Metabolomic Analysis Reveals Metabolic Conditionality Of Antibiotic Resistance In *Staphylococcus Aureus*

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Overview

Staphylococcus aureus (S. aureus) is an antibiotic-resistant pathogen second only to HIV in scope and significance in the U.S. today.[1] A major contributor to the clinical impact of S. aureus has been the rapid emergence of antibiotic resistance. Previously, we demonstrated that evolving vancomycin heteroresistance is accompanied by a systematic shift in the S. aureus metabolome. Untargeted profiling of the metabolomes of vancomycin-susceptible and vancomycin-heteroresistant isolates revealed changes in pathways of anaerobic metabolism. To evaluate the role of these pathways in supporting expression of heteroresistance we performed metabolomic and microbiological analyses of vancomycin-susceptible, -heteroresistant, and intermediate isolates under aerobic and anaerobic conditions. Results of these analyses indicate that expression of vancomycin resistance in S. aureus is suppressed by anaerobic conditions. Future studies examining the role of specific pathways of anaerobic metabolism in supporting vancomycin heteroresistance may identify novel antimicrobial targets aimed at suppressing resistance itself.

Introduction

Antibiotic resistance is a major area of unmet medical need. In the United States, this need has emerged nowhere more visibly than in the case of Staphylococcus aureus (S. aureus). Antibiotic-resistant S. aureus is a frequent cause of fatal infections and a public health problem second only to HIV/AIDS in scope and significance.[1] Existing knowledge indicates that antibiotic resistance is often mediated by biological gains-of-function and fitness costs that are compensated for by resistance-specific physiologic adaptations. The identities of such adaptations, however, remain lacking. Our work focuses on identifying the adaptations that accompany hetero-intermediate and intermediate vancomycin resistance in S. aureus. Knowledge of such adaptations may provide novel means of controlling the growing medical threat of antimicrobial resistance.

Methods

Bacterial strains and characterization: Serial S. aureus blood isolates from a clinical patients who failed vancomycin treatment due in evolving hetero-intermediate vancomycin resistance were obtained from The Clinical Microbiology Laboratory of the Weill Cornell Medical Center. Detailed genetic and microbiological characterizations of these isolates are described elsewhere.2 In addition, a random sampling of 5 unrelated clinical vancomycin-susceptible, -heteroresistant and –intermediate isolates was obtained from The Clinical Microbiology Laboratory of the Weill Cornell Medical Center. This study was approved by the IRB of Weill Cornell Medical Center under protocol number 0902010203.

Global Metabolite Profiling: S. aureus' intracellular metabolic pathways were studies using global metabolite profiling.

1. Bacterial cultivation:

Bacteria were cultivated in Brain Heart Infusion (BHI), and grown for metabolomic profiling on BHI agar using the filter culture methodology of Brauer et al.3 For both bacterial cultivation and growth on filters aerobic conditions were ensured by growth at 220rpm shaking incubation, while anaerobic conditions were ensured by growth in a 7L Mitsubishi Anaeropack. Bacteria were harvested in midlogarithmic phase of growth and metabolically quenched by immersion into a solvent mixture of acetonitrile:methanol:dH2O (40:40:20) pre-cooled to -40oC. Metabolites were extracted by mechanical lysis of bacteria in a MiniBeadBeater (BioSpec) followed by clarification of insoluble debris by centrifugation for 10 minutes at 14,000 rpm at 4□C. Bacterial biomass of individual samples was determined by measuring residual protein content.

2. Liquid chromatography-mass spectrometry: The mass spectrometer used for the studies was an Agilent

Accurate Mass 6220 TOF coupled to an Agilent 1200 LC system equipped with a solvent degasser, binary pump,

temperature-controlled auto-sampler and temperature-controlled column compartment containing a Cogent Diamond Hydride Type C silica column (150 mm × 2.1 mm). The flow rate was 0.4 ml/min with a gradient as follows: Solvent B (acetonitrile with 0.2% acetic acid) was held constant at 85% and solvent A (water with 0.2% acetic acid) was held constant at 15% for 2 minutes and decreased to 20% B over the next 12 minutes as detailed by Pesek et al.4 (Gradient 3). Dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution using an isocratic pump connected to a dual sprayer electrospray ionization source, operated in the positive-ion mode. ESI capillary voltage was set at 3500 V. The nebulizer pressure was set to 40 psig and the nitrogen drying gas was set to a flow rate of 10 L/min. The drying gas temperature was maintained at 250°C. The acquisition rate was 1.5 spectra/ sec and a stored mass range of m/z 50–1200. Data were collected in centroid mode in 2 GHz (extended dynamic range) mode. Detected masses were deemed identified metabolites on the basis of unique accurate mass retention time identifiers for masses exhibiting the expected distribution of accompanying isotopes.

3. Data processing and statistical analysis: Discovery metabolomic analysis used the Molecular Feature

Extraction algorithm of Agilent MassHunter Qualitative Analysis software to find individual metabolite

features on the basis of a chromatographically covariant ion families with a logical mass relationships.

Statistics and graphs were generated using Mass Profiler Professional software and the data was analyzed using principal components analysis and hierarchical clustering analysis.

Targeted metabolomic analysis used MassHunter Qualitative Analysis software "Find by Chromatography" algorithm and a customized Metlin database that contained pathway specific metabolites with retention times. Detected masses were deemed identified target metabolites on the basis of unique accurate mass retention time identifiers for masses exhibiting the expected distribution of accompanying isotopes. Statistical analysis was performed on an MS Excel platform using a student's t-test.

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Results and Discussion

Global metabolite profiles of 5 unrelated clinical vancomycin-susceptible, vancomycin-heteroresistant and vancomycin-intermediate S. aureus isolates were obtained and analyzed as described. These studies demonstrate that increasing vancomycin resistance in unrelated clinical isolates is associated with (1) conserved alterations in the global metabolite profile and (2) specific changes in arginine catabolism.

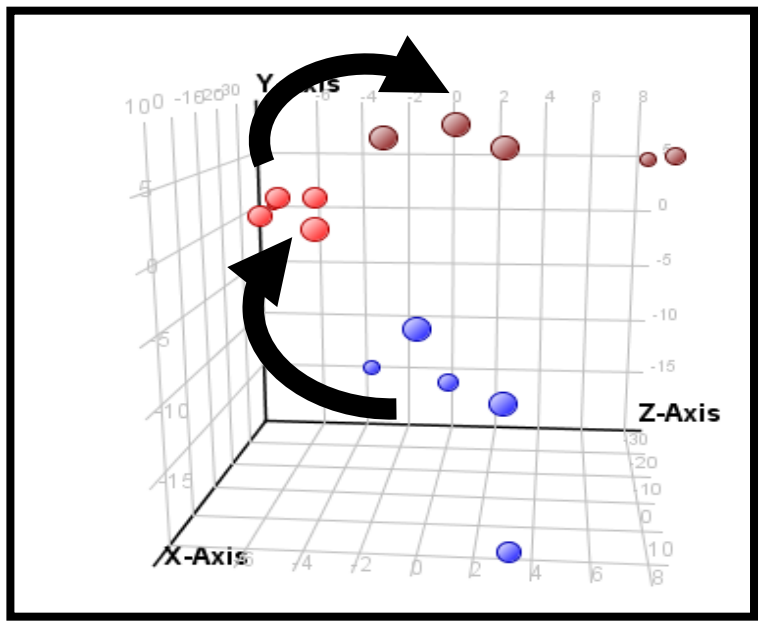

Figure 1. Principal components analysis of 5 randomly-selected clinical vancomycin-susceptible, -heteroresistant, and -intermediate isolates demonstrates a systematic skewing of the metabolome by resistance phenotype.

Figure 2. Hierarchical cluster tree of the same isolates as in (Figure 1).

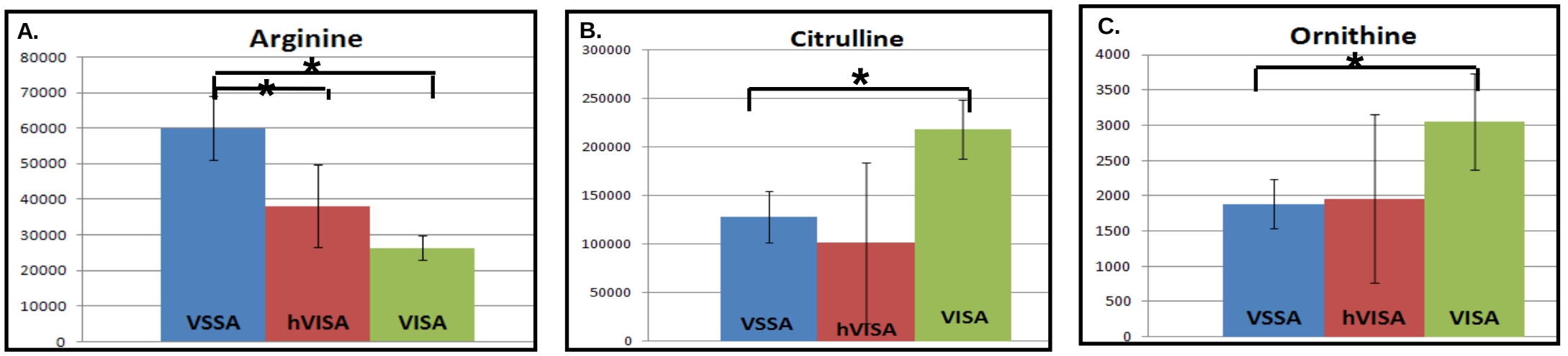

Figure 3. Targeted metabolomic analysis of intermediates of arginine catabolism demonstrates that increasing vancomycin resistance is accompanied by (a) decreased intracellular arginine pools with a corresponding increase in pools of (b) citrulline and (c) ornithine. Statistically significant differences denoted by (*).

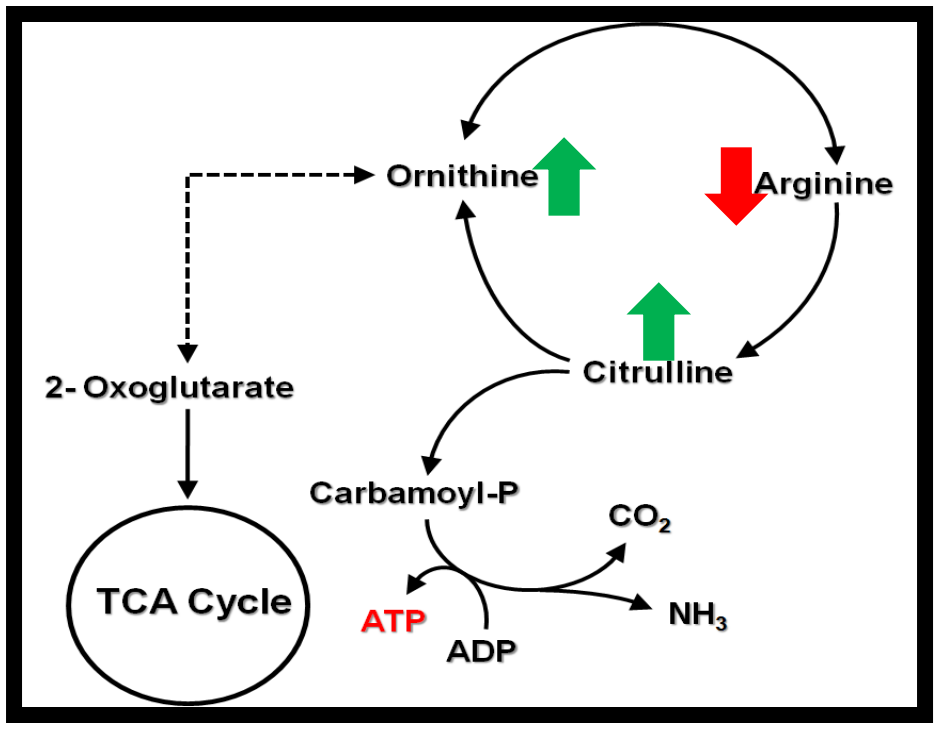

Figure 4. Proposed model of changes in arginine catabolism associated with increased vancomycin resistance in *S. aureus*. As vancomycin resistance increases, there is increased consumption of arginine pools (red arrow), with increased production of the downstream metabolites, citrulline and ornithine (green arrows).

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Growth curve analyses of isogenic and unrelated clinical S. aureus isolates with hetero-intermediate vancomycin resistance were performed under aerobic and anaerobic conditions. These studies demonstrate that expression of vancomycin heteroresistance is suppressed by growth under anaerobic conditions. Moreover, this suppression was accompanied by reciprocal changes in metabolites of the arginine deiminase pathway in the heteroresistant isolate from the clonal series.

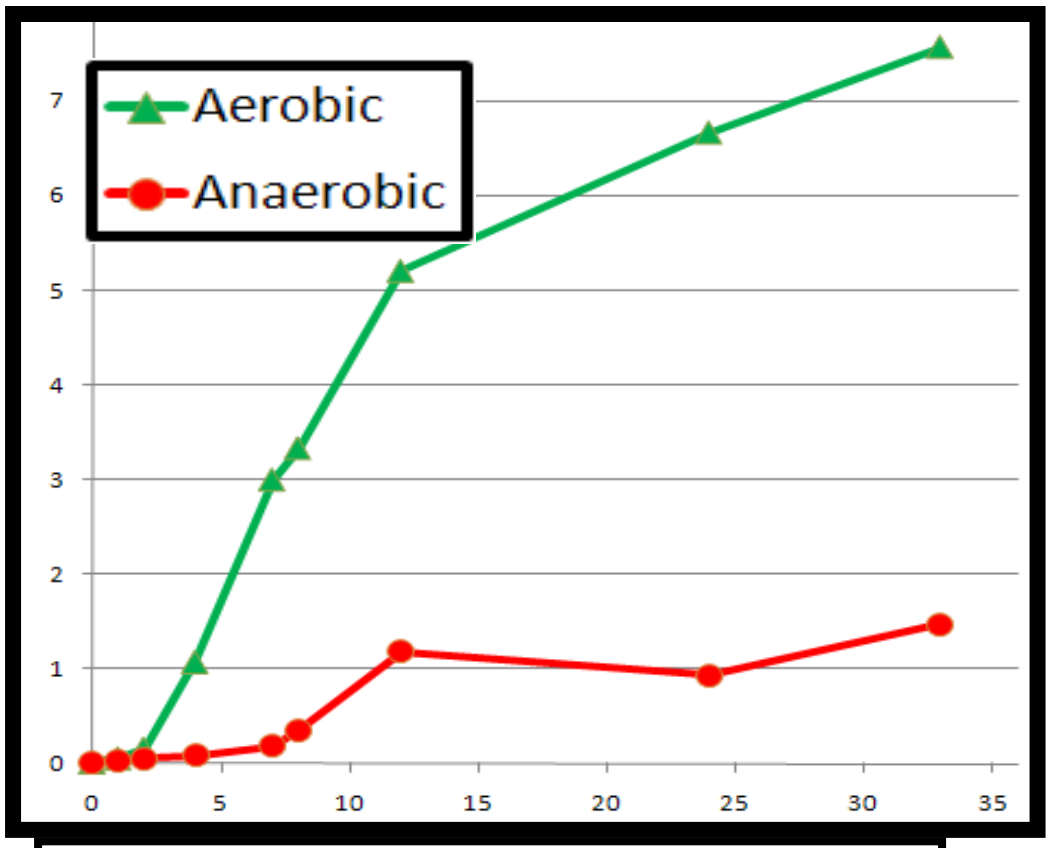


Figure 5. Growth of hVISA isolates B3 in enriched media with 2µg/mL vancomycin under aerobic vs. anaerobic conditions.

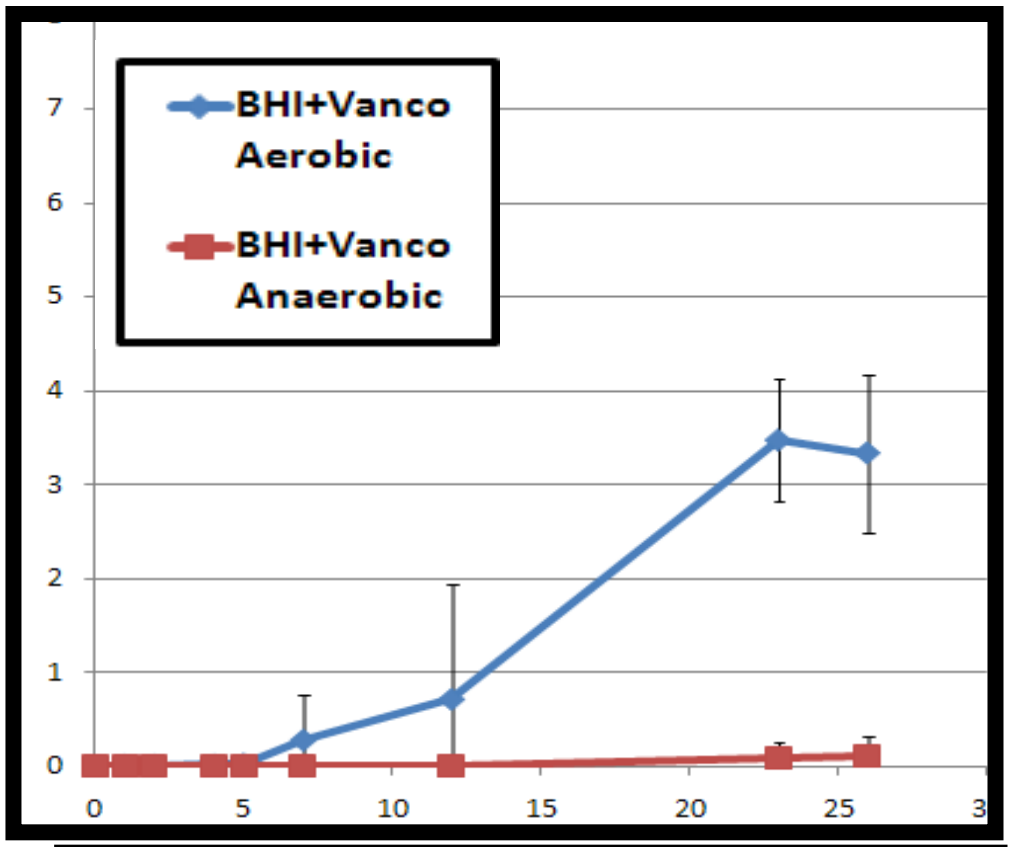
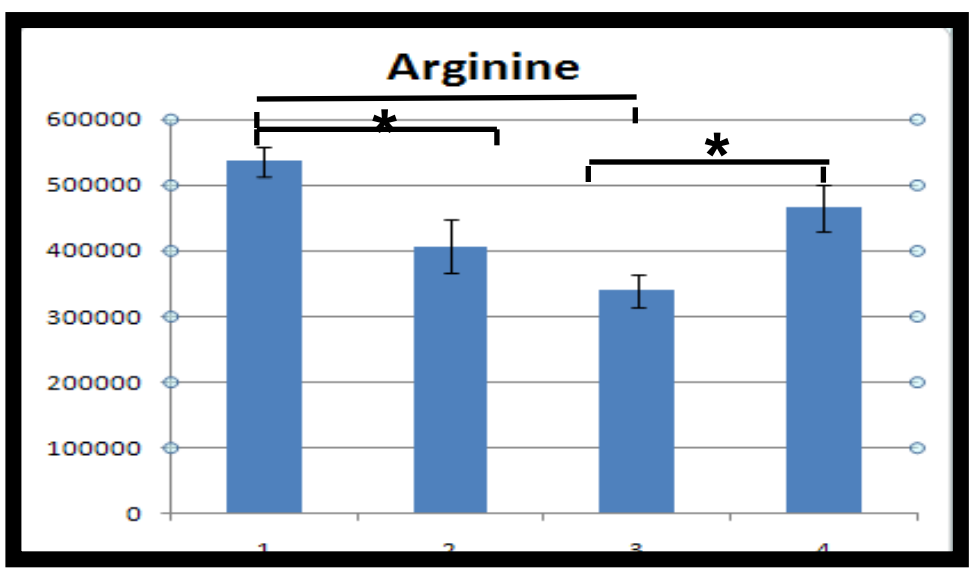


Figure 6. Averaged growth of 3 unrelated clinical vancomycin-heteroresistant isolates on enriched media with 2µg/mL vancomycin under aerobic conditions vs. anaerobic conditions



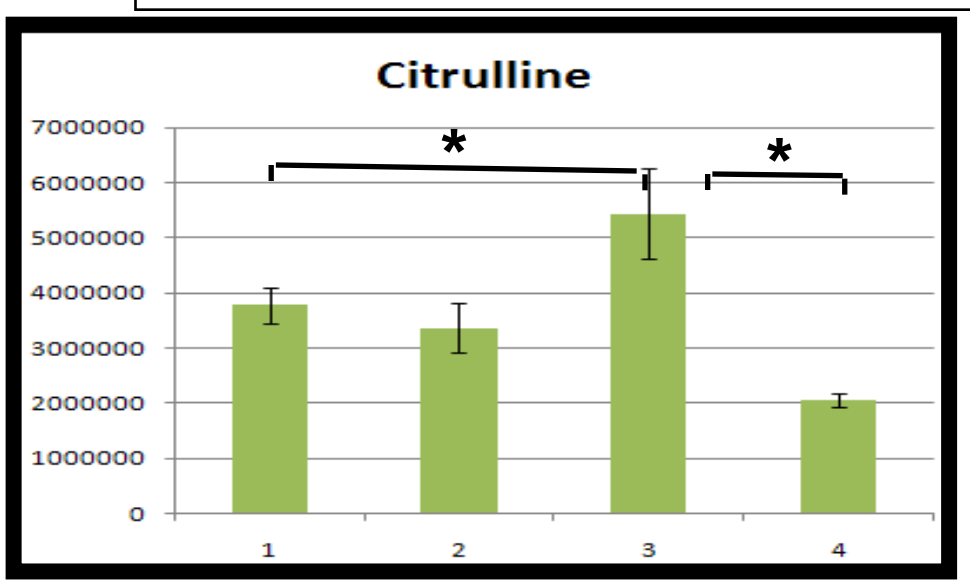


Figure 6. Suppression of vancomycin heteroresistance under anaerobic conditions is accompanied by reciprocal changes in metabolites of the arginine deiminase pathway in isolate B3. Statistically significant differences denoted by (*).

Conclusion

Taken together these studies indicate that S. aureus may utilize pathways of arginine catabolism in one of two ways: (1) under anaerobic conditions to support growth via the arginine deiminase pathway, (2) under anaerobic conditions, however, intermediates of arginine catabolism may be redirected to support vancomycin resistance. Knowledge of the specific metabolites/intermediates involved in supporting vancomycin resistance may identify novel chemotherapeutic targets aimed at preventing resistance outright.

References

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