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Characterization of *Staphylococcus aureus* Responses to Spermine Stress

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Abstract Spermine (Spm), a potent bactericidal polyamine, exerts a strong synergistic effect with β-lactams against methicillin-resistant *Staphylococcus aureus* (MRSA). To explore the Spm-based antibacterial targets in *S. aureus*, time course-dependent transcriptome analysis was conducted on Mu50 (MRSA) in the absence and presence of Spm. Genes in the *sigB* regulon and most ATP-producing pathways were found down-regulated when exposure to high dose Spm. In contrast, a number of genes for iron acquisition and regulation showed significant induction, indicating a specific connection between Spm and iron-depletion. The *tetM* gene for tetracycline (Tc) resistance exhibited most significant fold change among the listed genes. It was specifically upregulated by Tc and Spm but not by other ribosome-targeted drugs or other polyamines; however, such induction of *tetM* cannot confer resistance to Spm. A set of genes for osmotic balance, including *kdpABCDE* for potassium ion uptake and regulation, was also induced by Spm stress. Addition of KCl or NaCl, but not high concentration sucrose, was found to increase Spm MIC over 30-fold. In summary, transcriptome analysis demonstrated a specific pattern of response upon Spm exposure, suggesting Spm may alter the intracellular iron status and suppress the SigB regulon to exert its toxicity.

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Introduction

Polyamines are ubiquitous polycationic molecules essential for cell growth from bacteria to mammalian cells. A variety of biogenic polyamines has been discovered from different organisms: putrescine (Put) and spermidine (Spd) predominate in prokaryotic cells, whereas Spd and Spm are mainly found in eukaryotic cells [7]. The distinct distribution of Spm suggests that this tetra-amine may be associated with some new activities or specific regulations during evolution. Of interest, increased Spm levels have been demonstrated at inflammatory sites of infection and in cancer cells, suggesting the significance of Spm in cell proliferation and immune functions in human body [2, 48]. In contrast, many studies showed that exogenous Spm may become toxic to bacteria with accumulation, the inhibition of which is especially strong in *S. aureus* [15, 22]. Moreover, we also found that at low concentrations, Spm by itself has negligible effects while is able to sensitize bacteria to β-lactam antibiotics; such synergy is most potent in *S. aureus* [23]. However, the molecular target of Spm toxicity in *S. aureus* remains largely unknown.

In some bacteria, Spm toxicity can be alleviated by catabolic pathways. There are two common mechanisms that have been reported to initiate Spm degradation: glutamylation by γ-glutamylpolyamine synthetases (GPS) in *P. aeruginosa* [46] and acetylation by Spd/Spm acetyltransferase (SSAT) in *E. coli* and *B. subtilis*. In most *S. aureus* strains, there are no such Spm catabolic enzymes; the only exception is the USA300 lineage, which expresses

speG encoded SSAT to convert toxic Spm to non-toxic N-1 acetyl-Spm through acetylation, thus affording complete resistance to Spm. But for non-USA300 clones, *S. aureus* can be rapidly killed by physiological Spm concentrations [22].

To elucidate the molecular mechanism of Spm toxicity in *S. aureus*, we applied in this study the transcriptomic approach to investigate the cellular response to Spm stress. The results suggest that Spm may interfere with iron homeostasis and *sigB* regulon, and consequently led to the growth inhibition. Furthermore, in depth characterization of *tetM,* the gene that displayed the most significant fold change in Spm transcriptome analysis, demonstrated specific induction of *tetM* by Spm and Tetracycline, but not by other polyamines or similar ribosome-targeted antibiotics. Despite not contributing to Spm toxicity, these *tetM* derived data may have a potential impact on the current model of transcriptional attenuation and the dissemination of antimicrobial resistance determinant carried by conjugative transposon.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Staphylococcus aureus Mu50 (ATCC 700699), RN4220 (kindly provided by R. P. Novick), and *Escherichia coli* DH5α (Bethesda Research Laboratories) were employed in this study. Plasmids pCN38 and pCN56 (obtained from NARSA) are shuttle vectors of *E. coli* and *S. aureus* for gene cloning and promoter-*gfp* fusion, respectively [6]. Both *E. coli* and *S. aureus* strains were routinely cultured and maintained in the Luria–Bertani (LB) medium. When required, the LB medium was buffered with 20 mM Tris–HCl at the indicated pH. Antibiotics were added to the medium as necessary at the following concentrations: ampicillin, 100 μg ml^{-1} for *E. coli*; kanamycin, 25 μg ml^{-1} for *E. coli*, 50 μg ml^{-1} for *S. aureus*; erythromycin, 10 μg ml^{-1} for *S. aureus*; chloramphenicol, 10 μg ml^{-1} for *S. aureus*.

Transcriptional Profiling Conditions and Microarrays

Staphylococcus aureus Mu50 was grown in the buffered LB broth (pH8.0) at 37 °C to log-phase (OD_{600} of 0.5), and exogenous Spm was added to a final concentration of 10 mM. RNA samples were extracted with phenol (Fisher), digested with RNase-free DNase I (Pierce) to remove genomic DNA, and purified with RNeasy mini-columns (Qiagen). Synthesis, fragmentation, and terminal labeling of cDNA were carried out according to the protocols of the manufacturer (Affymetrix). Labeled cDNA was hybridized to the GeneChip *S. aureus* genome array. After scanning, the images were processed with GCOS 1.4 software (Affymetrix). Processed microarray data files have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE43230.

qRT-PCR

Total RNA was prepared as described above for microarrays after the cell cultures were treated with compounds as indicated in the text. Purified RNA was subjected to VeriQuest SYBR Green One-Step qRT-PCR (Affymetrix) using ABI 7500 Fast System (Applied Biosystems). The relative quantification of mRNA was determined by change in the expression level of the target transcripts relative to *gyrB* transcript (housekeeping gene) in accordance with the manufacturer's protocol. Data obtained are expressed as the mean fold change in transcript during stress condition relative to the compound-free control sample.

Construction of *tetM*-Promoter::*gfp* Fusion

A DNA fragment covering 705-bp upstream and 1-bp downstream of the *tetM* start codon was amplified by PCR from *S. aureus* Mu50 by primers 5′-CGC GGA TCC CGG ACT ACC AAC CCA AAC AGC TAG-3′and 5′-CCG GAA TTC TCA TGT GAT TTT CCT CCA TTC AA-3′. The PCR product was cloned in the *Bam*HI-*Eco*RI sites upstream of a promoterless *gfp* gene of the shuttle vector pCN56-CM (replace *ermC* from pCN56 with *cat194* from pCN38 to confer chloramphenicol resistance as selection marker). The correct construct pCNPtetM::gfp was introduced into RN4220 by electroporation and subsequently into Mu50.

Measurements of Promoter Activities

Bacteria carrying pCNPtetM::gfp were grown in LB (pH 8.0) with shaking at 37 °C to the exponential phase before exposure to polyamines (Spm, Spd or Put; 1 mM) or antibiotics translation inhibitors (tetracycline, chloramphenicol, erythromycin; 2 ng μl^{-1}). After 2 h, the cells were collected by centrifugation and suspended into the same volume of Tris–Cl buffer (pH8.0). The green fluorescence was then measured with a spectrofluorometer (EnSpire® Multimode Plate Readers, PerkinElmer) by excitation at a wavelength of 485 nm and detection of emission at 515 nm, and the growth curve was monitored by measuring the optical density at wavelength 600 nm (OD_{600}). The promoter activity was expressed as the ratio of relative fluorescence (RFU) over OD_{600}.

Cloning of the *tetM* Gene

A DNA fragment covering the *tetM* structural gene as well as 705-bp upstream and 668-bp downstream of *tetM* was amplified from genomic DNA of *S. aureus* Mu50 by the primer pair 5′-CGC GGA TCC CGG ACT ACC AAC CCA AAC AGC TAG-3′) and 5′-GGA AGA TCT TTA GAG CCG TTG GTT TAG CGA TTA-3′. The PCR product was digested by *Bam*HI and *Bgl*II, ligated to the *Bam*HI site of pUC18. One positive clone was confirmed by enzyme digestion and named as pUCtetM. The insert in pUCtetM was purified after *Bam*HI and *Eco*RI digestion and cloned into the same restriction sites of *E. coli–S. aureus* shuttle vector pCN38. The resulting plasmid pCN-tetM was confirmed by sequencing and introduced into *S. aureus* strain RN4220 by electroporation.

MIC Measurements

The broth micro-dilution method in 96-well titer plates was employed to determine MICs of antibiotics or spermine according to the CLSI guideline. Serial two-fold dilutions of the tested compound were prepared in the LB broth. When required, the LB medium was buffered with 20 mM Tris–HCl of the indicated pH. Fresh overnight cultures of each bacterial strain were diluted and inoculated with approximately 5×10^5 CFU per well. The plates were properly wrapped and incubated without shaking at 37 °C for 16 to 18 h. The MIC was defined as the lowest concentration of the agent that inhibited the growth of the bacteria as detected by unaided eyes. The MICs were determined in triplicate.

Results

Transcriptome Analysis of *S. aureus* in Response to High Dose Spm

For most *S. aureus* strains which do not possess SpeG, Spm can inhibit the bacterial growth in a dose-dependent manner [23]. To obtain a comprehensive view of the transcriptional changes by Spm, we conducted a series of independent microarray experiments in the absence (control group) and the presence (experimental group) of 10 mM Spm upon 15, 30, and 60 min exposures. To further identify genes of changes with statistical significance, we applied the following criteria to each of the control-experimental microarray data sets: (1) P value ≤ 0.05, (2) absolute fold change in transcript level ≥ 2.5, and (3) for up-regulation, genes in the experimental group of over 200 in signal intensity; for down-regulation, genes in the control group of over 200 in signal intensity. Of the over 3,000 ORFs in the genome of *S. aureus*, a list of 581 genes showing significant changes upon Spm treatment was compiled by this approach. To examine how these genes were distributed with regard to their physiological and biochemical functions, they were further classified according to COG functional categories (Table S1). By comparing with the profiles before Spm treatment, genes showing significant changes upon exposure to Spm predominantly participate in iron transport and metabolism, SigB regulon, and ATP production. Transcriptional profiles of representative genes are shown in Fig. 1, and below described some major findings.

Up-regulation

Iron Uptake and Metal-Dependent Transcriptional Regulators

As shown in Fig. 1, a number of genes encoding metal transporters (especially for iron) showed increased expression, indicative of possible dysregulation of metal homeostasis by Spm challenge. Iron acquisition by *S. aureus* is mainly mediated by siderophore or heme [17]. It has been reported that FhuABGD (ferric hydroxymate uptake) [38], HtsABC (heme transport system) [14], and SirABC (staphylococcal iron regulated transporter) [9] participate in siderophore import, while heme uptake go through the Isd system and the HtsBC permease [29, 43]. In specific, for siderophore import, staphyloferrin A is mediated by HtsABC and FhuA, staphyloferrin B by SirABC and FhuA, while xeno-siderophore by FhuABGD. Here, the *fhuABG, htsABC, and isdF* all showed 3–6-fold up-regulation by Spm. In addition, the *norA* gene encoding an iron-responsive efflux pump in *S. aureus* [12], also displayed over 2.5- fold increase. Moreover, four major metal-mediated repressors, Fur, PerR, Zur (three Fur family members) [19, 20, 24, 25], and MntR (DtxR-like protein) [21], all displayed induced expression. Although responding to different metal ions, these repressors share a similar mode of action: binding of metal ions to the regulators is essential for their DNA-binding activities in gene repression. Upon iron limitation, dissociation of F^{2+}-Fur complex derepresses the Fur regulon for siderophore synthesis, transport, and regulation (*fur* itself) [11]. Meanwhile, PerR, Zur, or MntR also can modulate the Fur regulon directly or indirectly, further expanding the regulatory capability on iron uptake and storage. In addition to iron-dependence, the PerR regulon (including *fur* and *perR* itself) can be activated by peroxide in order to modulate resistance of oxidative stress [19]. Collectively, these observations revealed a strong link of Spm toxicity to iron-restricted stress.

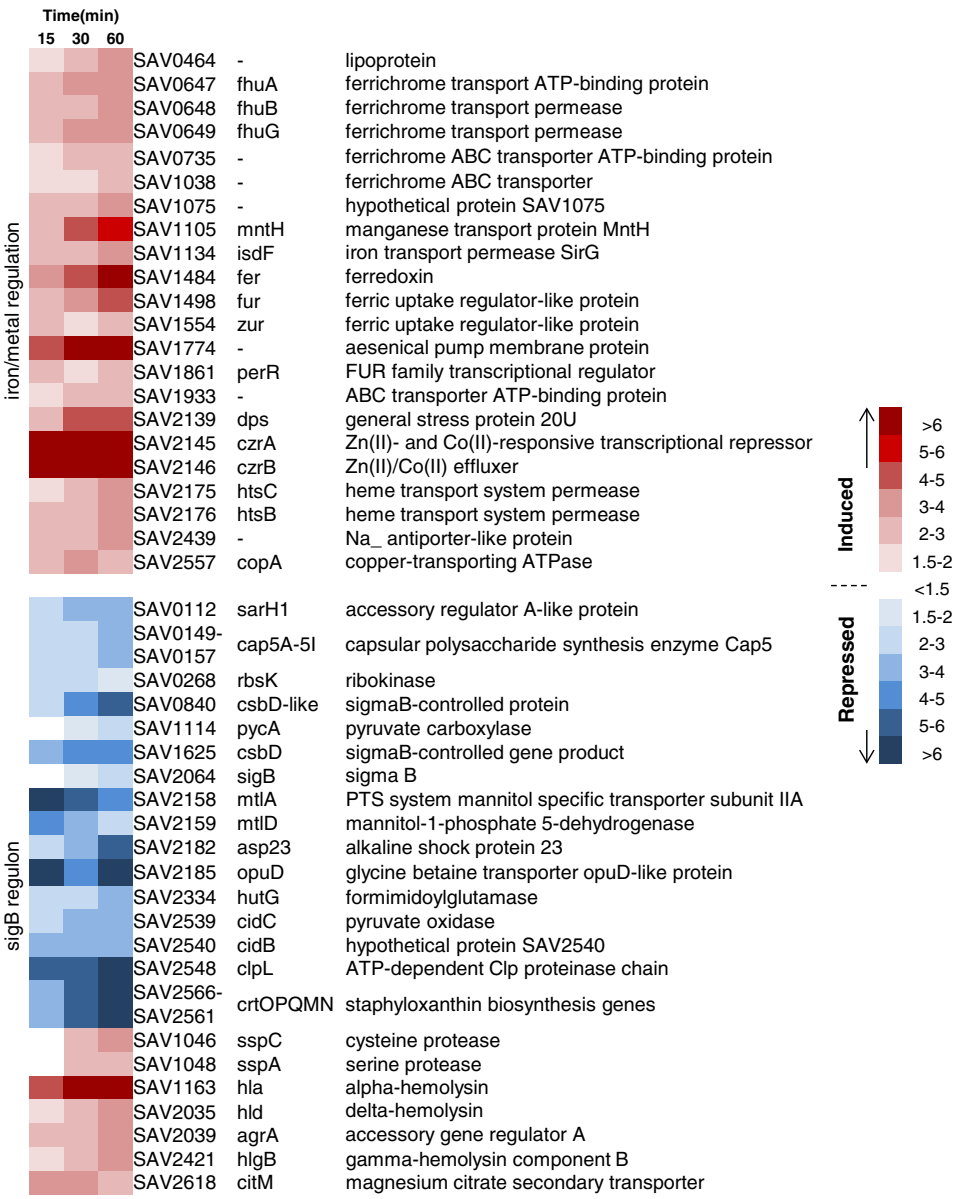


Fig. 1 Differential expression of iron-regulation and *sigB* regulon following Spm exposure. Microarray results are presented as the fold change of experimental group (samples collected at different time points after compared to control ($t = 0$, before Spm). Candidate genes were categorized according to functional class. Common name of the encoded protein is provided, and gene names are indicated in *parentheses*

Other Spm-Induced Gene Expression

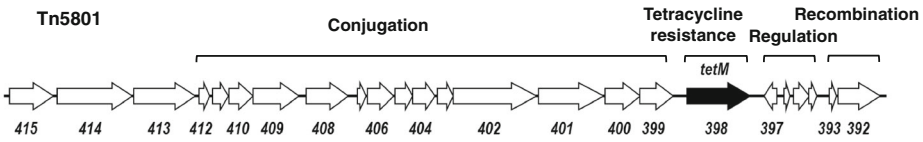
Genes related to metabolism and transport of certain amino acids, particularly those for arginine biosynthesis, showed significant induction by Spm. Up-regulation of these genes infers an increasing need of specific amino acids during Spm stress. Another notable finding was the induction of *potABCD* encoding components of a putative ABC transporter for polyamine uptake. Investigation of this operon was recently reported [47]. Besides, some Spm-inducible genes may likely contribute to osmotic tolerance, such as those encoding glycine betaine transporter (*opuD*), choline dehydrogenase (*betA*), glycine betaine/carnitine/choline ABC transporter (*opuCA/CB/CD*), and glycine aldehyde dehydrogenase (*gbsA*). Of interest, the *tetM,* gene (SAV0398) for tetracycline resistance, and the *kdpCBADE* operon (two copies in Mu50 genome, SAV0074-0070 and SAV2075-2079) for potassium uptake were also significantly induced by Spm (Table S1).

Down-Regulation

The SigB Regulon

One very striking event upon Spm stress was the down-regulation of Sigma B (SigB, σ^B) regulon (Fig. 1). A total of 251 genes were reported in the σ^B regulon of *S. aureus* [5], and 96 genes of this regulon were found responsive to high dose Spm. Among these genes 79 ORFs positively controlled by σ^B exhibited reduced expression, while 17 genes which are repressed by σ^B display an upregulated pattern in response to Spm stress (Table S1, Fig. 1).

a



b

Gene ID	Control signal	10mM Spm signal	1mM Spm signal	10mM Spm fold change	1mM Spm fold change
SAV0397	470	789	461	1.7	1.0
SAV0398	1683	13893	15716	**8.3**	**9.3**
SAV0399	262	403	156	1.5	0.6

Fig. 2 Schematic representation of Tn5801 from *S. aureus* strain Mu50 and the expression profiling of *tetM* with the flanking genes in response to Spm. **a** The ORFs within Tn5801 of *S. aureus* Mu50 are numbered SAV0392 to SAV0415 according to the original designations (GenBank accession no. BA000017). *tetM* is represented as *black arrow*. **b** Gene expression signals were from transcriptome analyses with Mu50 treated with high dose Spm (10 mM) for 15 min or low dose Spm (1 mM) for 120 min. GeneChip raw data are mean values from two independent sets of cultures. Expression change (*n*-fold) was the signal intensity ratio between Spm treated and untreated conditions

ATP Synthesis and Carbohydrate Metabolism/Transport

The addition of Spm to *S. aurues* decreased to different extents the expression of genes in major central metabolic pathways of energy production, including fermentation, glycolysis, gluconeogenesis, pyruvate conversion, pentose phosphate cycle, and TCA cycle. We also noticed (Table S1) a down-regulation of the membrane-bound ATP synthases (F0F1-ATPases). These data indicate an overall decreased activity or demand of energy production during Spm stress.

Many genes in carbohydrate metabolism and transport were down-regulated. In specific, expression of *ptsIH* for glucose uptake [34] was decreased, consistent with the reduced expression of genes in glycolysis and pentose phosphate cycle (the alternative glucose degradation pathway), as described above. Moreover, expression of the *lacABCDFEG* operon for lactose utilization was also decreased. The assimilation of lactose or galactose by *S. aureus* accumulates galactose 6-phosphate. This phosphorylated carbohydrate is the actual intracellular signal molecule for induction of the lactose (*lac*) genes, which can be further metabolized to triose phosphates used in the glycolysis [30]. Taken together, reduced expression of genes for sugar uptake and utilization was consistent with the pattern of down-regulation in ATP synthesis.

Spermine-Specific Induction of *tetM*

Among genes that were induced by 10 mM Spm, *tetM* exhibited the most significant fold change and high signals (over 8-fold). In *S. aureus* Mu50, *tetM* was identified on Tn5801 (Fig. 2a), which contains highly conserved ORFs in four functional modules: conjugation, regulation, recombination, and accessory genes such as *tetM* [10]. As shown in Fig. 2b, *tetM* was highly induced by exogenous Spm at either 1 mM or 10 mM. In comparison, Spm exerted no significant effect on the expression of other genes in Tn5801.

To substantiate the finding of Spm on *tetM* expression, and to test whether *tetM* was responsive to other polyamines, the transcript levels of *tetM* were measured by qRT-PCR. The cultures of Mu50 in the exponential phase were exposed to various polyamines (Spm, Spd, and Put; 1 mM), and the cells exposed to low dose Tc (2 ng μl^{-1}=4.16 μM) served as positive control of *tetM* induction. As shown in Fig. 3a, *tetM* expression was specifically induced by Spm and Tc to a comparable level but not by Spd or Put.

As described in Materials and Methods, a recombinant plasmid pCNPtetM::gfp was constructed to measure the promoter activity of *tetM* with gfp as the reporter gene. This plasmid was introduced into Mu50, and the effects of different polyamines and antibiotics were evaluated as shown in Fig. 3b. For polyamines, only Spm exerted an induction effect on *tetM* expression, consistent to the results of DNA microarrays and qRT-PCR. For ribosome-targeted antibiotics, only Tc was able to induce the *tetM* promoter; chloramphenicol and erythromycin showed no effect.

It is of interest to understand why Spm can induce *tetM* transcription and whether the expression of TetM is able to

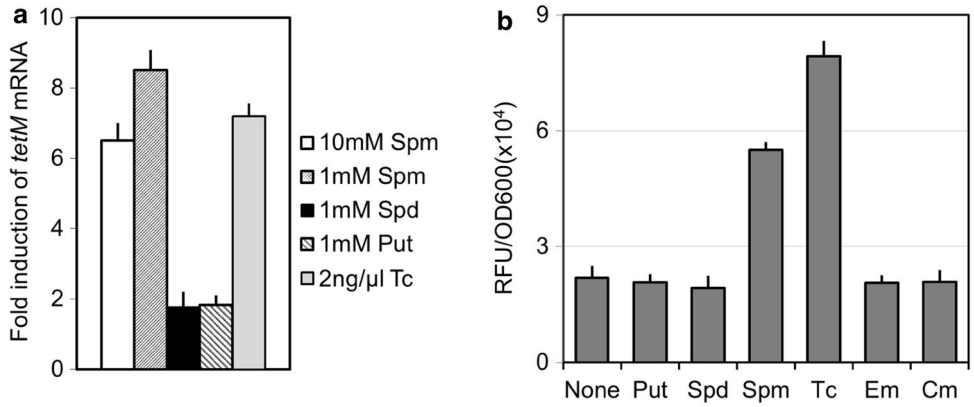


Fig. 3 *tetM* responses to polyamines and protein inhibitors. **a** qRT-PCR analysis of *tetM* in response to different polyamines. The fold change represents the difference between Ct of drug-free condition (control) and Ct of the compound-supplement condition. **b** Promoter activities of P*tetM*. *S. aureus* strain Mu50 harboring pCNPtetM::gfp was grown for 2 h in the presence or absence of polyamine (1 mM of Put, Spd, Spm) or antibiotics (2 ng μl^{-1} of Tc, Em or Cm). *Tc* tetracycline, *Em* erythromycin, *Cm* chloramphenicol. Promoter activities were expressed as relative fluorescence versus OD_{600} of the culture. Standard deviations are derived from at least two independent cultures

Table 1 Effects of the *tetM* gene on MICs of tetracycline (Tc) and spermine (Spm)

Host strain[a]	Plasmid	MIC	
		Tc (μg/mL)	Spm (mM)
S. aureus Mu50	None	100	2
S. aureus RN4220	None	1.5	2
S. aureus RN4220	pCNtetM[b]	100	2
E. coli DH5α	None	1.5	ND
E. coli DH5α	pCNtetM	25	ND

ND not determined

[a] *S. aureus or E. coli* strains with or without *tetM* expressing vector

[b] pCNtetM, pCN38 with *tetM* under the control of its endogenous promoter, served as *tetM* expressing vector

confer resistance to Spm. First, to test whether the TetM is indeed responsible for Tc resistance, the *tetM* gene including its regulatory region from Mu50 was cloned and transformed into *E. coli* DH5α as well as *S. aureus* RN4220 where *tetM* was absent. In the subsequent drug-susceptibility test, MIC of Tc in RN4220 harboring *tetM* increased more than 60- fold in comparison to its host, reaching to a level comparable to that in Mu50; similarly, *tetM* was able to raise the tolerance of *E. coli* to Tc, but to a lesser extent (Table 1).

Since Spm toxicity also applies to strains of *S. aureus* without the *tetM* gene, it is unlikely that TetM is the molecular target of Spm toxicity. However, it was possible that TetM expression might promote Spm resistance. To test this hypothesis, Spm MIC was determined in *tetM* expressing strains of *S. aureus* (chromosome-borne as in Mu50 and plasmid-borne as in RN4220/tetM carrying a recombinant *tetM*-bearing plasmid). As revealed in Table 1, both RN4220/tetM and Mu50 showed no difference on the Spm sensitivity in spite of *tetM* expression, indicating that the up-regulation of *tetM* by Spm is not a defensive mechanism to Spm toxicity.

Although *tetM* may not play any role in Spm toxicity (Table 1), such specific induction by Spm is exceptionally interesting. Given that Tc acts by binding to the 30S subunit of ribosomes, we investigated the *tetM* response to other drugs against translation by measurements of the *tetM* promoter activities. As shown in Fig. 2b, unlike Tc, erythromycin and chloramphenicol were unable to induce *tetM*, suggesting *that tetM* exclusively responds to Tc and Spm, but not other ribosome-targeted drugs or other polyamines.

Increase of Spm Tolerance by KCl and NaCl

Another interesting observation revealed from our transcriptome data is the induction of *kdpABCDE* locus upon Spm exposure. The *kdp* genes, which are widely distributed among bacteria, originally were considered for potassium uptake and regulation [13], while recent lines of evidence also showed that the Kdp system is involved in virulence of some bacteria including *S. aureus* [33, 45]. In *E. coli*, the KdpABC is a high-affinity K^+ uptake ATPase, and its expression is regulated by the KdpDE two-component system in response to K^+ limitation or turgor pressure [18].

The connection between Spm and K^+ homeostasis was originally described in eukaryotic systems, where Spm enters and occludes the transmembrane pore domain of inwardly rectifying potassium channel (Kir) [37, 44]. In bacteria, despite of less complexity, many potassium transport systems (like Kdp-ATPase, Trk) have been shown

to behave similarly to the eukaryotic countparts. Given this, we proposed that in *S. aureus* Spm may block the K^+ uptake in a similar manner as that in the eukaryotic potassium channel, and therefore supplement with a high concentration of K^+ may increase Spm resistance by counteracting Spm obstruction on the uptake channel. To test this, Spm MIC in Mu50 was measured in LB containing 300 mM KCl. Interestingly the addition of KCl indeed increased Spm MIC from 1 mM to 32 mM.

It should be noted that aside from K^+ limitation, high osmolarity also has been proposed to serves as the signal for induction of the *kdp* operon [3]. To test whether the induction of *kdp* by Spm was due to osmolarity upshift, we also measured Spm MIC in the presence of other osmotic shock agents - 300 mM NaCl and 600 mM sucrose. Surprisingly, like KCl, NaCl also enhanced the tolerance of Mu50 to Spm with MIC of 32 mM; nonetheless, osmotic shock with 600 mM sucrose showed no effect.

Discussion

Spm Toxicity and Iron Limitation

In this study, we analyzed changes in the transcriptome of *S. aureus* Mu50 upon Spm stress. The stimulation of iron acquisition, including iron uptake genes (*fhuABG* 4-fold, *htsABC* 12- fold) and metal-specific regulators (Fur, PerR, Zur, MntH 3-fold) was particularly of note, indicative of a specific connection between Spm and iron-depletion. Similarly, early reports have indicated the effects of low iron stress and siderophore production on spermine biosynthesis [4]. In fact, Spm has been known to have iron chelating properties by forming ternary complexes between Fe^{2+} ions and phosphate groups [26–28], which can contribute to polyamine anti-oxidant activity through inhibiting Fe^{2+} auto-oxidation and in consequence hydroxyl radical generation [31, 40, 41]. Therefore, the interesting changes seen with the up-regulation of metallo-regulators Fur, Zur, and MntR could directly result from metal chelation. In addition, the antioxidant properties of Spm would explain the lack of regulation on PerR-dependent antioxidant components (like *katA*) since more iron is being transported, alongside the metal chelation reducing Fenton chemistry. Taken together, we hypothesize that Spm may induce stress through iron starvation in *S. aureus*.

Iron is essential for bacterial growth due to its involvement in multiple metabolic processes, including respiration [heme-containing cytochromes, (Fe–S)-containing ferredoxins] and key enzymes in central metabolism [(Fe–S)-containing enzymes in TCA, like SdhA, CitB, CitG] [16, 17]. Thus, the potential sequestration of iron by Spm may lead to deficiency on electron transport and energy production. Of note, a recent report shows that a number of spontaneous spermine-resistant mutants of *S. aureus* are small colony variants with deficiency on menadione synthesis. Mutations affecting the generation of menadione and associated electron transport may compromise Spm toxicity [22]. From our transcriptome data, genes for iron acquisition and for the biosynthesis of cytochromes showed significant induction under Spm stress, inferring a correlation between heme/iron and Spm. Hence, both heme/iron and menaquinine, two indispensable components for electron transport chain, seem to have connections with Spm. It also suggests the possible effect of Spm on cellular respiration and energy production through these connections of unknown nature.

Collectively, we propose a model for Spm toxicity as described below. Spm may generate iron-depleted conditions or change the intracellular iron status by iron chelating properties. Such iron starvation signals are sensed by metal-specific regulators (Fur, PerR, MntR, Zur), which initiate derepression of iron uptake systems. Even though low dose Spm is sufficient to induce iron uptake, the delivered impact may be sequestered by redirecting central metabolism, thus explaining why low dose Spm has no inhibition on *S. aureus*. Upon high dose Spm, the potential detrimental effect resulting from iron limitation might be overwhelming, which leads to the instant inhibition on cell growth. Furthermore, the fact that high dose Spm is able to turn off the SigB regulon may cause the cells even more difficult to survive under Spm stress.

Down-Regulation of SigB Regulon by Spm

The alternative sigma factor σ^B of *S. aureus* is in charge of the general stress response, expression of virulence determinants, and modulation of antibiotic resistance. It controls a large regulon, either directly by recognizing conserved σ^B promoter sequences (e.g., *asp23*, *clpL*) or indirectly via σ^B-dependent elements (e.g., RNAIII, *agrA)* [32, 36]. In specific, σ^B has been shown to influence the expression of several global regulators, including up-regulation of SarA, SarS (SarH1) as well as down-regulation of RNAIII and *agr* regulatory system, all of which exhibited an altered pattern of expression by Spm in our study. Of note, the function of σ^B in virulence factor production is exactly the opposite from that of RNAIII/*agr* quorum sensing circuits. Therefore, the increased expression of exoprotein and toxin genes under Spm stress might be a consequence of lower σ^B and/or higher RNA III transcription.

From the results of transcriptome analysis, it was very clear that the σ^B regulon was turned off under Spm stress. Conserved in most bacteria, the major physiological function of the σ^B regulon was to protect the cells when under stress. However, it was surprising that Spm stress in

fact shuts down the σ^B regulon. We considered this effect could be very detrimental to the cells. This novel finding warrants future investigation on the molecular mechanism of Spm effects on σ^B.

Potential Mechanisms of *tetM* Induction by Tetracycline and Spm

Tetracycline (Tc) functions by binding to a specific region in the 16 s rRNA of the 30S ribosomal subunit and preventing access of aminoacyl-tARNA (aa-tRNA) to the ribosomal A site [8]. The ribosomal protection protein TetM can release Tc binding and rescue the ribosome to return to the elongation cycle [42]. The mechanism for *tetM* regulation has been proposed through transcriptional attenuation [39]. A "leader peptide" immediately upstream of *tetM*, contains rare amino acids; therefore, the ribosome pauses at these positions due to the shortage of rare aminoacyl-tRNAs (aa-tRNAs), leading to retarded translation. The non-synchronism of RNA polymerase and ribosome makes the transcription terminated at the stem-loop termination sites which are upstream of *tetM* start codon, resulting in no expression of *tetM* when Tc is absent. When cells are exposed to Tc which retards the translation, there is a backup of charged tRNAs, including the rare ones needed for the *tetM* leader peptide. The increased availability of these aa-tRNAs allows for coupled translation and transcription, thus enables disruption of the step-loop terminator. Transcription then proceeds into *tetM*, and more TetM were expressed to protect ribosome from Tc attack.

Spm also can bind to 16S rRNA in the 30S subunit [1]. This determined Spm-binding site partially overlaps with the Tc binding site. The effects of Spm on ribosomal functions were further characterized in many aspects: (1) Spm can enhance the binding of aa-tRNA to the A site to ribosome; (2) Spm cross-linking to rRNA reduces the translational accuracy and therefore increases the probability of using "wrong aa-tRNA" for translation in the lack of right cognate aa-tRNA; (3) Spm had no impact on the peptide bond formation [1]. Based on these, along with the transcriptional attenuation model for Tc regulation on *tetM*, as well as the specific induction of *tetM* by Spm and Tc, it was reasonable to propose that a) Spm may behave like Tc by inhibiting translation to induce *tetM*; or b) Spm may facilitate aa-tRNA incorporation to 30S binding site thus bypass the transcriptional attenuation of *tetM* and enhance its expression. As a small molecule, Tc or/and Spm may use riboswitch to achieve the regulatory action. Considering the fact that Spm does not restrain the aa-tRNA binding as mentioned above, the former mechanism is less favorable. Alternatively, given the complicated structure of *tetM* mRNA and Tc/Spm small molecules, it is interesting to speculate riboswitch as potential mechanism of *tetM* regulation by Tc and/or Spm instead of the current model of coupled transcription-translation as described above.

Protection of Spermine Stress by KCl/NaCl

In this study, expression of the *kdpCBAFDE* operon was found inducible by exogenous Spm. Since Spm was reported to block the Kdp-like channel of potassium uptake [37, 44], we hypothesized that growth inhibition of *S. aureus* by Spm might be related to potassium uptake, and that induction of the *kdp* operon might be the result of low potassium inside the cells. Although addition of potassium (300 mM KCl) indeed increased Spm MIC up to 32-fold, a similar fold of protection against Spm can also be observed in the presence of 300 mM NaCl but not by 600 mM of sucrose. Since high osmolarity by NaCl or sucrose was reported to induce high levels of *kdp* expression [35], it was apparent that induction of the *kdp* operon alone cannot protect the cells from Spm stress, and the mechanism of NaCl/KCl protection against Spm stress remained to be answered.

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References

1. Amarantos I, Zarkadis IK, Kalpaxis DL (2002) The identification of spermine binding sites in 16S rRNA allows interpretation of the spermine effect on ribosomal 30S subunit functions. Nucleic Acids Res 30(13):2832–2843
2. Amendola R, Cervelli M, Fratini E, Polticelli F, Sallustio DE, Mariottini P (2009) Spermine metabolism and anticancer therapy. Curr Cancer Drug Targets 9(2):118–130
3. Ballal A, Basu B, Apte SK (2007) The Kdp-ATPase system and its regulation. J Biosci 32(3):559–568
4. Bergeron RJ, Weimar WR (1991) Increase in spermine content coordinated with siderophore production in *Paracoccus denitrificans*. J Bacteriol 173(7):2238–2243
5. Bischoff M, Dunman P, Kormanec J, Macapagal D, Murphy E, Mounts W, Berger-Bachi B, Projan S (2004) Microarray-based analysis of the *Staphylococcus aureus* sigmaB regulon. J Bacteriol 186(13):4085–4099. doi:10.1128/JB.186.13.4085-4099.2004
6. Charpentier E, Anton AI, Barry P, Alfonso B, Fang Y, Novick RP (2004) Novel cassette-based shuttle vector system for gram-positive bacteria. Appl Environ Microbiol 70(10):6076–6085. doi:10.1128/AEM.70.10.6076-6085.2004
7. Cohen SS (1998) A guide to the polyamines. Oxford University Press, New York
8. Connell SR, Tracz DM, Nierhaus KH, Taylor DE (2003) Ribosomal protection proteins and their mechanism of tetracycline resistance. Antimicrob Agents Chemother 47(12):3675–3681
9. Dale SE, Sebulsky MT, Heinrichs DE (2004) Involvement of SirABC in iron-siderophore import in *Staphylococcus aureus*.

J Bacteriol 186(24):8356–8362. doi:10.1128/JB.186.24.8356-8362.2004
10. de Vries LE, Christensen H, Skov RL, Aarestrup FM, Agerso Y (2009) Diversity of the tetracycline resistance gene tet(M) and identification of Tn916- and Tn5801-like (Tn6014) transposons in *Staphylococcus aureus* from humans and animals. J Antimicrobiol Chemother 64(3):490–500. doi:10.1093/jac/dkp214
11. Delany I, Spohn G, Pacheco AB, Ieva R, Alaimo C, Rappuoli R, Scarlato V (2002) Autoregulation of *Helicobacter pylori* Fur revealed by functional analysis of the iron-binding site. Mol Microbiol 46(4):1107–1122
12. Deng X, Sun F, Ji Q, Liang H, Missiakas D, Lan L, He C (2012) Expression of multidrug resistance efflux pump gene norA is iron responsive in *Staphylococcus aureus*. J Bacteriol 194(7):1753–1762. doi:10.1128/JB.06582-11
13. Epstein W (2003) The roles and regulation of potassium in bacteria. Prog Nucleic Acid Res Mol Biol 75:293–320
14. Grigg JC, Cooper JD, Cheung J, Heinrichs DE, Murphy ME (2010) The *Staphylococcus aureus* siderophore receptor HtsA undergoes localized conformational changes to enclose staphyloferrin A in an arginine-rich binding pocket. J Biol Chem 285(15):11162–11171. doi:10.1074/jbc.M109.097865
15. Grossowicz N, Razin S, Rozansky R (1955) Factors influencing the antibacterial action of spermine and spermidine on *Staphylococcus aureus*. J Gen Microbiol 13(3):436–441
16. Haley KP, Skaar EP (2012) A battle for iron: host sequestration and *Staphylococcus aureus* acquisition. Microb Infect/Institut Pasteur 14(3):217–227. doi:10.1016/j.micinf.2011.11.001
17. Hammer ND, Skaar EP (2011) Molecular mechanisms of *Staphylococcus aureus* iron acquisition. Annu Rev Microbiol 65:129–147. doi:10.1146/annurev-micro-090110-102851
18. Heermann R, Jung K (2010) The complexity of the 'simple' two-component system KdpD/KdpE in Escherichia coli. FEMS Microbiol Lett 304(2):97–106. doi:10.1111/j.1574-6968.2010.01906.x
19. Horsburgh MJ, Clements MO, Crossley H, Ingham E, Foster SJ (2001) PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. Infect Immun 69(6):3744–3754. doi:10.1128/IAI.69.6.3744-3754.2001
20. Horsburgh MJ, Ingham E, Foster SJ (2001) In *Staphylococcus aureus*, fur is an interactive regulator with PerR, contributes to virulence, and Is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. J Bacteriol 183(2):468–475. doi:10.1128/JB.183.2.468-475.2001
21. Horsburgh MJ, Wharton SJ, Cox AG, Ingham E, Peacock S, Foster SJ (2002) MntR modulates expression of the PerR regulon and superoxide resistance in *Staphylococcus aureus* through control of manganese uptake. Mol Microbiol 44(5):1269–1286
22. Joshi GS, Spontak JS, Klapper DG, Richardson AR (2011) Arginine catabolic mobile element encoded speG abrogates the unique hypersensitivity of *Staphylococcus aureus* to exogenous polyamines. Mol Microbiol 82(1):9–20. doi:10.1111/j.1365-2958.2011.07809.x
23. Kwon DH, Lu CD (2007) Polyamine effects on antibiotic susceptibility in bacteria. Antimicrob Agents Chemother 51(6):2070–2077. doi:10.1128/AAC.01472-06
24. Lee JW, Helmann JD (2007) Functional specialization within the Fur family of metalloregulators. Biometals: an international journal on the role of metal ions in biology, biochemistry, and medicine 20(3–4):485–499. doi:10.1007/s10534-006-9070-7
25. Lindsay JA, Foster SJ (2001) Zur: a Zn(2+)-responsive regulatory element of *Staphylococcus aureus*. Microbiology 147(Pt 5):1259–1266
26. Lomozik L, Jastrzab R (2003) Copper(II) complexes with uridine, uridine 5′-monophosphate, spermidine, or spermine in aqueous solution. J Inorg Biochem 93(3–4):132–140
27. Lomozik L, Jastrzab R (2003) Non-covalent and coordination interactions in Cu(II) systems with uridine, uridine 5′-monophosphate and triamine or tetramine as biogenic amine analogues in aqueous solutions. J Inorg Biochem 97(2):179–190
28. Marzabadi MR, Llvaas E (1996) Spermine prevent iron accumulation and depress lipofuscin accumulation in cultured myocardial cells. Free Radical Biol Med 21(3):375–381
29. Mazmanian SK, Skaar EP, Gaspar AH, Humayun M, Gornicki P, Jelenska J, Joachmiak A, Missiakas DM, Schneewind O (2003) Passage of heme-iron across the envelope of *Staphylococcus aureus*. Science 299(5608):906–909. doi:10.1126/science.1081147
30. Mcclatchy E Jr (1963) Induction of lactose utilization in *Staphylococcus aureus*. J Bacteriol 86(6):1211–1215
31. Mozdzan M, Szemraj J, Rysz J, Stolarek RA, Nowak D (2006) Anti-oxidant activity of spermine and spermidine re-evaluated with oxidizing systems involving iron and copper ions. Int J Biochem Cell Biol 38(1):69–81. doi:10.1016/j.biocel.2005.07.004
32. Pane-Farre J, Jonas B, Forstner K, Engelmann S, Hecker M (2006) The sigmaB regulon in *Staphylococcus aureus* and its regulation. Int J Med Microbiol 296(4–5):237–258. doi:10.1016/j.ijmm.2005.11.011
33. Parish T, Smith DA, Kendall S, Casali N, Bancroft GJ, Stoker NG (2003) Deletion of two-component regulatory systems increases the virulence of Mycobacterium tuberculosis. Infect Immun 71(3):1134–1140
34. Postma PW, Lengeler JW, Jacobson GR (1993) Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. Microbiol Rev 57(3):543–594
35. Price-Whelan A, Poon CK, Benson MA, Eidem TT, Roux CM, Boyd JM, Dunman PM, Torres VJ, Krulwich TA (2013) Transcriptional profiling of *Staphylococcus aureus* during growth in 2 M NaCl leads to clarification of physiological roles for Kdp and Ktr K+ uptake systems. mBio 4(4): e00407–e00413. doi: 10.1128/mBio.00407-13
36. Shaw LN, Aish J, Davenport JE, Brown MC, Lithgow JK, Simmonite K, Crossley H, Travis J, Potempa J, Foster SJ (2006) Investigations into sigmaB-modulated regulatory pathways governing extracellular virulence determinant production in *Staphylococcus aureus*. J Bacteriol 188(17):6070–6080. doi:10.1128/JB.00551-06
37. Shin HG, Lu Z (2005) Mechanism of the voltage sensitivity of IRK1 inward-rectifier K+ channel block by the polyamine spermine. J Gen Physiol 125(4):413–426. doi:10.1085/jgp.200409242
38. Speziali CD, Dale SE, Henderson JA, Vines ED, Heinrichs DE (2006) Requirement of *Staphylococcus aureus* ATP-binding cassette-ATPase FhuC for iron-restricted growth and evidence that it functions with more than one iron transporter. J Bacteriol 188(6):2048–2055. doi:10.1128/JB.188.6.2048-2055.2006
39. Su YA, He P, Clewell DB (1992) Characterization of the tet(M) determinant of Tn916: evidence for regulation by transcription attenuation. Antimicrob Agents Chemother 36(4):769–778
40. Tadolini B (1988) The influence of polyamine-nucleic acid complexes on Fe^{2+} autoxidation. Mol Cell Biochem 83(2):179–185
41. Tadolini B (1988) Polyamine inhibition of lipoperoxidation. The influence of polyamines on iron oxidation in the presence of compounds mimicking phospholipid polar heads. Biochem J 249(1):33–36
42. Taylor DE, Chau A (1996) Tetracycline resistance mediated by ribosomal protection. Antimicrob Agents Chemother 40(1):1–5
43. Wright JA, Nair SP (2012) The lipoprotein components of the Isd and Hts transport systems are dispensable for acquisition of heme by *Staphylococcus aureus*. FEMS Microbiol Lett 329(2):177–185. doi:10.1111/j.1574-6968.2012.02519.x

44. Xie LH, John SA, Weiss JN (2002) Spermine block of the strong inward rectifier potassium channel Kir2.1: dual roles of surface charge screening and pore block. J Gen Physiol 120(1):53–66
45. Xue T, You Y, Hong D, Sun H, Sun B (2011) The *Staphylococcus aureus* KdpDE two-component system couples extracellular K+ sensing and Agr signaling to infection programming. Infect Immun 79(6):2154–2167. doi:10.1128/IAI.01180-10
46. Yao X, He W, Lu CD (2011) Functional characterization of seven gamma-Glutamylpolyamine synthetase genes and the bauRABCD locus for polyamine and beta-Alanine utilization in *Pseudomonas aeruginosa* PAO1. J Bacteriol 193(15):3923–3930. doi:10.1128/JB.05105-11
47. Yao X, Lu CD (2014) Functional Characterization of the *potRABCD* Operon for Spermine and Spermidine Uptake and Regulation in *Staphylococcus aureus*. Curr Microbiol. doi:10.1007/s00284-014-0556-1
48. Zhang M, Wang H, Tracey KJ (2000) Regulation of macrophage activation and inflammation by spermine: a new chapter in an old story. Crit Care Med 28(4 Suppl):N60–N66