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The *Clostridium difficile* quorum-sensing molecule alters the *Staphylococcus aureus* toxin expression profile

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Treatment of *Staphylococcus aureus* infections has become increasingly challenging due to the rise of antibiotic resistant strains. Therefore, the development of antibiotic-independent treatments that supplement or provide an alternative approach to traditional therapies is greatly needed. One promising approach is the development of 'antipathogenic' therapies that inhibit bacterial virulence. Quorum sensing is a bacterial communication mechanism in which increasing cell densities precipitate changes in gene expression, allowing bacteria to regulate essential processes [1]. The accessory gene regulator (Agr) quorum sensing system is a Gram-positive specific mechanism that is conserved among all staphylococcal species and similar across a large number of bacteria [2]. It is mediated by small, secreted autoinducing peptides (AIP) that are usually produced constitutively during growth and increasing bacterial densities are accompanied by a concomitant increase in AIP concentrations that regulate gene expression [1].

S. aureus strains possess any one of four variations of AIPs (groups I to IV) that affect genes that regulate a variety of functions [3], including genes encoding potent pore-forming toxins, immunoevasive compounds, superantigens, and tissue-degrading enzymes associated with severe clinical outcomes [1]. Thus, inhibiting the quorum sensing mechanism may improve clinical outcomes.

Recently, a *C. difficile* Agr-like quorum sensing peptide (TI signal) was shown to regulate toxin production [4]. Due to its similarity with the *S. aureus* AIPs, we examined the effect of the TI signal on *S. aureus* global gene expression using RNA sequencing (RNA-seq). *S. aureus* USA300 (TCH1516, a community-acquired methicillin-resistant strain belonging to Agr group I) was cultured in tryptic soy broth (TSB) containing 0 (control), 2.85, or 22.80 mg of the TI signal for 24 hours at 37°C. Differential gene expression was conducted by RNA-Seq (SeqWright Genomic Services, Houston, TX.) Total RNA was isolated using RNEasy (Qiagen) and quantified on a Nanodrop ND-1000 (Nanodrop, Wilmington, DE). RNA samples were subjected to two rounds of prokaryotic Ribo-zero rRNA depletion (Illumina, San Diego, CA) and their integrity evaluated using an Agilent BioAnalyzer RNA Pico chip (Agilent, Santa Clara, CA). cDNA was synthesized using a TruSeq sample

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Supplementary Material

Table S1. RNA-Seq data.

preparation kit and sequenced on the Illumina Hiseq 2000 platform (2×100 bp). Sequencing and statistical analyses were performed using the DNAnexus software (DNAnexus, Inc., Mountain View, CA). The data were expressed as fold-change in gene expression compared to the control untreated group (Table S1). Fold-changes were statistically significant ($p < 0.0005$) for 28 of the 500 genes for at least one of the TI signal concentrations tested (Table S1). Furthermore, the protein expression profiles of strains of different Agr types treated with TI signal were similar, suggesting that the TI signal alters *S. aureus* protein expression independent of the AIP type (Figure S1).

Because the alpha-toxin gene (*hla*) was significantly affected by the TI signal (Table S1), we next examined the impact of the TI signal on alpha toxin (strain NRS178) and the Pantone Valentine LukS subunit (LukS-PV) production (strain TCH1516). Bacterial cultures were prepared as above in the presence (0.068 ng-1.39 µg) or absence of the TI signal for either 24 or 48 h at 37°C, respectively. Supernatants were collected and subjected to SDS-PAGE using 4–20% Tris-Glycine precast gradient gels (BioRad, Hercules, CA) and transferred onto nitrocellulose paper. Recombinant LukS-PV [5] and Hla (IBT Bioservices) were used as positive controls. Super Block (Pierce, Rockford, IL) was used to block nonspecific binding and dilute the primary and secondary antibodies. Blots were incubated with each antibody for 1 h at room temperature. Blots were washed with 0.05% TBS-T 3 times for 5 minutes each between incubations. The membranes were probed using either rabbit anti-LukS-PV or anti-Hla (IBT Bioservices) at a 1:2,000 dilution followed by a goat anti-rabbit alkaline phosphatase (AP)-labeled secondary antibody (1:7,000 dilution) (Invitrogen, Frederick, MD) [5]. Bands corresponding to respective toxins were visualized following development of the blots with NBT/BCIP (Pierce). The results demonstrated that the TI signal decreased LukS and Hla production levels in a dose-dependent manner (Figure 1A). The TI signal also affected the expression profile of Staphylococcus protein A (SpA) (Figure 1A). The observed changes in Hla and LukS production were not due to growth impairment since the doses of the TI signal associated with the lowest CFUs did not correspond to the doses associated with reduced LukS-PV and Hla production (Figure 1B). Furthermore, the TI signal had no discernible effect on biofilm formation (data not shown).

The present study demonstrated that the *C. difficile* quorum sensing peptide affected the gene and protein expression profiles of different *S. aureus* strains. Moreover, the TI signal inhibited the production of Hla and LukS-PV toxins important in *S. aureus* pathogenesis suggesting that the TI signal may be promising as an ‘antipathogenic’ therapy for *S. aureus* infections. Our investigation is ongoing to elucidate the mechanism and to further examine how we could leverage the potential of TI signal to combat *S. aureus* infections.

Supplementary Material

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FIG 1.

The effect of the TI signal on toxin production and growth. A) TI signal affects LukS-PV and Hla expression profiles of *S. aureus* strains TCH1516 (upper panel) and NRS178 (lower panel). Respective strains were cultured in the absence or presence of decreasing

concentrations of TI for 24 h and 48 h, respectively. Supernatants were subjected to SDS-PAGE and subsequently transferred onto nitrocellulose, probed with either anti-LukS-PV or anti-Hla followed by a goat anti-rabbit AP-conjugated antibody. Blots probed with secondary alone did not show any detectable banding patterns other than non-specific binding to SpA. Each experiment was repeated twice and shown are representative blots. B) *S. aureus* growth in the presence of the TI signal. *S. aureus* strains (a) TCH1516 and (b) NRS178 were cultured in the absence or presence of decreasing concentrations of TI signal for 24 h and 48 h, respectively. Pellets were collected and resuspended in sterile PBS, diluted 1:10⁷ and 100 µL plated in triplicate onto mannitol salt agar and cultured aerobically at 37°C. Colonies were counted 24 h later. The data are expressed as the mean CFU per time point tested of three separate experiments for all TI concentrations shown. Graphs were generated and statistical analyses conducted using GraphPad Prism. A Student's t test was used to assess statistical differences between CFUs obtained following culture in the presence or absence of the TI signal. No statistical differences between CFUs was observed.