Quantitative NMR Metabolite Profiling of Methicillin-Resistant and Methicillin-Susceptible \textit{Staphylococcus aureus} Discriminates between Biofilm and Planktonic Phenotypes

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ABSTRACT: Wound bioburden in the form of colonizing biofilms is a major contributor to nonhealing wounds. \textit{Staphylococcus aureus} is a Gram-positive, facultative anaerobe commonly found in chronic wounds; however, much remains unknown about the basic physiology of this opportunistic pathogen, especially with regard to the biofilm phenotype. Transcriptomic and proteomic analysis of \textit{S. aureus} biofilms have suggested that \textit{S. aureus} biofilms exhibit an altered metabolic state relative to the planktonic phenotype. Herein, comparisons of extracellular and intracellular metabolite profiles detected by $^1$H NMR were conducted for methicillin-resistant (MRSA) and methicillin-susceptible (MSSA) \textit{S. aureus} strains grown as biofilm and planktonic cultures. Principal component analysis distinguished the biofilm phenotype from the planktonic phenotype, and factor loadings analysis identified metabolites that contributed to the statistical separation of the biofilm from the planktonic phenotype, suggesting that key features distinguishing biofilm from planktonic growth include selective amino acid uptake, lipid catabolism, butanediol fermentation, and a shift in metabolism from energy production to assembly of cell-wall components and matrix deposition. These metabolite profiles provide a basis for the development of metabolite biomarkers that distinguish between biofilm and planktonic phenotypes in \textit{S. aureus} and have the potential for improved diagnostic and therapeutic use in chronic wounds.

KEYWORDS: \textit{Staphylococcus aureus}, biofilm, NMR metabolomics

INTRODUCTION

An estimated $58 billion in medical costs are associated with chronic wound complications afflicted over 18 million diabetics in the United States; 1 24% of diabetics can expect to undergo limb amputation within their lifetime as the result of a chronic, nonhealing wound. The social and economic burdens of these types of wounds are severe and growing rapidly.

While chronicity of the wound likely results from multiple factors such as dysfunctional circulation and compromised immunity, wound bioburden in the form of bacterial biofilm is a major contributing factor in the shift from acute to chronic wound. Bacterial biofilms are structured communities of cells that adhere to a surface and display phenotypic heterogeneity. In the case of the wound bed, the surface is biotic with the bacterial biofilm adhering to devitalized tissue. The chronic wound biofilm persists at the solid surface–air interface and is sustained via exudate seeping from the wound, resulting in a complex nutritional environment. Relatively little is known about the metabolism of these bacterial communities and whether there exists potential small molecule biomarkers associated with their metabolism that could be of diagnostic, prognostic, or therapeutic use.

One of the most common, opportunistic, bacterial colonizers found across multiple types of chronic wounds is the Gram-positive \textit{Staphylococcus aureus}, which can be either methicillin-resistant (MRSA) or methicillin-susceptible (MSSA). \textit{S. aureus} is a facultative anaerobe that can grow by utilizing either oxygen or nitrate for respiration or by mixed acid fermentation. Despite the prevalence of \textit{S. aureus} in chronic wounds, the basic physiology of this opportunistic pathogen is still poorly understood, especially with regard to the biofilm phenotype.

Within biofilms, bacterial cells can experience significant environmental heterogeneity, and these microenvironments appear to be related to virulence. It has been postulated that altered metabolism contributes to the higher tolerance of bacterial biofilms to therapeutic agents and, while biofilms have traditionally been regarded as metabolically dormant, recent transcriptomic and proteome analyses of \textit{S. aureus}...
biofilms indicate that cells within a biofilm have active, though altered, metabolic activity relative to planktonic growth.\textsuperscript{9a,12} These investigations are, however, distantly removed from direct detection of phenotype. In contrast, metabolomic analysis of small molecule metabolites present in both the extracellular and intracellular environments provides a more direct assessment of the defining characteristics of cellular phenotype.\textsuperscript{13} For example, Zhu and coworkers\textsuperscript{14} investigated the role of selective amino acid uptake by biofilms, suggesting that cells within a biofilm do not have ready access to external electron acceptors necessitating organic acid-producing fermentative strategies and that ammonia generation by arginine deiminase enzymatic activity offsets pH decreases due to accumulation of these organic acids. However, mutational analysis demonstrated that arginine deiminase is not essential for \textit{S. aureus} biofilm growth.\textsuperscript{14} How selective uptake of amino acids by the biofilm impacts biofilm physiology remains an open question, warranting further investigation.

Correlations between virulence and metabolism have been observed at the transcriptomic, proteomic, and metabolic levels for \textit{S. aureus}\textsuperscript{12,14,15} and strain-dependent differences in the biofilm forming capacity of \textit{S. aureus} have been demonstrated, suggesting a correlation between metabolic activity and pathogenicity;\textsuperscript{16} however, direct time course comparison of metabolic changes between strains that exhibit different virulence traits and between planktonic and biofilm growth conditions have not been performed. In the present study, we have utilized quantitative \textsuperscript{1}H NMR spectroscopy to detect and identify both intracellular and extracellular water-soluble small-molecule metabolites. The metabolic profiles of a methicillin-resistant and a methicillin-susceptible \textit{S. aureus} strain, grown both as biofilm and planktonic cell cultures over extended time periods, were characterized. The model biofilm culturing system used here mimics a chronic wound environment by growing the biofilm colonies at a solid surface–air interface with nutrients absorbed from growth media in a fashion similar to biofilms extracting nutrients from seeping exudate of a chronic wound.\textsuperscript{17} This culturing strategy is in stark contrast with previous metabolic comparisons between biofilm and planktonic cultures of \textit{S. aureus} that used a closed-system, flow-cell model of biofilm growth, in which oxygen exchange with air is limited.\textsuperscript{14}

The ability to readily quantify metabolites confers an advantage to NMR metabolomics\textsuperscript{18} and facilitates the use of unsupervised, orthogonal projection-based, statistical analyses such as principal component analysis (PCA).\textsuperscript{19} PCA analysis yields insights into metabolic relationships between different bacterial phenotypes without biasing the statistical clustering output of those phenotypes. Using PCA analysis, it was possible to differentiate between pathogenic (MRSA) and nonpathogenic (MSSA) strains of \textit{S. aureus} based on metabolite profiles. In addition, it was possible to distinguish between \textit{S. aureus} biofilm and planktonic phenotypes using PCA analysis of metabolite profiles in a complex growth medium. This study lays the groundwork for assessing the efficacy of therapeutic strategies based on small-molecule targets identified through metabolomics approaches for \textit{S. aureus} biofilm colonization of chronic wounds, while also gaining insights into metabolic strategies that characterize biofilm physiology.

### EXPERIMENTAL PROCEDURES

#### Bacterial Strains, Growth Conditions, and Sampling

Two phylogenetically distinct strains of \textit{S. aureus} were used in this study: the methicillin-resistant (MRSA) clinical wound isolate \textit{S. aureus} \textit{10943}\textsuperscript{17,20} and the methicillin-susceptible (MSSA) laboratory strain \textit{S. aureus} ATCC 6538.\textsuperscript{17,20b,c,21} Growth media for both planktonic and biofilm cultures consisted of tryptic soy broth (TSB) (Fluka Analytical). Inocula for both planktonic and biofilm growth conditions consisted of batch cultures grown in TSB at 37 °C to an optical density reading of 1.7 at 600 nm (\textit{OD}_{600nm}). Aliquots (1 mL) were collected for serial dilution, drop plating, and calculation of colony forming units (cfu).

For planktonic studies, inoculum cultures were diluted 1:100 in fresh TSB and cultured at 37 °C in 1 L flasks shaking at 150 rpm. Planktonic cultures were grown under aerobic conditions with flask-to-medium volume ratios of 3:1. Cultures (10 mL) of cells and supernatant were harvested every 2 h up to 12 h, then at 24 and 48 h post inoculation. Biofilm growth was cultured as previously described.\textsuperscript{17} In brief, tissue culture inserts (Millipore Millicell, 0.4 μm pore size) were inoculated with five 10 μL droplets of overnight inoculum culture (∼10\textsuperscript{8} cfu/mL) and grown for 72 h at 37 °C, at which point the biofilms had reached linear growth, which is referred to here as the T0 biofilm growth point.\textsuperscript{17} Although pore size on the tissue culture insert did not prevent bacterial cells from escaping into the growth medium in the well below, the biofilms constituted the primary growth phenotype for the cultures. To maintain biofilm viability, we refreshed growth media every 24 h. Once biofilms reached linear growth phase (T0), biofilms were collected every 24 h, up to 72 h (referred to as T24, T48, and T72 in the text). For each biofilm growth time point, spent supernatant was collected from the plate well, and biofilms were harvested from the insets by gently pipetting with 1 mL of sterile PBS to dislodge the biofilms and were immediately centrifuged at 4700 rpm for 10 min at 25 °C to pellet the cells. As with planktonic samples, biofilm supernatant and pellet were immediately flash-frozen in liquid nitrogen and stored at −80 °C. In addition, sham controls (i.e., TSB media only with no bacterial inoculation) were included on each plate to assay for leaching of plate materials into media as well as loss of volatile compounds from media due to culture conditions. For all growth conditions, samples were harvested in technical triplicates and repeated in biological duplicates.

#### NMR Sample Preparation

NMR samples were prepared from duplicate experiments with triplicate technical replicates for each growth condition and each time point. Metabolites were extracted as previously described.\textsuperscript{21a,22} Although leakage of intracellular metabolites into the extracellular environment has been reported for certain metabolism quenching procedures,\textsuperscript{23} limited loss of organic acids during sample preparation and statistical reliability across methods\textsuperscript{21a} indicated that the cold methanol extraction method is most suited for harvesting and extracting metabolites from our samples. In brief, supernatants were filtered through an extensively prewashed centrifuge filter (with sterile-filtered water) with a 3 kDa molecular weight cutoff (Millipore Amicon) prior to lyophilization overnight at room temperature. Cell pellets were washed in 60% ice-cold methanol (Sigma-Aldrich) and centrifuged at 5000 rpm for 10 min. Pellets were resuspended in a 2:1 ice-cold methanol/chloroform solution (Sigma-Aldrich) prior to cell lysis by sonication.
A 1:1 aqueous chloroform solution (Sigma-Aldrich) was added, samples were gently mixed, and aqueous layers were collected by centrifugation and transferred by pipetting to a clean microcentrifuge tube. Aqueous layer samples were lyophilized overnight at room temperature. Lyophilized samples were stored at −20 °C until further use. For 1H NMR, lyophilized samples were resuspended in 500 μL of NMR buffer (10 mM NaH2PO4/Na2HPO4 containing 0.25 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid [DSS] in 100% D2O, pH 7) and transferred to 5 mm Wilmad NMR tubes.

**NMR Analysis**

1H NMR spectra were acquired at 298 K (25 °C) on a Bruker 600-MHz (1H Larmor frequency) AVANCE III solution NMR spectrometer equipped with a SampleJet automatic sample loading system, a 5 mm triple resonance (1H, 15N, 13C) liquid-helium-cooled TCI probe (cryoprobe), and Topspin software (Bruker version 3.2). One-dimensional 1H NOESY experiments were performed using the Bruker supplied noesypr1d pulse sequence with 256 scans, 1H spectral window of 9600 Hz. FIDs were collected in 32K data points, with a dwell time interval of 52 μsec amounting to an acquisition time of ∼1.7 s, using a 2 s relaxation recovery delay between acquisitions and a NOESY mixing time period of 50 ms. Pulse sequence settings were based on standard recommendations by the Chenomx guide for recording 1D 1H NMR spectra of small molecule metabolites.

Spectral processing and analysis was performed using the Chenomx NMR software (version 7.6) (Chenomx). For each sample, NMR spectra were phased and baseline-corrected, and a line broadening function of 0.5 to 1.5 Hz was applied according to recommended Chenomx protocols and previously reported metabolomics analysis methods. Variable line broadening was applied to each sample to account for small field strength NMR fluctuations. 1H total correlation spectroscopy (TOCSY) spectra were transferred to 5 mm Wilmad NMR tubes.

Two phylogenetically distinct strains of *S. aureus* were grown under identical planktonic and biofilm conditions over time courses to comprehensively quantify metabolic differences between planktonic and biofilm phenotypes. *S. aureus* 10943 is a community-acquired, methicillin-resistant (CA-MRSA) clin-

**RESULTS**

### Separation of MRSA and MSSA *S. aureus* Strains Based on Metabolite Profiles

Two phylogenetically distinct strains of *S. aureus* were grown under identical planktonic and biofilm conditions over time courses to comprehensively quantify metabolic differences between planktonic and biofilm phenotypes. *S. aureus* 10943 is a community-acquired, methicillin-resistant (CA-MRSA) clin-

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ical isolate from a chronic wound. For comparison, the common, nonvirulent methicillin-susceptible (MSSA) laboratory strain *S. aureus* 6538 (Rosenbach) was also investigated. Phylogenetic separation of these two strains demonstrates that each strain inhabits distinct branches of the *S. aureus* genetic family tree and exhibits distinct degrees of virulence. *S. aureus* 10943 most closely aligns with CA-MRSA strain *S. aureus* USA300 TCH1516 as well as related MRSA strains such as *S. aureus* USA300 FPR3757 and *S. aureus* TW20. *S. aureus* 6538 most closely clusters phylogenetically to a related *S. aureus* Rosenbach strain, *S. aureus* ATCC 51811, as well as the MSSA laboratory strain *S. aureus* Newman (Figure 1A).26

![Figure 1](image)

Figure 1. Two phylogenetically distinct strains of *S. aureus* have unique pigmentation. (A) Phylogenetic separation of *S. aureus* 6538 and *S. aureus* 10943 indicates that *S. aureus* 10943 is most closely related to common CA-MRSA strains, while *S. aureus* 6538 is most closely related to non-MRSA strains. Blue lines indicate phylogenetic branches associated with methicillin resistance and red lines indicate phylogenetic branches associated with *S. aureus* species with no known antibiotic resistance. Black lines indicate parent phylogenetic branches.26 (B) Two phylogenetically dissimilar strains of *S. aureus* were inoculated onto tissue culture inserts and grown as biofilms in six-well tissue culture plates at 37 °C. Every 24 hours, feeder medium was refreshed within the plate well. Images represent mature biofilms that have been cultured a total of 72 h to reach linear growth phase (referred to as the T0 biofilm growth time point). Left panel depicts the chronic wound isolate, MRSA strain *S. aureus* 10943 and right panel depicts the lab-adapted, MSSA strain *S. aureus* "Rosenbach" ATCC 6538.

The two strains exhibited similar growth profiles. The planktonic cultures were grown under aerobic batch conditions in nonmodified TSB at 37 °C in 1 L shaking flasks. The biofilms were grown in an aerobic-modified tissue culture model that mimics growth conditions found in a chronic wound. While no visible growth differences between the strains were observed in the planktonic cultures, distinct pigmentation differences were noticeable in the biofilm cultures (Figure 1B). Pigmentation in *S. aureus* has been correlated to virulence. Thus, the observable difference in biofilm pigmentation between the strains (Figure 1B, right and left panels, respectively) corroborates with the known clinical significance of *S. aureus* 10943 as a MRSA strain.

Growth profiles for *S. aureus* 10943 and *S. aureus* 6538 batch cultures grown in TSB were similar based on optical density (OD<sub>600 nm</sub>) readings (Figure 2A) and transitioned through the growth phases at very similar rates, that is, exhibiting exponential growth between 2 and 6 h, transitioning to stationary phase between 8 and 10 h and maintaining stationary phase through the 48 h experimental time course. *S. aureus* 10943 and *S. aureus* 6538 biofilm cultures also exhibited very similar growth profiles with slightly higher optical densities (OD<sub>600 nm</sub>) for *S. aureus* 6538 biofilm at all time points; however, this OD<sub>600 nm</sub> difference was not statistically significant. The OD<sub>600 nm</sub> readings for all biofilm time points suggested the biofilms reached a linear growth phase beginning at the T0 biofilm time point (Figure 2A).

Metabolic transitions accompanying different growth phases in planktonic cultures correlated with glucose availability. Glucose concentrations were measured by quantitative 1H NMR for each sample at each time point; the values correspond to the extracellular glucose concentrations (mM) normalized to the viable cell counts (log or absolute number). TSB medium glucose concentration was measured to be 19.24 ± 0.99 mM. The transition from exponential growth to stationary phase was characterized by the complete exhaustion of glucose, which occurred within 8 h of inoculation (Figure 2B). During biofilm growth experiments both strains also exhibited nearly identical profiles for glucose consumption with the glucose being completely consumed within each 24 hour time point, that is, before the biofilm medium was refreshed (Figure 2B).

Both *S. aureus* 10943 and *S. aureus* 6538 planktonic cultures exhibited a drop in medium pH during exponential growth (Figure 2C). For example, the extracellular pH drops from pH 7.13 at inoculation to a pH of 5.21 at stationary phase and from pH 7.17 to pH 5.96 for *S. aureus* 10943 and *S. aureus* 6538, respectively. In contrast with the planktonic cultures, the biofilm cultures exhibited increased bulk pH levels in the spent medium; the *S. aureus* 10943 medium increased from an initial pH of 7.67 to pH 8.24 and *S. aureus* 6538 medium increased from pH 7.18 to 7.99.

Intracellular and extracellular metabolites were identified and quantified for both *S. aureus* 10943 and *S. aureus* 6538 strains grown under planktonic and biofilm conditions. Samples were collected over an extended time course ranging from exponential growth to late-stationary phase for the planktonic cultures and every 24 hours, up to 72 h, for the biofilm cultures, which demonstrated a linear growth phenotype. Metabolism was quenched and metabolites extracted using an ice-cold aqueous methanol/sonification protocol, as described in the Experimental Procedures. Extracted metabolites were identified and quantified using 1D 1H NMR, and NMR spectral features were assigned to particular metabolites by spectral pattern fitting to reference spectra of small-molecule metabolites annotated in the Chenomx (version 7.6) metabolomics database. Of the greater than 300 verified compounds in the Chenomx library, more than 120 water-soluble compounds from the sample supernatant and 40 water-soluble compounds...
from cell pellets were identified in at least one sample from at least one growth condition.

Differences in the two phylogenetically distinct *S. aureus* strains 10943 and 6538, grown both as biofilm cultures and planktonic cultures were distinguished based on PCA of their respective metabolite profiles. PCA utilizes orthogonal transformation of correlated metabolite profiles into linearly uncorrelated PCs to separate samples according to distinct patterns, and provides a quantitative basis for distinguishing the *S. aureus* strains (Figure 3A–D). Both the MRSA (i.e., *S. aureus* 10943) and MSSA (i.e., *S. aureus* 6538) strains were separated along the principal component 1 (PC1) axis for both the intracellular and extracellular metabolite profiles of the planktonic cultures as well as the extracellular metabolite profiles of the biofilm cultures (Figure 3A–C, respectively). PC1 accounted for ~50% of the statistical variability within the samples, suggesting the two strains used distinguishably different metabolic strategies; however, the biofilm intracellular metabolite profiles clustered nearly identically (Figure 3D).

**Quantitative Metabolite Profiles Can Distinguish between *S. aureus Biofilm Growth and Planktonic Cell Cultures in Phylogenetically Distinct Strains of S. aureus***

PCA comparison of both intracellular and extracellular metabolite profiles for both strains was analyzed concomitantly (Figures 4 and 5) to establish whether biofilm and planktonic phenotype of *S. aureus* can be quantitatively distinguished based on metabolic differences, irrespective of strains and growth stages (i.e., exponential, stationary, linear). PCA analysis of intracellular or extracellular metabolite profiles resulted in biofilm samples for both strains clustering into a single quadrant with no overlap of biofilm phenotype with planktonic phenotype (Figures 4 and 5). Because of biofilm metabolite profiles segregating into a single quadrant (lower, left quadrant, Figures 4 and 5), the combination of PC1 and PC2 identified those metabolites for which correlation coefficients are most significantly associated with the biofilm phenotype of *S. aureus* (Tables 1 and 2). As is conventional for many biological analyses, a 0.4 threshold for the correlation coefficients was used here to identify the most significant metabolite contributors to the PCA variations. In addition, correlation coefficient values in bold indicate which metabolites are most significantly related to the PCs from which 2D PCA scores plot are built, further highlighting the statistical significance of these metabolites as contributing variables for sample distinction (Tables 1 and 2).

Amino acid profiles suggest that distinct selective amino acid uptake may be a key feature differentiating between biofilm and planktonic cultures in *S. aureus*, regardless of strain. Interestingly, PCA factor loadings analysis demonstrated that distinct amino acid profiles for both intracellular and extracellular pools of metabolites contribute significantly to the separation of the biofilm samples for both strains, including amines such as arginine (Table 1), hydroxy acids such as serine (Table 1), amido acids such as asparagine (Tables 1 and 2), and aromatic amino acids such as histidine (Table 2). Arginine metabolism has been suggested to play an important role in biofilm survival and the factor loadings analysis conducted here suggests that arginine catabolism is an important feature of the biofilm phenotype (Table 1). Indeed, amino acid catabolism may be a significant component of biofilm metabolism, as multiple metabolites associated with amino acid degradation were identified in at least one sample from at least one growth condition.
acid degradation contribute significantly to the statistical separation of the biofilm cultures in the PCA scores plots shown here, including metabolites associated with alanine, aspartate, cysteine, isoleucine, methionine, serine, threonine,
Tables 1 and 2.) The correlation coefficient numbers in bold indicate metabolites for which the statistical relatedness of the variable is most correlated to the PC, as indicated by the square of the cosine.

and histidine metabolic pathways (Tables 1 and 2). Whether *S. aureus* biofilms are selectively utilizing amino acids or catabolizing whichever small molecule is most available remains to be established. The PCA factor loadings analysis suggests that biofilms opt for the latter option because metabolites associated with catabolism of other amino acids, such as glycine, tryptophan, and lysine, do not contribute significantly to the separation of the biofilm samples on the PCA scores plots. (See Tables 1 and 2.)

Secondary energy sources also appear to be important in distinguishing the biofilm phenotype from its planktonic counterpart. Both intracellular and extracellular metabolites associated with lipid catabolism such as glycerol (Table 2), glycerate (Table 2), malonate (Table 2), and propionate (Table 1) contribute to the statistical separation of the biofilms into a single quadrant of the PCA scores plots. Malonate is also a product of pyrimidine degradation in some bacteria. While genes for this pathway have been sequenced in staphylococcus species, a functional pathway has yet to be demonstrated; however, in support of a role for purine and pyrimidine catabolism in biofilm formation, multiple metabolites associated with catabolism of these molecules were identified as contributing to the discrimination between biofilm and planktonic phenotypes (Tables 1 and 2). In addition, pyrimidine nucleotides serve as precursors for synthesis of ticoic acids and peptidoglycan in *S. aureus*, and may indicate biofilm metabolic investment in cell-wall synthesis and matrix deposition.

In addition, biofilms may effectively utilize alternative carbohydrate metabolic pathways once glucose has been consumed because hexose catabolism of galactose and mannose contributes to biofilm separation (Table 2). The correlation coefficient for acetoin indicates that this ketone contributes significantly to distinction between biofilms and planktonic cultures irrespective of *S. aureus* strains and highlights the importance of butanediol fermentation in biofilms, as has been suggested.14

Other studies have suggested that upregulation of glycolysis in biofilms is not directed toward production of energy but instead directs metabolic flux to other metabolic pathways engaged in the production of cell-wall components and matrix deposition.12 To this effect, correlation coefficients for metabolites associated with cell-wall synthesis such as N-acetylglucosamine and UDP-N-acetylgulosamine are shown here to contribute significantly to segregating the biofilm samples into a single quadrant of the PCA scores plot (Figure S).

*S. aureus* Biofilm Phenotype Is Distinguished Both by Adaptable Energy Production and Investment into Small Molecules Important for Sessile Survival

Using the information embedded in correlation coefficients of key metabolites, PCA score plots indicated which metabolic activities might most distinguish the biofilm phenotype from its planktonic counterpart (summarized in Table 6). Glucose consumption by the biofilm suggests that while glucose is available, glycolysis is active (Figure 2B); however, once glucose is consumed, the biofilms appear to readily switch to alternative energy sources. As noted by others,14 relatively high intracellular concentrations of acetoin, a metabolic precursor to 2,3-butanediol, indicate that butanediol fermentation is part of a mixed acid fermentation strategy employed by the biofilms.

The fermentative metabolite profile suggests that microaerobic and anaerobic environments exist within the biofilm, as has been previously suggested.12,14,21 Also as previously reported,14,21,14 selective uptake of amino acids may differentiate between biofilm and planktonic cultures; however, in our study, the patterns of intracellular and extracellular amino acids are most significantly correlated with amino acid fermentation through the Stickland reaction,14 in which one amino acid serves as an electron donor and one amino acid serves as an electron acceptor. For example, both *S. aureus* 10943 and *S. aureus* 6538 biofilms selectively transported isolucine, an electron donor, into the cytosol, while concomitantly increasing intracellular pools of sarcosine, an electron acceptor, as well as secreting relatively high levels of.

Table 1. Factor Analysis for Intracellular Metabolites

| metabolite          | PC1   | PC2   |
|---------------------|-------|-------|
| acetate             | 0.962 | −0.027|
| creatine            | 0.961 | −0.038|
| caprylate           | 0.955 | −0.024|
| acitone             | 0.933 | −0.042|
| formate             | 0.928 | −0.082|
| 2-hydroxybutyrate   | 0.876 | 0.024 |
| choline             | 0.866 | 0.084 |
| betaine             | 0.858 | 0.156 |
| acetoin             | −0.828| 0.124 |
| lactate             | 0.811 | 0.023 |
| methanol            | 0.797 | −0.139|
| glutamate           | 0.790 | 0.023 |
| alanine             | 0.743 | 0.232 |
| ornithine           | 0.725 | 0.355 |
| succinate           | −0.713| 0.237 |
| aspartate           | −0.584| 0.171 |
| arginine            | −0.458| 0.713 |
| acetocetate         | −0.450| 0.247 |
| leucine             | −0.442| 0.746 |
| glutamine           | −0.433| 0.752 |
| creatinine          | −0.389| 0.595 |
| isovalerate         | −0.367| 0.537 |
| 2-hydroxyisovalerate| −0.364| 0.695 |
| glutarate           | −0.361| 0.569 |
| 3-hydroxybutyrate   | −0.344| 0.662 |
| 4-aminobutyrate     | −0.322| 0.581 |
| isobutyrate         | −0.322| 0.611 |
| serine              | −0.290| 0.512 |
| cystathionine       | −0.289| 0.631 |
| asparagine          | −0.289| 0.629 |
| trans-aconitate     | −0.285| 0.604 |
| threonate           | −0.261| 0.439 |
| propionate          | −0.243| 0.481 |
| 2-hydroxylglutarate | −0.222| 0.466 |
| creatine phosphate  | −0.222| 0.466 |
| isoctirate          | −0.222| 0.466 |
| sarcosine           | −0.222| 0.466 |
| succinylacetone     | −0.222| 0.466 |
| 3-hydroxyphenylacetate| −0.203| 0.503 |
| acetamide           | −0.203| 0.503 |
| percent total variance | 30.37%  | 17.44% |
| cumulative percent variance | 47.81% |
### Table 2. Factor Analysis for Extracellular Metabolites

| metabolite                  | correlation coefficient<sup>a,b</sup> | metabolite                  | correlation coefficient<sup>a,b</sup> |
|-----------------------------|----------------------------------------|-----------------------------|----------------------------------------|
|                            | PC1       | PC2       | PC1       | PC2       |
| 4-aminobiphturate           | 0.948     | 0.259     | trehalose | 0.502     | 0.380     |
| uridine                    | 0.947     | 0.281     | serotonin| 0.474     | 0.049     |
| choline                    | 0.947     | 0.257     | urea      | 0.468     | 0.071     |
| 2-hydroxybutyrate          | 0.945     | 0.273     | N6-acetyllysine | 0.465     | 0.044     |
| isopropanol                | 0.941     | 0.267     | isobutyrte | 0.464     | −0.242    |
| GTP                        | 0.930     | 0.268     | glutarate | 0.449     | −0.258    |
| 5-hydroxynindole-3-acetate | 0.894     | 0.292     | asparagine | −0.446    | 0.832     |
| isocaprate                 | 0.892     | 0.206     | alanine   | −0.445    | 0.849     |
| cysteine                   | 0.890     | 0.288     | nicotinurte | 0.442     | 0.766     |
| NADH                       | 0.887     | 0.302     | glycerol  | −0.438    | 0.828     |
| dimethylamine              | 0.882     | 0.274     | threonine | −0.437    | 0.742     |
| 2-deoxyinosine             | 0.881     | 0.265     | O-phosphocholine | 0.434     | 0.021     |
| citrate                    | 0.880     | 0.156     | sn-glycero-3-phosphocholine | 0.432     | 0.070     |
| glycine                    | 0.878     | 0.067     | propylene glycol | −0.422    | 0.057     |
| ethanolamine               | 0.874     | 0.296     | 2-hydroxyisobutyrte | −0.418    | 0.789     |
| putrescine                 | 0.865     | 0.225     | serine    | −0.396    | 0.705     |
| AMP                        | 0.863     | 0.081     | lysine    | 0.387     | 0.832     |
| 2-aminoobutyrte            | 0.845     | 0.198     | thymine   | 0.381     | −0.523    |
| NADPH+                     | 0.824     | 0.206     | leucine   | −0.374    | 0.862     |
| NADPH                      | 0.818     | 0.276     | glutamine | −0.349    | 0.677     |
| sucrose                    | 0.817     | 0.299     | proline   | −0.346    | 0.672     |
| O-phosphoethanolamine      | 0.816     | 0.291     | 4-hydroxyphenylactate | 0.337     | −0.437    |
| arginine                   | 0.803     | 0.038     | aspartate | −0.326    | 0.879     |
| nicotinate                 | −0.803    | 0.150     | riboflavin | −0.307    | 0.740     |
| creatinine                 | 0.801     | 0.226     | anserine  | −0.249    | 0.539     |
| methanol                   | 0.797     | 0.252     | phenylalanine | −0.238    | 0.753     |
| myo-inositol               | 0.792     | 0.273     | hypoxanthine | −0.229    | 0.503     |
| O-acetylcholine            | 0.788     | −0.027    | tryptophan | 0.221     | 0.701     |
| carnitine                  | 0.781     | 0.237     | cystine   | −0.210    | −0.632    |
| butyrate                   | 0.780     | 0.264     | glycerate | −0.205    | −0.680    |
| ethanol                    | 0.748     | 0.186     | phenylacete | −0.203    | −0.636    |
| 5-hydroxytryptophan        | −0.739    | 0.603     | N-acetylgucosamine | −0.202    | −0.578    |
| N-acetylseryotonin         | −0.739    | 0.603     | homocysteine | −0.200    | −0.591    |
| 4-aminoobutyrte            | −0.723    | 0.552     | 2-‘-deoxyadinosine | −0.199    | 0.757     |
| trimethylamine N-oxide     | −0.719    | 0.277     | uracil    | −0.196    | −0.540    |
| ATP                        | 0.710     | 0.276     | carnosine | −0.190    | −0.543    |
| oxypurinol                 | 0.698     | 0.262     | cystathionine | −0.184    | −0.493    |
| urocanate                  | −0.692    | 0.000     | malonate  | −0.182    | −0.479    |
| gluconate                  | 0.685     | 0.134     | maleate   | 0.116     | 0.911     |
| betaine                    | −0.675    | 0.561     | isovalerate | −0.114    | −0.666    |
| methionine                 | −0.674    | 0.552     | S-adenosylhomocysteine | −0.110    | 0.829     |
| creatine phosphate         | 0.669     | 0.160     | valine    | −0.107    | 0.516     |
| glutathione                | 0.662     | 0.014     | pyroglutamate | −0.096    | 0.931     |
| sarcosine                  | 0.661     | 0.071     | biotin    | −0.086    | −0.463    |
| glutamate                  | 0.655     | −0.026    | 2-phenylpropanate | −0.086    | −0.463    |
| pyridoxine                 | −0.652    | 0.627     | galactose | −0.086    | −0.463    |
| ornithine                  | 0.643     | 0.113     | 4-pyridoxate | −0.086    | −0.463    |
| lactate                    | 0.643     | 0.119     | UDP-N-acetylgucosamine | −0.086    | −0.463    |
| succinate                  | 0.633     | 0.122     | 2-hydroxy-3-methylvalerate | −0.086    | −0.463    |
| tyrosine                   | −0.623    | 0.266     | 3-aminobutyrate | −0.086    | −0.463    |
| ADP                        | 0.607     | 0.067     | mannose   | −0.086    | −0.463    |
| O-phosphoserine            | −0.598    | 0.667     | nicotinic acid adenine dinucleotide | −0.086    | −0.463    |
| acetoine                   | −0.575    | 0.672     | xanthosine | −0.086    | −0.463    |
| N-acetyltirosine           | 0.560     | 0.055     | 6-hydroxynicotinate | −0.086    | −0.463    |
| melatonin                  | 0.559     | 0.083     | cholate   | −0.086    | −0.463    |
| 3-methylxanthine           | 0.551     | 0.209     | galactitol | −0.086    | −0.463    |
| methylsuccinate            | 0.544     | −0.029    | trans-4-hydroxy-L-proline | −0.086    | −0.463    |
| isoleucine                 | −0.544    | 0.719     | glucose   | −0.070    | 0.733     |
ammonia (data not shown), a byproduct of amino acid catabolism. This suggests that amino acid catabolism serves as an important source of energy for the biofilm phenotype and that amino acid uptake may not be specific, as previously suggested, but rather is an adaptive strategy to environmental conditions and nutrient availability, a finding consistent with other data.

It has been hypothesized that S. aureus biofilms adapt to strongly reduced conditions through production of reduced organic acids like lactate and alcohols including butanediol. While butanediol fermentation was observed in both S. aureus 10943 and S. aureus 6538 biofilms (Figures 4 and 5), additional intracellular metabolites of importance to the PCA analysis included compounds associated with poly-β-hydroxybutyrate (PHB) synthesis and degradation. To regulate NADH and NAD+ levels, PHB serves as both an electron and carbon sink in bacteria. Relatively high intracellular pools of 3-hydroxybutyrate and acetoacetate, metabolites associated with

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### Table 2. continued

| metabolite        | PC1     | PC2     | metabolite        | PC1    | PC2     |
|-------------------|---------|---------|-------------------|--------|---------|
| histidine         | −0.530  | 0.595   | 3-hydroxisovalerate| 0.033  | −0.618  |
| γ-methylhistidine | −0.529  | 0.731   | adenosine         | 0.020  | 0.673   |
| percent total variance |         | | cumulative percent variance |          | 30.37%  | 21.73%  |

“Significance of correlation coefficient set at threshold of 0.4. Correlation coefficient numbers in bold indicate metabolites for which the statistical relatedness of the variable is most correlated to the PC as indicated by the square of the cosine.”

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Figure 6. Schematic representation of central metabolism and secondary metabolic activity in S. aureus characteristic of the biofilm phenotype. Bar charts represent fold changes in metabolite concentrations for biofilm and stationary planktonic samples, respectively, normalized to the metabolite concentrations in each respective exponential planktonic culture for select intracellular and extracellular biofilm metabolites that contribute to statistical separation of the biofilm phenotype from the planktonic phenotype irrespective of strain. S. aureus 10943 is indicated by blue bars and S. aureus 6538 is indicated by red bars. Dark bars indicate planktonic samples and light bars indicate biofilm samples. Data shown are representative samples from duplicate experiments.

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PHB synthesis and degradation, were measured as S. aureus maintains a favorable redox balance through appropriate NADH to NAD+ ratios (Tables 1 and 2).

Finally, in the biofilm model used here,17 the biofilms reach a linear phase and do not significantly increase in biomass over time (Figure 2A); however, the biofilms Consumate and secrete significant amounts of metabolites, suggesting a very dynamic metabolic state. As previously proposed, the output of this metabolic activity may be cell-wall synthesis in response to cellular turnover and deposition of EPS components.12b,33 In support of this hypothesis, the PCA analyses shown here (Figures 4 and 5) indicate that alternative hexose utilization contributes significantly to the statistical separation of the biofilm and planktonic phenotypes. While hexose sugars can be catabolized via glycolysis, both mannose and galactose may be channeled into the production of rhamnose, a component found in exopolysaccharide repeating units.34

Cell-wall synthesis entails the building of murein monomers from precursors UDP-N-acetylglucosamine and UDP-N-acetylMuramylate, both synthesized in the cytoplasm of S. aureus.35 Both N-acetylglucosamine and its precursor UDP-N-acetylglucosamine were metabolites for which PCA correlation coefficients indicated that secretion of these metabolites is an important characteristic of both S. aureus 10943 and S. aureus 6538 biofilm phenotypes (Table 2). While selective uptake of alanine by the biofilms may be important for amino acid fermentation via the Stickland reactions, as previously mentioned, it may also play a significant role as a precursor for the synthesis of cell-wall components as well as EPS deposition. These results suggest that flexible metabolic activity specific to the biofilms not only includes the previously reported energy production from mixed acid fermentation and TCA activity but also involves significant energy expenditure for maintenance of a proper redox balance, synthesis of cell-wall components, and EPS matrix deposition (Figure 6).

DISCUSSION

Nonhealing of wounds such as diabetic and pressure ulcers is, in part, due to the persistence of bacterial biofilm-based infections;4,10,36 however, metabolic contributions to persistence of the bacteria in the wound remain an area of investigation.37 Specifically, there is interest in identifying small-molecule biomarkers that distinguish between biofilm and planktonic phenotypes, which could be used as a noninvasive, prognostic tool indicating bacterial biofilm colonization in a wound.20b,36 In addition, such profiles could provide insights into physiological differences between biofilm and planktonic cell cultures, which could be exploited to therapeutic advantage. It has been speculated that pathogenicity in bacteria is the result of an evolutionary drive to obtain nutritional resources.38 While traditional antibiotics have been designed against planktonically grown bacteria and to treat metabolically active bacteria, bacteria in a biofilm are metabolically different from planktonic bacteria,39 therefore, the design of novel therapies for wounds necessitates considering and accounting for the unique ability of the biofilm to resist treatment, especially through adaptive metabolic changes. Because of the correlation between chronicity in wounds and bacterial biofilm contamination, a number of biofilm-targeted antimicrobials have emerged including the iron-binding innate immune molecule lactoferrin;40 however, metabolic characterization of the biofilm phenotype has the potential to uncover many other biofilm-specific targets for the development of novel wound therapeutics.

Previous transcriptomics and proteomics analyses of S. aureus biofilm and planktonic cultures12,14 demonstrated that, when grown as a biofilm, this facultative anaerobe switches to fermentative metabolism within the biofilm. Consistent with these observations, our data demonstrate that for both strains of S. aureus anaerobic metabolism is induced in the biofilm cultures and suggest that mixed acid fermentation is active, contributing to a biomarker profile that distinguishes between biofilm and planktonic phenotypes.

Selective amino acid uptake profiles have been reported for S. aureus biofilm cultures when compared to planktonic cultures.14,21a While statistical PCA analysis of the biofilm and planktonic cultures did identify both intracellular and extracellular metabolite patterns that distinguished between the biofilm and planktonic phenotypes, regardless of strain type, these profiles did not exactly match those previously reported.14,21a Whereas Zhu and coworkers4 reported selective uptake of glutamine, serine, proline, glycine, threonine, and arginine, and Wu and coworkers21a reported selective uptake of arginine, we observed metabolic trends that distinguish between biofilm and planktonic phenotype based on differential intracellular pools of arginine, aspartate, glutamine, leucine, and serine and extracellular pools of alanine, asparagine, histidine, isoleucine, methionine, threonine, and tyrosine. These results suggest that while amino acid metabolism by the biofilm is important, the specific profile of amino acids transported into the cytosol is not a discriminant in and of itself of biofilm versus planktonic modes of growth.

Metabolic discrimination of biofilms independent of strain type is due in part to different intracellular concentrations of amino acids that function as electron donors (alanine, leucine, isoleucine, histidine) and electron acceptors (leucine and sarcosine) for amino acid fermentation by the Stickland reaction.30 These data suggest that while selective amino acid uptake profiles do indicate significant amino acid utilization by S. aureus in response to redox needs, these profiles may not be specific to any particular set of amino acids and may rather reflect the opportunistic scavenging of the bacteria for whichever electron donor or acceptors may most readily be available.

Furthermore, metabolic discrimination of biofilms as established by PCA analysis of different metabolite expression patterns suggests that S. aureus biofilms might utilize secondary metabolic pathways to address redox stress. For example, when oxygen is limited, the synthesis of poly-β-hydroxybutyrate (PHB) could serve as an efficient electron sink. Degradation of PHB would contribute to cellular redox balance by reducing NAD2 to NADH concomitantly with the breakdown of PHB to acetocoeate and entry of acetate into the TCA cycle (Figure 6).

Although PHB was detected in S. aureus nearly 50 years ago,41 the complete metabolic pathway for both synthesis and degradation of this polymer has yet to be firmly established in S. aureus. Putative S. aureus genes for PHB biosynthesis and degradation have been identified and annotated to be associated with virulence; however, demonstration of a functional PHB metabolic pathway in S. aureus remains to be accomplished.32 The question of whether S. aureus biofilms selectively utilize this pathway as a mechanism to maintain an appropriate intracellular redox balance warrants further investigation.
Consistent with previous observations,12a,b metabolic investment in synthesis of cell-wall components and EPS deposition contributes significantly to the statistical separation of the biofilm and planktonic phenotypes in the PCA analysis (Figures 4 and 5). Despite having reached linear growth phase, the biofilm cultures exhibit significant central metabolism activity without a significant increase in the number of viable cells. Strain-independent PCA statistical grouping of metabolite without a significant microbial susceptibility to antibiotics and cell-wall disrupting acids play a major role in host-pathogen interactions and alterations in cell-wall components can significantly affect microbial susceptibility to antibiotics and cell-wall disrupting agents.33

CONCLUSIONS

While identification of a single, robust small molecule biomarker that distinguishes between biofilm and planktonic cultures would have significant translational research appeal for clinical diagnostics, it is unlikely that such a universal metabolite biomarker exists despite encouraging data to the contrary.20b Indeed, the NMR metabolomics analysis presented here indicates that even within a single bacterial species significant differences in metabolite patterns can be observed for both biofilm and planktonic phenotypes. Despite the complexity of such an analysis and by using a comprehensive experimental approach that included phylogenetically distinct strains of S. aureus and metabolite sampling through time courses that account for multiple growth phases, we have demonstrated herein the ability to distinguish biofilm from planktonic cultures based on distinct metabolite profiles. While the NMR metabolomics approach presented is robust, the research strategy incorporated only two strains of a single species; it will be of significant interest to explore whether comparable metabolite profile analyses can distinguish between biofilms and planktonic cultures of other Gram-positive opportunistic pathogens, as well as Gram-negative pathogens and potentially mixed species biofilms. In addition to demonstrating the use of global metabolite profiling for discriminating between S. aureus biofilm and planktonic cultures, the contribution of metabolite variables to the statistical separation of the biofilm phenotype from its planktonic counterpart in the PCA analyses presented here sheds light on some tantalizing areas of bacterial metabolism for further investigation and indicates how little is known about the physiology and metabolic characteristics of this important common, opportunistic pathogen.

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Notes

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ABBREVIATIONS

CA-MRSA, community-acquired methicillin-resistant Staphylococcus aureus; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; EPS, extracellular polymeric substance; MRSA, methicillin-resistant Staphylococcus aureus; MSSA, methicillin-susceptible Staphylococcus aureus; NOESY, nuclear Overhauser enhancement spectroscopy; PC, principal component; PCA, principal component analysis; PHB, poly-β-hydroxybutyrate; TCA, tricarboxylic acid; TSB, tryptic soy broth

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