulence factors in *S. aureus* ATCC 700699 treated with FADDI-019. Of the 208 genes that were differentially expressed during the FADDI-019 treatment, 68 were downregulated. The main pathways repressed by FADDI-019 treatment are involved in virulence. The *saeSR* genes, which code for the SaeSR two-component system, were downregulated 3.89-fold and 4.03-fold, respectively (Fig. 5G). The *saeQ* and *saeP* genes, which encode regulatory proteins that increase the phosphatase activity of SaeS, were repressed 4.19-fold and 8.88-fold, respectively (Fig. 5G). In

![FIG 4](image-url)

(A) PCA results for all data, with individual samples plotted on principal component 1 (horizontal axis) and principal component 2 (vertical axis). (B) Graphical representation of the gene enrichment process using Degust. Individual differentially regulated genes are represented as horizontal red lines. The vertical axes represent the log₂ fold change of all genes at each time point in control and treated conditions (indicated above each axis) relative to 15 min of treatment with FADDI-019. Gray boxes indicate the gating cutoffs used to enrich the gene list for genes that are greater than 1 log₂ fold differentially expressed throughout the control samples but >1 log₂ fold differentially expressed after 60 min.
addition to the repression of virulence pathways, FADDI-019 treatment also had significant effects on known pathogenicity determinants in *S. aureus*. Four enterotoxin genes, *yent2*, *sei*, *sem*, and *seo*, which encode proteins that are the causative agents of toxic shock, were downregulated 2.09-fold, 2.67-fold, 2.19-fold, and 2.94-fold, respectively (Fig. 5H) (36). The import of polyamines has been linked to a number of processes, including signaling and infection (37, 38). The putrescine/spermidine importer *potABCD* was repressed by FADDI-019 treatment. The *potABCD* genes were significantly downregulated, by 2.85-fold, 2.96-fold, 2.80-fold, and 2.56-fold, respectively, relative to their expression in the untreated control (Fig. 5I).

Identification of a folate metabolism chokepoint. We used a systems biology approach to identify metabolic chokepoints based on the transcriptomic response to FADDI-019 treatment. Pathway analysis using the transcriptome data identified that each amino acid biosynthesis system was overexpressed (Fig. 6A). Importantly, the folate metabolic pathways were also overexpressed (Fig. 6B). Tetrahydrofolate is a key metabolite for amino acid biosynthesis (39); our pathway analysis indicated that amino acid metabolism is dependent on folate metabolism and that the tetrahydrofolate synthesis pathway is a suitable chokepoint to inhibit both processes. Sulfamethoxazole is an antimicrobial that inhibits the tetrahydrofolate metabolic pathway (Fig. 5C) (40–42). We measured the MICs of sulfamethoxazole in combination with FADDI-019 against *S. aureus*. Table 2 shows the MICs of FADDI-019 and sulfamethoxazole against *S. aureus* ATCC 700699. Sulfamethoxazole alone had a MIC greater than 128 mg/liter, indicating that it had no antimicrobial activity against *S. aureus* ATCC 700699. In combination, 16 mg/liter sulfamethoxazole decreased the FADDI-019 MIC substantially, to 4 mg/liter, against *S. aureus* ATCC 700699.
DISCUSSION
Polymyxins have been used as a last-line therapeutic option against Gram-negative pathogens (43–45). However, as their mode of action requires the initial binding to lipid A in the outer membrane, polymyxin B and colistin are inactive against Gram-positive bacteria (3, 46). Furthermore, resistance to polymyxins is frequently acquired in vitro within 24 h of treatment by loss or modification of lipid A (16, 17, 47–50). Previously, we have shown that a novel polymyxin, FADDI-019, has antimicrobial activity against Gram-positive bacteria (3, 9). FADDI-019 was designed with a major alteration from the clinically available polymyxin B and colistin. FADDI-019 has a D-octylglycine at position 6, whereas polymyxin B and colistin have D-phenylalanine and D-leucine residues, respectively, at this location (3, 9). Hence, the hydrophobicity of FADDI-019 at position 6 is greater than that of polymyxin B or colistin. The predicted effect of this difference
is to increase the hydrophobic reach of the molecule at position 6. Previously, we have shown that both polar and hydrophobic residues are important for the action of polymyxins against Gram-negative bacteria (3). The activity of FADDI-019 against Gram-positive bacteria indicated that increased hydrophobicity is crucial for the antimicrobial activity of the core polymyxin structure against Gram-positive bacteria. It is evident that FADDI-019 has a wider antimicrobial spectrum than do polymyxin B and colistin (3). Time-kill assays revealed that no regrowth of S. aureus ATCC 700699 was observed after treatment, which is in contrast to the killing of polymyxins against Gram-negative bacteria, where regrowth of polymyxin-resistant variants is frequently observed even within 12 h (26, 27). In addition to the aforementioned properties of FADDI-019, we have previously reported that, in rodent models, FADDI-019 has a tolerability equivalent to that of colistin and polymyxin B after intravenous (0.75 mg/kg of body weight) and subcutaneous (40 mg/kg) administration (3). FADDI-019 also lacks hemolytic activity, similar to polymyxin B and colistin. However, it should be noted that the MIC of FADDI-019 against S. aureus is between 4- and 8-fold higher than that of polymyxin B or colistin against Gram-negative bacteria (26, 51) and that this may limit the use of FADDI-019 as a therapeutic.

The analysis of cell morphology by SEM after treatment revealed that cell blebbing was not induced by FADDI-019 at 1× or 4× MIC, although morphology changes were evident at 8× MIC. Membrane blebbing is a common feature of polymyxin-treated Gram-negative bacteria (52, 53), so the absence of membrane blebbing is suggestive of an alternative killing mechanism. To further investigate the effect of FADDI-019 treatment, we examined the membrane polarity of S. aureus ATCC 700699 cells by flow cytometry. FADDI-019 depolarized the cell membrane within 15 min, indicating that, despite the lack of membrane blebbing, FADDI-019 interacted with the S. aureus cell membrane. Our results show that the antimicrobial mechanism of FADDI-019 against S. aureus is different from previously characterized polymyxin mechanisms against Gram-negative pathogens (5). Furthermore, the lack of lipid A, absence of membrane blebbing, and extensive membrane depolarization are suggestive of an alternative target of FADDI-019.

Analysis of transcriptomic changes caused by FADDI-019 treatment showed that a number of major pathways associated with vancomycin resistance were upregulated. The vraSR two-component system, which was originally characterized for its role in vancomycin resistance, is responsible for coordinating the response to antimicrobials that target the cell wall (54). Transcriptomic studies that assayed the effects of ovisporin-1, temporin L, and dermaseptin K4-S4 against S. aureus ATCC 25904 have been reported previously (21, 22). Unlike the cyclic FADDI-019, ovisporin-1, temporin L, and dermaseptin K4-S4 are all linear alpha-helical CAMPs. The upregulation of vancomycin resistance genes was also observed with alpha-helical CAMP treatments against S. aureus (22), which suggests that cell wall homeostasis has a key role in the response to all CAMPs that show antimicrobial activity against Gram-positive bacteria. Amino acid biosynthesis genes and peptide import systems were also upregulated in response to alpha-helical CAMP treatments (21). The predominant amino acid biosynthesis pathway upregulated by FADDI-019 treatment was the leucine, isoleucine, and valine superpathway. Components of other amino acid pathways (e.g., glutamine, methionine, serine, and tryptophan) were also upregulated (Fig. 5E). In addition to amino acid biosynthesis, the OppABCDF machinery, which imports tripeptides from the environment (55), was upregulated in response to FADDI-019. The OppABCDF machinery is

| Strain             | FADDI-019 | SMX | FADDI-019 + SMX |
|--------------------|-----------|-----|-----------------|
| S. aureus ATCC 700698 | 16        | >128 | 4/16           |
| S. aureus ATCC 700699 | 16        | >128 | 4/16           |

SMX, sulfamethoxazole.
important for quorum sensing and nutrition (55). Opp transporters have been shown to supply bacteria with exogenous peptides that serve as amino acid resources. The activation of the Opp transport system is therefore synonymous with amino acid production. The role of amino acid biosynthesis and peptide import in the response to CAMPs is currently not well understood. However, in response to FADDI-019 treatment, we observed the strongest activation of gene expression, compared to all other differentially expressed genes, in amino acid biosynthesis genes. Taken together, we propose that the increase in vancomycin resistance gene expression and cell wall metabolism drives the need for increased amino acid production and import of tripeptides.

FADDI-019 also significantly repressed genes involved in the virulence of S. aureus. The repression of saeRS and the SaeRS-regulated virulence factor sbi by other, alpha-helical CAMPs, i.e., ovisporin-1, temporin L, and dermaseptin K4-S4, has been shown previously (21, 22). However, other known SaeRS-regulated genes (e.g., coa, fnbB, and saeQP), which were similarly repressed in our study, were not repressed by those alpha-helical CAMPs in S. aureus ATCC 25904 (21, 22). These differences are most likely due to differences in the mode of action between FADDI-019 and alpha-helical CAMPs and/or biological variability between S. aureus strains. In addition to the repression of SaeRS-regulated genes, four enterotoxin genes were also dramatically repressed by FADDI-019 treatment. Interestingly, this response is also specific to FADDI-019 and has not been reported for other CAMPs. Our data suggest that, in addition to killing S. aureus ATCC 700699, it is very likely that FADDI-019 reduced the virulence of the infecting bacterial cells as a secondary effect. When making comparisons between strains, it is important to note that the strain used in this study is a vancomycin-intermediate S. aureus (VISA), whereas S. aureus ATCC 25904 is vancomycin susceptible. VISA strains have been shown to have mutations in regulatory genes, including vraSR, which alter the transcription profile and result in intermediate vancomycin resistance (56, 57). The SaeSR two-component system is not known to be differentially regulated in different VISA strains (56).

Our transcriptomic analysis of S. aureus ATCC 700699 during FADDI-019 exposure demonstrated that a large number of essential metabolism processes, including folate biosynthesis, were upregulated (Fig. 5D). Some of these changes have been previously observed with alpha-helical CAMPs (22); however, the upregulation of folate biosynthesis genes was not reported with alpha-helical-CAMP treatment in that study. We hypothesized that these perturbations are important for S. aureus ATCC 700699 during FADDI-019 treatment. Pathway analysis (Fig. 5B) revealed that tetrahydrofolate production would be a suitable chokepoint target for a synergistic combination with FADDI-019. Indeed, as a test of this hypothesis, we subsequently demonstrated that FADDI-019 and sulfamethoxazole in combination were synergistic, with substantial reductions in the MICs of both compounds (Table 2). Our systems approach, exemplified here, holds much promise for identifying novel approaches to identify rational antimicrobial combinations in the future.

In conclusion, in an era of burgeoning multidrug resistance and diminishing therapeutic options, it is crucial that every effort must be made to discover antibacterial compounds against problematic pathogens. This is the first study reporting the transcriptomics of an unexpected antibacterial activity of a novel synthetic polymyxin analog against Gram-positive S. aureus. Importantly, our systems approach using transcriptomics has shown that pathway analysis of responses to drugs has a significant potential in predicting synergistic antibiotic combinations.

MATERIALS AND METHODS

Bacterial strains, culturing conditions, and susceptibility tests. S. aureus ATCC 700699 (Mu50, VISA), S. aureus ATCC 700698 (Mu3, heterogeneous VISA), A. baumannii ATCC 17978, A. baumannii ATCC 19606, and P. aeruginosa ATCC 27853 were grown at 37°C on Mueller-Hinton (MH) agar plates or in cation-adjusted Mueller-Hinton broth (CAMHB) (Ca²⁺ at 22.5 mg/liter and Mg²⁺ at 11.25 mg/liter). No antibiotic selection was used unless stated otherwise. The MICs of FADDI-019, colistin (Sigma-Aldrich, Castle Hill, Australia), polymyxin B (Sigma-Aldrich), and sulfamethoxazole (Sigma-Aldrich) were determined by broth microdilution in CAMHB according to the Clinical and Laboratory Standards Institute protocol (n = 3).
The synergistic effect by FADDI-019 with sulfamethoxazole was examined using a checkerboard method (see Table S2 in the supplemental material) (58).

**Time-kill studies.** The time-kill kinetics of FADDI-019 against S. aureus ATCC 700699 was examined in three replicates at 2×, 4×, and 8× MIC with a log-phase broth culture (optical density at 600 nm [OD₆₀₀] of 0.4, 10⁴ CFU/ml) (59). Viable bacteria were enumerated on nutrient agar plates from samples collected at 0, 0.5, 1, 2, 4, 6, and 10 min after treatment. Colonies were counted following overnight incubation at 37°C, and the lower limit of detection was 20 CFU/ml.

**Preparation of cells for SEM.** Cultures of S. aureus ATCC 700699 were incubated at 37°C with aeration until the OD₆₀₀ reached 0.4. FADDI-019 was added to a final concentration of 1×, 4×, or 8× MIC, and cultures were incubated for an hour at 37°C. An untreated sample and a sample treated with 99.5 mg/liter polymyxin B were used as controls. After the incubation, cells were pelleted by centrifugation at 5,000 × g for 10 min. Cells were fixed by the addition of 2.5% glutaraldehyde for 1 min and then washed three times using phosphate-buffered saline (PBS) (pH 7.4). The bacterial cultures were air dried onto polyethyleneimine-coated coverslips and immersed for an hour in 2.5% glutaraldehyde in PBS. The slides were then washed with PBS three times, and dehydration was done using 10% increments of ethanol in water from 0 to 100% for 10 min at each step. The coverslips were dried using a Balzers critical point dryer (Balzers, Liechtenstein, Germany) prior to mounting on 20-mm aluminum stubs with double-sided carbon tabs. The cells were coated with gold and imaged with a Philips XL30 field-emission scanning electron microscope (SEM; Philips, Eindhoven, Holland) at the University of Melbourne (Victoria, Australia).

**Flow cytometry.** Cultures (10 ml) of S. aureus ATCC 700699 were incubated at 37°C until the OD₆₀₀ reached 0.4 and then treated with either polymyxin B (99.5 mg/liter) or FADDI-019 at 4× MIC. Samples (300 μl) were taken from each culture at 0, 15, and 60 min after treatment and incubated for 10 min with DiOC₂(3) (3,3’-diethyloxacarbocyanine iodide; Thermo Fisher, Victoria, Australia). Samples (300 μl) were analyzed by flow cytometry (NovoCyte; ACEA Biosciences, Inc.) with the laser set to emit at 488 nm, and fluorescence was measured in the red and green channels. All incidents were plotted on a two-dimensional (2-D) scatterplot using their respective fluorescence intensities in each channel.

**RNA-Seq transcriptomics.** S. aureus ATCC 700699 was grown to an OD₆₀₀ of 0.4 from an initial absorbance of 0.005, and FADDI-019 was added to a final concentration of 4× MIC. Samples were collected at 0, 15, and 60 min posttreatment and preserved with RNAprotect (Qiagen, United States) following the manufacturer’s instructions. Cells were pelleted by centrifugation at 5,000 × g for 10 min at 4°C. RNA was isolated using an RNeasy minikit (Qiagen) in accordance with the manufacturer’s instructions. RNA sequence reads were independently aligned with the genome sequence of S. aureus ATCC 700699 (NCBI accession number NC_002758.2) using Subread (Victorian Bioinformatics Consortium, http://www>vicbioinformatics.com/software.shtml) (60, 61). The RNA sequence data from biological replicates were analyzed using the voom and limma linear modeling methods via the Descude interactive Web-based RNA-seq visualization software (http://www>vicbioinformatics.com/deugust/). Differentially expressed genes were defined as those with a change in expression of >2-fold and a corresponding false discovery rate (FDR) of <0.05. The gene ontology terms and pathways were annotated via BioCyc and KEGG. Principal component analysis was performed using the R statistical computing package (62). For pathway analysis, genes shown to be differentially expressed at 15 min after treatment relative to their expression in the control were mapped onto the metabolic network of S. aureus ATCC 700699 (biocyc.org). Pathways containing multiple differentially expressed genes were selected for chokepoint analysis (biocyc.org).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://dx.doi.org/10.1128/mSphere.00119-16.

Table S1, PDF file, 0.4 MB.
Table S2, PDF file, 0.04 MB.

**ACKNOWLEDGMENTS**

We are grateful to Simon Crawford (University of Melbourne, Parkville) for his technical support regarding SEM experiments. We are also grateful to Trevor Wilson (Monash Health Translational Precinct, Clayton, Australia) for library preparation and RNA sequencing.

J.L., T.V., R.L.N., P.E.T., and K.D.R. are supported by a research grant from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (R01 AI098771), and J.L. and T.V. are also supported by R01 AI111965. J.L. is an Australian NHMRC Senior Research Fellow. T.V. is an Australian NHMRC Industry Career Development Research Fellow.
REFERENCES
1. Hancock RE. 1997. Peptide antibiotics. Lancet 349:418–422. http://dx.doi.org/10.1016/S0140-6736(97)08051-7.
2. Hancock RE, Lehner R. 1998. Cationic peptides: a new source of anti-biotics. Trends Biotechnol 16:82–88. http://dx.doi.org/10.1016/S0167-7999(97)01156-6.
3. Velkov T, Rosen KD, Nation RL, Thompson PE, Li J. 2013. Pharmacology of polymyxins: new insights into an “old” class of antibiotics. Future Microbiol 8:711–724. http://dx.doi.org/10.2127/fmb.13.39.
4. Azad MA, Huang JX, Cooper MA, Roberts KD, Thompson PE, Nation RL, Li J, Velkov T. 2012. Structure-activity relationships for the binding of polymyxins with human alpha-1-acid glycoprotein. Biochem Pharmacol 84:278–291. http://dx.doi.org/10.1016/j.bcp.2012.05.004.
5. Powers JP, Hancock RE. 2003. The relationship between peptide structure and antibacterial activity. Peptides 24:1681–1691. http://dx.doi.org/10.1016/j.peptides.2003.08.023.
6. Meredith JJ, Dufoeur A, Bruch MD. 2009. Comparison of the structure and dynamics of the antibiotic peptide polymyxin B and the inactive nonapeptide in aqueous trifluoroethanol by NMR spectroscopy. J Phys Chem B 113:544–551. http://dx.doi.org/10.1021/jp080739x.
7. Brogden KA. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3:238–250. http://dx.doi.org/10.1038/nrmicro1098.
8. Hancock RE, Diamond G. 2000. The role of cationic antimicrobial peptides in innate host defences. Trends Microbiol 8:402–410. http://dx.doi.org/10.1016/S0966-842X(00)01823-0.
9. Velkov T, Roberts KD, Nation RL, Wang J, Thompson PE, Li J. 2014. Teaching “old” polymyxins new tricks: new-generation lipopeptides targeting gram-negative “superbugs.” ACS Chem Biol 5:544–551. http://dx.doi.org/10.1021/cb500080r.
10. Rabanal F, Grau-Campistany A, Vila-Farrés X, Gonzalez-Linares J, Borràs M, Vila J, Manresa A, Cajal Y. 2015. A biospiered peptide scaffold with high antibiotic activity and low in vivo toxicity. Sci Rep 5:10558. http://dx.doi.org/10.1038/srep10558.
11. Gallardo-Godoy A, Muldoon C, Becker B, Elliott AG, Lash LH, Huang JX, Butler MS, Pelington R, Kavanagh AM, Ramu S, Phetsang W, Bialevich MA, Cooper MA. 2016. Activity and predicted nephrotoxicity of synthetic antibiotics based on polymyxin B. J Med Chem 59:1068–1077. http://dx.doi.org/10.1021/acs.jmedchem.5b01592.
12. Grau-Campistany A, Manresa A, Pujol M, Rabanal F, Cajal Y. 2016. Tryptophan-containing lipopeptide antibiotics derived from polymyxin B with activity against Gram positive and Gram negative bacteria. Biochim Biophys Acta 1858:333–343. http://dx.doi.org/10.1016/j.bbamem.2015.01.013.
13. Macfarlane EL, Kwasnicka A, Hancock RE. 2000. Role of Pseudomonas aeruginosa PhoP-PhoQ in resistance to antimicrobial cationic peptides and aminoglycosides. Microbiology 146:2543–2554. http://dx.doi.org/10.1099/00221287-146-10-2543.
14. Dalebroux ZD, Matamouros S, Whittington D, Bishop RE, Miller SI. 2014. PhoQ regulates acidic glycerophospholipid content of the Salmonella Typhimurium outer membrane. Proc Natl Acad Sci U S A 111:1963–1968. http://dx.doi.org/10.1073/pnas.1316901111.
15. Llobet E, Campos MA, Giménez P, Moranta D, Bengoechea JA. 2011. Analysis of the networks controlling the antimicrobial- peptide-dependent induction of Klebsiella pneumoniae virulence factors. Infect Immun 79:3718–3722. http://dx.doi.org/10.1128/IAI.00226-11.
16. Lee H, Hsu FF, Turk J, Groisman EA. 2004. The PmrA-regulated pmrC gene mediates phosphoethanolamine modification of lipid A and poly-myxin resistance in Salmonella enterica. J Bacteriol 186:4124–4133. http://dx.doi.org/10.1128/JB.186.13.4124-4133.2004.
17. Arroyo LA, Herrera CM, Fernandez L, Hanksin JV, Trent MS, Hancock RE. 2011. The pmrCAB operon mediates polymyxin resistance in Acinetobacter baumannii ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. Antimicrob Agents Chemother 55:3374–3375. http://dx.doi.org/10.1128/AAC.00256-11.
18. Cheng HY, Chen YF, Peng HL. 2010. Molecular characterization of the PhoPQ-PmID-PmrAB mediated pathway regulating polymyxin B resistance in Klebsiella pneumoniae CG43. J Biomed Sci 17:60. http://dx.doi.org/10.1186/1423-0127-17-60.
19. Kim SY, Choi HJ, Ko KS. 2014. Differential expression of two-component systems, pmrAB and phoPQ, with different growth phases of Klebsiella pneumoniae in the presence or absence of colistin. Curr Microbiol 69:37–41. http://dx.doi.org/10.1007/s00284-014-0549-0.
20. Helander IM, Kato Y, Kilpeläinen I, Kostiainen R, Lindner B, Namkina K, Sugiyama T, Yokochi T. 1996. Characterization of lipopolysaccharides of polymyxin-resistant and polymyxin-sensitive Klebsiella pneumoniae O3. Eur J Biochem 237:272–278. http://dx.doi.org/10.1111/j.1432-1033.1996.0272nx.
21. Pietiäinen M, Gardemeister M, Mecklin M, Leskelä S, Sarvas M, Kontinen VP. 2005. Cationic antimicrobial peptides elicit a complex stress response in Bacillus subtilis that involves ECF-type sigma factors and two-component signal transduction systems. Microbiology 151:1577–1592. http://dx.doi.org/10.1099/mic.0.27761-0.
22. Pietiäinen M, François P, Hyryläinen HL, Tangomo M, Sass V, Sahl HG, Schrenzel J, Kontinen VP. 2009. Transcriptome analysis of the responses of Staphylococcus aureus to antimicrobial peptides and characterization of the roles of vraDE and vraSR in antimicrobial resistance. BMC Genomics 10:429. http://dx.doi.org/10.1186/1471-2164-10-429.
23. Henry R, Vithanage N, Harrison P, Seemann T, Coustts S, Moffatt JH, Nation RL, Li J, Harper M, Adler B, Boyde JD. 2012. Colistin-resistant, lipopolysaccharide-deficient Acinetobacter baumannii responds to lipopolysaccharide loss through increased expression of genes involved in the synthesis and transport of lipoproteins, phospholipids, and poly-beta-1,6-N-acetylgalactosamine. Antimicrob Agents Chemother 56:59–69. http://dx.doi.org/10.1128/AAC.00519-11.
24. Henry R, Crane B, Powell D, Deveson Lucas D, Li Z, Aranda J, Harrison P, Nation RL, Adler B, Harper M, Boyde JD, Li J. 2015. The transcriptomic response of Acinetobacter baumannii to colistin and dorfopenalone and in combination in an in vitro pharmacokinetics/pharmacodynamics model. J Antimicrob Chemother 70:1303–1313. http://dx.doi.org/10.1093/jac/dku536.
25. Li J, Turnidge J, Milne R, Nation RL, Coulthard K. 2001. In vitro pharmacodynamic properties of colistin and colistin methanesulfonate against Pseudomonas aeruginosa isolates from patients with cystic fibrosis. Antimicrob Agents Chemother 45:781–785. http://dx.doi.org/10.1128/AAC.45.3.781-785.2001.
26. Poudyal A, Howden BP, Bell JM, Gao W, Owen RJ, Turnidge JD, Nation RL, Li J. 2008. In vitro pharmacodynamics of colistin against multidrug-resistant Klebsiella pneumoniae. J Antimicrob Chemother 62:1311–1318. http://dx.doi.org/10.1093/jac/dkn425.
27. Tan CH, Li J, Nation RL. 2007. Activity of colistin against heteroresistant Acinetobacter baumannii and emergence of resistance in an in vitro pharmacokinetic/pharmacodynamic model. Antimicrob Agents Chemother 51:3434–3435. http://dx.doi.org/10.1128/AAC.01571-06.
28. Li J, Rayner CR, Nation RL, Owen RJ, Spelman D, Tan KE, Liolios L. 2006. Heteroresistance to colistin in multidrug-resistant Klebsiella pneumoniae. Antimicrob Agents Chemother 50:2946–2950. http://dx.doi.org/10.1128/AAC.01103-06.
29. Jothi R, Balaji S, Wuster A, Grochow JA, Gsponer J, Przytycka TM, Aravind L, Babu MM. 2009. Genomic analysis reveals a tight link...
from the two-component regulatory system SaeRS on global gene expression in two different Staphylococcus aureus strains. J Bacteriol 188: 7742–7758. http://dx.doi.org/10.1128/JB.00555-06.

40. Jeong DW, Cho H, Jones MB, Shatzkes K, Chart H, Bou G, Livermore DM, Woodford N. 2011. Phosphoethanolamine modification of lipid A in colistin-resistant variants of Acinetobacter baumannii mediated by the pmrAB two-component regulatory system. Antimicrob Agents Chemother 55: 3370–3379. http://dx.doi.org/10.1128/AAC.00078-11.

41. Moffatt JH, Harper M, Mansell A, Crane B, Boyce JD. 2013. Lipopolysaccharide-deficient Acinetobacter baumannii shows altered signaling through toll-like receptors and increased susceptibility to the host antimicrobial peptide LL-37. Infect Immun 81:684–689. http://dx.doi.org/10.1128/IAI.00362-12.

42. Owen RJ, Li J, Nation RL, Spelman D. 2007. In vitro pharmacodynamics of colistin against Acinetobacter baumannii clinical isolates. J Antimicrob Chemother 59:473–477. http://dx.doi.org/10.1093/jac/dkl513.

43. Rahman M, Berditsch M, Hawecker J, Arakadani MF, Gerthsen D, Ulrich AS. 2010. Damage of the bacterial cell envelope by antimicrobial peptides gramicidin S and PGLA as revealed by transmission and scanning electron microscopy. Antimicrob Agents Chemother 54:3132–3142. http://dx.doi.org/10.1128/AAC.00124-10.

44. Belcheva A, Golemi-Kotra D. 2008. A close-up view of the VraSR two-component system. A mediator of Staphylococcus aureus response to cell wall damage. J Biol Chem 283:12354–12364. http://dx.doi.org/10.1074/jbc.M710010200.

45. Gardan R, Besset C, Guillot A, Gilton C, Monnet V. 2009. The oligopeptide transport system is essential for the development of natural competence in streptococcus thermophiles strain LMD-9. J Bacteriol 191: 4647–4655. http://dx.doi.org/10.1128/JB.00257-09.

46. Howden BP, Peleg AY, Stinear TP. 2014. The evolution of vancomycin intermediate Staphylococcus aureus (VISA) and heterogenous-VISA. Infect Genet Evol 21:575–582. http://dx.doi.org/10.1016/j.meegid.2013.03.047.

47. Sieradzki K, Leski T, Dick J, Borio L, Tomasz A. 2003. Evolution of a vancomycin-intermediate Staphylococcus aureus strain in vivo: multiple changes in the antibiotic resistance phenotypes of a single lineage of methicillin-resistant S. aureus under the impact of antibiotics administered for chemotherapy. J Clin Microbiol 41:1687–1693. http://dx.doi.org/10.1128/JCM.41.6.1687-1693.2003.

48. Zhou Z, Ribeiro AA, Lin S, Cotter RJ, Miller SJ, Raetz CR. 2001. Lipid A modifications in polymyxin-resistant Salmonella typhimurium: PMRdependent 4-amino-4-deoxy-L-arabinose, and phosphoethanolamine incorporation. J Biol Chem 276:8311–8312. http://dx.doi.org/10.1074/jbc.M001062000.

49. Becero A, Llober J, Aranda J, Begochea JA, Doumith M, Hornsey M, Dhanji H, Chart H, Bou G, Livermore DM, Woodford N. 2011. Phosphoethanolamine modification of lipid A in colistin-resistant variants of Acinetobacter baumannii mediated by the pmrA2 component regulatory system. Antimicrob Agents Chemother 55: 3370–3379. http://dx.doi.org/10.1128/AAC.00078-11.

50. Jombart T, Boucher D, Melton AG, Strömbäck B, Westerhuis JA, Chennells MM, Frouard L, Rutter M, Galloway D, Haddrill SR, Deiner S, Trinchet C, Pybus OG. 2011. Unlock linear model analysis tools for RNA-seq read counts. Genome Biol 12:R24. http://dx.doi.org/10.1186/gb-2011-12-3-r24.

51. Ogüt MF, Callen JS, Maltby P, Cuchillo D, Britton SK, Nunney L, Gordon JL, Gogarten JP, Beveridge TJ. 2012. Evolution of consensus and noise on the transcriptome in Escherichia coli. Genome Biol 13:R71. http://dx.doi.org/10.1186/gb-2012-13-9-r71.
Author/s:
Zhao, J; Cheah, S-E; Roberts, KD; Nation, RL; Thompson, PE; Velkov, T; Du, Z; Johnson, MD; Li, J

Title:
Transcriptomic Analysis of the Activity of a Novel Polymyxin against Staphylococcus aureus

Date:
2016-07-01

Citation:
Zhao, J; Cheah, S-E; Roberts, KD; Nation, RL; Thompson, PE; Velkov, T; Du, Z; Johnson, MD; Li, J, Transcriptomic Analysis of the Activity of a Novel Polymyxin against Staphylococcus aureus, MSPHERE, 2016, 1 (4)

Persistent Link:
http://hdl.handle.net/11343/233520

File Description:
Published version