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Drug-Resistant *Staphylococcus aureus* Strains Reveal Distinct Biochemical Features with Raman Microspectroscopy

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Abstract

Staphylococcus aureus (*S. aureus*) is a leading cause of hospital-acquired infections, such as bacteremia, pneumonia, and endocarditis. Treatment of these infections can be challenging since strains of *S. aureus*, such as methicillin-resistant *S. aureus* (MRSA), have evolved resistance to antimicrobials. Current methods to identify infectious agents in hospital environments often rely on time-consuming, multistep culturing techniques to distinguish problematic strains (i.e., antimicrobial resistant variants) of a particular bacterial species. Therefore, a need exists for a rapid, label-free technique to identify drug-resistant bacterial strains to guide proper antibiotic treatment. Here, our findings demonstrate the ability to characterize and identify microbes at the subspecies level using Raman microspectroscopy, which probes the vibrational modes of

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Author Contributions

The authors contributed equally for the preparation of this manuscript.

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A summary of *S. aureus* strains evaluated using Raman microspectroscopy; the differences in *S. aureus* pigmentation for the strains studied (PDF)

molecules to provide a biochemical “fingerprint”. This technique can distinguish between different isolates of species such as *Streptococcus agalactiae* and *S. aureus*. To determine the ability of this analytical approach to detect drug-resistant bacteria, isogenic variants of *S. aureus* including the comparison of strains lacking or expressing antibiotic resistance determinants were evaluated. Spectral variations observed may be associated with biochemical components such as amino acids, carotenoids, and lipids. Mutants lacking carotenoid production were distinguished from wild-type *S. aureus* and other strain variants. Furthermore, spectral biomarkers of *S. aureus* isogenic bacterial strains were identified. These results demonstrate the feasibility of Raman microspectroscopy for distinguishing between various genetically distinct forms of a single bacterial species *in situ*. This is important for detecting antibiotic-resistant strains of bacteria and indicates the potential for future identification of other multidrug resistant pathogens with this technique.

Graphical Abstract

Keywords

Staphylococcus aureus; Raman microspectroscopy; drug-resistant bacteria; identification; principal component analysis; quadratic discriminant analysis

Healthcare-associated infections (HAIs), i.e., infections that patients obtain while receiving medical or surgical treatment in a healthcare facility, are a major threat to patient health. They have led to high rates of morbidity and mortality and annual healthcare costs of nearly \$45 billion.¹ The most common HAIs in U.S. acute care hospitals include but are not limited to pneumonia, surgical site infections, bloodstream infections, and gastrointestinal illness.² Among surgical complications, wound infections account for 29% of these events and over 10% of all adverse events.³

Infections caused by *Staphylococcus aureus* (*S. aureus*) are of particular interest since they are a major burden to U.S. hospitals specifically for high-risk patients. In one study, hospitalized patients with an *S. aureus* infection experienced three times the length of hospital stay, three times the amount of total charges, and five times the risk of in-hospital death compared to patients that did not have this type of infection.⁴ The development of antibiotic-resistant strains such as methicillin-resistant *S. aureus* (MRSA) further contribute to this growing problem by creating an additional barrier against treatment. Methicillin resistance is mediated by the staphylococcus cassette chromosome (SCC mec), which encodes for penicillin binding protein PBP2a.^{5,6} Although the number of nosocomial infections by *S. aureus* increased by 62% per year, the number of MRSA infections in hospitals increased by 119% per year from 1999 to 2005.⁷ This rapid increase in antibiotic

resistance motivates the need of pathogen surveillance for early detection of outbreaks in hospital and community associated infections.

One subpopulation of bacteria that causes persistent infections in patients with acute and chronic illnesses are bacterial variants of *S. aureus* called small-colony variants (SCVs). These SCVs display increased tolerance to particular classes of antibiotics such as aminoglycosides and therefore often arise in response to therapeutic intervention.⁸ These SCVs, which take on fastidious growth requirements, present with a phenotype that is different from their parent strain characterized by colonies with decreased pigmentation that are approximately one-tenth the size of wildtype *S. aureus* colonies.

The current approach for detecting and identifying bacterial strains like *S. aureus* in clinical samples involves a multistep process to evaluate specific targets of the sample. Typically, samples are cultured on blood agar and characterized on the basis of morphological features, as seen through microscopy, and biochemical tests that evaluate biosynthesis pathways. Identification of bacteria at the species level and determination of antibiotic susceptibility requires additional targeted biochemical testing (e.g., catalase test, coagulase test, and deoxyribonuclease (DNase) test to identify *S. aureus*) since specificity is critical for proper diagnosis and treatment. These techniques are time-consuming due to incubation times and subsequent manual tests requiring interpretation of biochemical reactions. In addition to atypical colony morphologies, SCVs require extended culture periods and various biochemical assays to accurately detect and identify. These tests, which evaluate the response from various biochemical reactions, may be difficult to interpret due to altered metabolic pathways of SCVs.⁹ Furthermore, the gold standard for determining whether bacteria are susceptible/resistant to specific antimicrobials is through antimicrobial susceptibility testing (AST), which evaluates the isolated bacteria with various antimicrobials for growth inhibition. Specific strategies that promote growth and minimize reversion to normal colony phenotypes have also been developed to test AST from *S. aureus* SCVs, commonly seen in chronic infections.¹⁰ Again, these approaches can be time-consuming (days to weeks), involve bacterial cultures that have limited growth, and are challenging to interpret especially for mutant strains. More recently, a custom-built automated dark-field microscope was used to acquire time-lapse images of bacteria flowing in a multichannel fluidic cassette to evaluate phenotypical parameters to identify bacterial pathogens from patients affected by ventilator-associated pneumoniae¹¹ and characterize various *S. aureus* strains associated with antibiotic resistance and susceptibility.¹² Although this approach obtained 100% sensitivity and 97% specificity for detection of high-risk pneumoniae organisms, it required expert image interpretation to analyze antibiotic susceptibility testing.

To obtain near 100% specificity for bacterial identification, researchers routinely rely on polymerase chain reaction (PCR) to amplify deoxyribonucleic acid (DNA) from bacteria.¹³ However, PCR is dependent on target genes, that may not be available for specific bacterial mutants, is vulnerable to contamination, and is not able to distinguish between live versus dead bacteria in a clinical sample.¹⁴ These factors negatively affect the sensitivity of PCR for accurate identification of bacteria.

Optical techniques such as fluorescence spectroscopy have previously been used for detecting bacteria in a saline solution.¹⁵ Although the authors report identification of *S. aureus*, fluorescence fingerprints of *S. aureus* and other strains presented overlapping features. More recently, a similar study evaluated fluorescence spectroscopy for characterization of clinically important bacteria, including *S. aureus*, and found that tryptophan could be used for detecting bacteria but not for specifically identifying *S. aureus*.¹⁶ Fluorescence spectroscopy is unable to provide specific biochemical features important for discrimination of bacteria and may be further complicated with intrinsic fluorophores that may saturate system detectors. An accurate biochemical characterization of these strains is critical for accurate detection and discrimination among other species and isogenic variants. Although other UV wavelengths could be used as an excitation source to detect the resonance effect of the absorption band at 478 nm¹⁷ for staphyloxanthin, the main pigment in WT *S. aureus*, there is an increased risk of deleterious effects such as fluorescence saturation and breaking of covalent bonds. This could cause misrepresentation of the biochemical profile of the bacterial strain and make it challenging for accurately discerning bacteria at the species level. Therefore, there is a need for a rapid (seconds to minutes), noninvasive technique able to accurately detect and identify bacterial species and drug-resistant strains to guide proper treatment.

Raman spectroscopy (RS) is an inelastic light scattering technique that provides molecular specificity and has been used extensively for characterizing bacteria.^{18–22} Its ability to provide accurate and reproducible spectral information of a sample, perform rapid measurements, and create a biochemical profile of a sample have paved the way for biological applications. Biochemical features such as proteins, DNA, lipids, and polysaccharides have been detected using RS to probe bacterial components at the single-cell level.²³ Using confocal Raman microspectroscopy, metabolic changes in microorganisms have been characterized on the basis of the high spatial resolution ($\sim 1 \mu\text{m}$) the technique provides. Studies have been performed to evaluate changes in cell culture composition such as an increase in polysaccharides during the cell cycle process, highlighting the diversity in microbial subpopulations.^{24,25} Researchers have also investigated the biochemical response of antibiotics at varied concentrations on bacteria using Raman microspectroscopy.²⁶ Furthermore, bacterial strains at the subspecies level have been characterized using Raman microspectroscopy and successfully classified with a support vector machine (SVM) algorithm.^{27,28} This study builds upon previous research as one of the first to our knowledge to investigate isogenic variants of bacteria of clinical importance using Raman microspectroscopy. More specifically, the hypothesis that Raman microspectroscopy could discriminate wild-type versus single gene mutant strains of the same species of bacteria using a label-free, noninvasive approach was tested and evaluated. The goal of this work is to utilize Raman microspectroscopy to characterize the spectral features of different isolates of the same species and demonstrate the ability of this technique to distinguish between them. Specifically, isogenic and small colony variants of *S. aureus* were investigated *in situ* using spectral regions for analysis. On the basis of our results, we show that Raman microspectroscopy can be used to identify genetic variants of *S. aureus*, discriminate between methicillin-resistant and methicillin-sensitive strains, and determine biochemical features important for discrimination of various bacterial strains.

RESULTS AND DISCUSSION

RS Can Differentiate Two Virulent Strains of Group B *Streptococcus* (GBS)

Raman microspectroscopy was used to characterize and differentiate various bacterial species such as wild-type *S. aureus* (WT JE2), *Streptococcus agalactiae*, commonly known as group B *Streptococcus* (GBS), and *Haemophilus influenzae* (*H. influenzae*) (Figure 1). The strain WT JE2 presents two main peaks at 1159 and 1523 cm^{-1} resembling carotenoid bands, which we tentatively assigned as C–C stretching and C=C stretching, respectively, on the basis of previous RS measurements of *S. aureus*²⁹ (Figure 1). In addition to WT JE2, two strains of GBS were spectrally measured (GBS 1084 and GBS 37). For GBS 1084, two unique peaks at 1121 and 1504 cm^{-1} were tentatively assigned as C–C stretching and C=C stretching, respectively, on the basis of a similar 12 double bonded polyene³⁰ (Figure 1). These resonantly enhanced peaks may be related to the GBS pigment that is composed of a 676-Da ornithine rhamno-polyene with a linear chain of 12 conjugated double bonds.³¹ As can be seen in Figure 1, these two narrow peaks for GBS 1084 are red-shifted in the Raman spectrum compared to similar bands (carotenoids) for WT JE2. The decrease in stretching frequency may be due to a higher conjugation of the 12 double bonds in GBS 1084 pigment compared to the pigmentation in WT JE2 (staphyloxanthin) that does not contain as much conjugation throughout the molecule. A frequency red-shift caused by conjugation length was previously found in Raman spectra of *t*-butyl capped polyenes for higher *N*-enes, where *N* is the number of double bonds of a molecular structure.³⁰ For GBS 37, a nonpigmented strain, various biochemical features assigned as pyrimidine ring breathing (783 cm^{-1}) and C–O–O symmetric and asymmetric stretching in peptidoglycan (1379 cm^{-1}) were observed (Figure 1). *H. influenzae* shows distinct Raman features such as tyrosine ring breathing (852 cm^{-1}) and CH₂ fatty acids twisting (1299 cm^{-1}) that are important for identification compared to the other bacterial spectra³² (Figure 1). The dramatic spectral differences between the two strains of GBS, *H. influenzae*, and WT JE2 indicate the potential of Raman microspectroscopy to distinguish bacterial isolates at the subspecies level. This finding motivates the application of this technique to discriminate single genetic variations in *S. aureus* mutants.

Genetically Modified Strains of *S. aureus* Are Distinguished from WT

Various *S. aureus* mutants (WT JE2, *crtM*, *ispA*, SAUSA300–0918, *mecA*, and *fntA*) were studied using Raman microspectroscopy. To test the ability of using this technique to distinguish between single gene mutations, Raman spectra from *S. aureus crtM* are analyzed. This mutant was chosen as a positive control since deletion of *crtM* disrupts biosynthesis of the carotenoid staphyloxanthin, which is responsible for the golden pigment of *S. aureus* and predicted to contribute to the two main *S. aureus* Raman peaks at 1159 and 1523 cm^{-1} . Figure 2 clearly shows the absence of these Raman peaks in the *crtM* mutant compared with the strong presence of these features in WT JE2 spectra, which supports the assignment to this specific carotenoid pigment that differs between mutant samples (Figure 2). These carotenoids not only are an important factor for the cell membrane's integrity but also play a role in the virulence of *S. aureus*.³³ Another mutant chosen for this study includes *ispA*, an unpigmented *S. aureus* strain predicted to display a similar Raman profile to that of *crtM* due to the lack of staphyloxanthin production. The

phenotypic profile of this mutant was visually indistinguishable from that of *crtM* (Figure 3A). Finally, to assess whether a unique lipid signature could be detected in *S. aureus* using Raman microspectroscopy, *SAUSA300-0918*, a putative lipid metabolism mutant, was compared to the parental strain. To quantify the various Raman peaks seen in the *S. aureus* mutants, peak ratios of mean normalized intensities, highlighted by the gray bands in Figure 3A, were calculated. The lipid mutant strain (*SAUSA300-0918*) was evaluated using a peak ratio of 876 to 1004 cm^{-1} (asymmetric stretching $\text{N}+(\text{CH}_3)_3/\text{phenyl ring}$ breathing as part of phenylalanine). The Raman peak at 876 cm^{-1} has been shown to be relevant for characterizing membrane lipids, specifically phosphatidylcholine.³⁴ This lipid peak ratio demonstrates a significant ($p < 0.0001$) decrease when *SAUSA300-0918* is compared to *S. aureus* mutants and WT JE2 (Figure 3B). Since this gene is part of the glycerolipid metabolism pathway in *S. aureus*, deletion of the gene could negatively impact lipid production related to cell wall composition.

To determine the differences in pigmentation in *S. aureus* mutants, the peak ratio of 1523 to 1004 cm^{-1} (carotenoid/phenylalanine) was analyzed. This peak ratio shows a statistically significant ($p < 0.0001$) increase in pigmentation due to staphyloxanthin in WT JE2 as compared to *ispA*, *crtM*, and the other *S. aureus* mutants (Figure 3C). In addition, the lack of pigmentation in *ispA* and *crtM* due to their respective genetic mutations is confirmed using the peak ratio described. These results confirm the ability of Raman microspectroscopy to interrogate bacterial colonies and distinguish between strains of *S. aureus* with a one-gene mutation. On the basis of these findings, we sought to determine whether or not antibiotic-resistant and sensitive mutants could be distinguished using Raman microspectroscopy *in situ* as this ability would have significant clinical implications.

Antibiotic-Resistant *S. aureus* Strains Can Be Identified Using RS

Clinical relevance of this technology was evaluated by comparing methicillin-sensitive mutants *mecA* and *fntA* to their methicillin-resistant parental strain using Raman microspectroscopy. A full-spectrum analysis of *S. aureus* mutants was performed using principal component analysis (PCA), a nonsupervised statistical method that reduces high-dimensional data by converting it to an orthogonal vector space based on projections (principal components) that explain the most variance. The results from this approach show that the unpigmented strains (*crtM* and *ispA*) can be distinguished from methicillin-sensitive strains (*mecA* and *fntA*), *SAUSA300-0918*, and WT JE2 (data not shown) with high accuracy. The new coordinates for the original spectral data determined from PCA were then input into a quadratic discriminant analysis (QDA) classifier to demonstrate the ability of Raman microspectroscopy to distinguish between the strains described.

While a full-spectrum analysis can be used to reduce high-dimensional data to a few linear combinations of variables called principal components, the biochemical relevance of these components is unknown. To identify spectral features important for discrimination, correlation coefficients (loadings) of the components scores were used to determine how much of the variation of each variable is explained by each principal component (PC). Rather than using a single wavenumber peak, a more biochemically rich comparison can be made by utilizing regions of wavenumbers identified on the basis of the magnitude of the

correlation coefficients computed for each feature. Combining the selected spectral regions from the PCA loadings that explain larger variances in the data minimizes overfitting, therefore creating a more reliable model for identification of biomarkers important for discrimination.

For the *S. aureus* mutants, the highest PC1 correlation coefficient was observed at 1523 cm^{-1} followed by that at 1159 cm^{-1} as determined by plotting the correlation coefficients (PC loadings) versus the Raman shift (Figure 4A). Since these two wavenumbers both described pigmented versus unpigmented *S. aureus* mutants, the highest PC1 feature was used to distinguish between these strains (1523 cm^{-1}). The highest PC2 correlation coefficient was located at 781 cm^{-1} , and the second highest was at 910 cm^{-1} . The spectral regions of analysis identified for the *S. aureus* mutant data were determined by evaluating the magnitudes of the correlation coefficients of PC1 and PC2. The first spectral region of interest was 765–934 cm^{-1} (region 1), which contained PCA correlation coefficients that were at least 50% of the second highest correlation coefficient in PC2 located at 910 cm^{-1} (Figure 4B). This threshold was part of the selection criteria for a spectral region. Since the second highest PC1 correlation coefficient (1159 cm^{-1}) was not used for analysis, the next highest PC2 correlation coefficient, 1431–1464 cm^{-1} , was selected as the second spectral region of interest (region 2) (Figure 4B). The third spectral region of interest (region 3) was 1495–1544 cm^{-1} , based on the maximum PC1 feature (1523 cm^{-1}) (Figure 4B).

A subsequent PCA using singular value decomposition (PCA-SVD), which has a higher precision in calculating the eigenvectors by not using the covariance matrix, was calculated on the basis of the determined spectral regions. The scores from this analysis were used to fit a quadratic discriminant analysis (QDA) model for each spectral region. A quadratic discriminant analysis (QDA) based on PCA singular value decomposition (PCA-SVD) was implemented to discriminate between the *S. aureus* mutants and classify spectra on the basis of a statistically robust approach. Since the decision boundaries for specific spectral regions may be nonlinear, a quadratic function analysis was used. Boundaries generated from the QDA fit based on spectral region 1 (765–934 cm^{-1}) present 100% classification of wild-type (WT) JE2, methicillin-sensitive strains (*fmtA* and *mecA*), *SAUSA300-0918*, and nonpigmented strains (*crtM* and *ispA*) when compared to each other (Figure 5A,B). These results were based on PC1 and PC2, which explained 71.60% and 17.40% of the data within this spectral region, respectively. Various biochemical features in region 1 are assigned to cytosine (782 cm^{-1}), tyrosine (853 cm^{-1}), and C-O-C stretching and teicuronic acid (907 cm^{-1}) found in the cell wall of Grampositive that characterize each of the *S. aureus* mutants (Figure 5A). Region 2 (1431–1464 cm^{-1}) shows 100% discrimination with boundaries based on the QDA model that successfully separate nonpigmented strains (*crtM* and *ispA*), methicillin-sensitive (*fmtA* and *mecA*) and *SAUSA300-0918* strains, and WT JE2 compared to each other (Figure 5C,D). For region 2, PC1 and PC2 explained 97.90% and 1.07% of the variance, respectively. This spectral region was dominated by CH_2/CH_3 bending (1456 cm^{-1}) (Figure 5C).

Region 3 (1495–1544 cm^{-1}) of interest for the *S. aureus* mutants presents boundaries based on the QDA model that present 100% discrimination of nonpigmented strains (*crtM* and *ispA*) compared to the rest of the *S. aureus* mutants investigated (Figure 5E,F). Within this

spectral region of interest, PC1 and PC2 explained 99.90% and 0.03% of the variance in the data, respectively. The high percentage of variance explained by PC1 is related to the dominating Raman peak known to be due to the tentatively assigned carotenoid staphyloxanthin (1523 cm^{-1}) (Figure 5E). Similar biochemical features resembling carotenoids have also been detected in *Mycoplasma pneumoniae* and were used for strain identification using Raman spectroscopy.³⁵ The findings motivated us to compare spectral features of methicillin-resistant to methicillin-sensitive *S. aureus*.

Initially, WT JE2, a methicillin-resistant isolate of *S. aureus* was compared to Newman, a methicillin-sensitive *S. aureus* strain. From the Raman spectra of these strains, two distinguishing peaks can be observed at 1159 and 1523 cm^{-1} , both related to carotenoid features (Figure 6A). Another peak that presents changes in intensity includes 1456 cm^{-1} (CH_2/CH_3 bending). Peak ratios of 1456 cm^{-1} to 1004 cm^{-1} are significantly ($p < 0.0001$) lower for WT JE2 when compared to Newman (Figure 6B). In addition, a peak ratio of 1523 cm^{-1} to 1004 cm^{-1} shows that WT JE2 is significantly ($p < 0.0001$) greater when compared to Newman (Figure 6C). This was similarly observed with the carotenoid peak at 1159 cm^{-1} . A decrease in pigmentation production is a characteristic phenotypical feature seen in SCVs,³⁶ which is confirmed by the comparison of the carotenoid peak ratio between WT JE2 and Newman. In addition, the Amide III-bending (C–N) at 1290 cm^{-1} is significantly greater in intensity for WT JE2 compared to Newman. Furthermore, spectral intensity differences were seen in the previously described carotenoid peaks (1159 and 1523 cm^{-1}) and CH_2/CH_3 bending peak (1456 cm^{-1}) when *S. aureus* mutants *fmtA* and *mecA* were compared to WT JE2 and Newman. These spectral bands potentially indicate lower carotenoid concentration and higher lipid (triacylglycerol) concentration for Newman compared to WT JE2, *fmtA*, and *mecA*. These differences in the Raman spectra provided insight into biochemical factors that could be used to differentiate methicillin-sensitive from methicillin-resistant *S. aureus* strains.

Small-Colony Variants (SCVs) Can Be Distinguished from WT Newman Strain

Since results strongly indicate that Raman microspectroscopy can distinguish biochemical signatures of methicillin resistance or sensitivity in *S. aureus*, other types of antibiotic tolerance were investigated. Analysis was extended to the clinically relevant small-colony variant (SCV) phenotype, which is intrinsically resistant to aminoglycoside antibiotics. The SCV phenotype conveyed by three different types of mutations was compared to their parental strain, the methicillin-sensitive strain Newman.³⁷ The SCV mutations chosen for this analysis were a double cytochrome deletion *cyd* *gox*, as well as the more clinically relevant variants lacking heme (*hemB*) or menaquinone (*menB*) biosynthesis.

Raman spectra of SCVs with regions showing spectral differences are highlighted by gray bands and quantified using peak ratios (Figure 7A). The first peak ratio of 781 cm^{-1} to 1004 cm^{-1} (pyrimidine ring breathing as part of deoxyribonucleic acid (DNA)/phenylalanine) was significantly lower ($p < 0.0001$) for Newman when compared to the other SCVs (Figure 7B). Another peak ratio of interest was the 1524 cm^{-1} to 1004 cm^{-1} (assigned as carotenoid/phenylalanine), which shows Newman at a significantly higher Raman intensity ($p < 0.0001$) compared to the other three SCVs (Figure 7C). The lower Raman intensity at 1524 cm^{-1} for

the SCVs was expected since they are defective in their pigment production⁸ compared to Newman.

Evaluation of SCVs using their full-spectrum for PCA shows variation based on PC1 (93.3%) and PC2 (2.21%) between Newman, *cyd qox*, *hemB*, and *menB* (data not shown). Following the same approach as the *S. aureus* mutants, the magnitudes of the correlation coefficients from PC1 and PC2 of the SCVs were used to identify spectral regions for subsequent analysis. The highest PC1 correlation coefficient is located at 1524 cm^{-1} , and the second highest is at 1159 cm^{-1} (Figure 8A). These are the same Raman peaks that were identified from the *S. aureus* mutant data. Since both of these features are characteristic of carotenoids, only the highest PC1 feature (1524 cm^{-1}) was included as part of the analysis. The next spectral region of interest for analysis was defined by the third highest PC1 feature at 781 cm^{-1} , which was also present in PC2 as the highest correlation coefficient for the SCV data. Since the feature with the second highest PC2 correlation coefficient (1522 cm^{-1} , Figure 8A) was previously selected from PC1, the third highest PC2 feature located at 1019 cm^{-1} was used for spectral region analysis. The spectral SCV regions identified for analysis are 772–800 cm^{-1} (region 1), 1012–1029 cm^{-1} (region 2), and 1500–1558 cm^{-1} (region 3) (Figure 8B).

A QDA analysis using PCA-SVD of each of the spectral regions of interest was again implemented to discriminate among SCVs. For region 1 (772–800 cm^{-1}) of the SCV data, the QDA boundaries provided 100% classification of Newman from the rest of the SCVs (Figure 9A,B). Within spectral region 1, PC1 and PC2 explained 99.20% and 0.35% of the variance in the data, respectively. This was mainly dependent on the Raman peak that dominates this spectral region located at ~781 cm^{-1} (assigned as pyrimidine ring breath as part of DNA), which was significantly lower in Newman compared to the other SCVs (Figure 9A). Region 2 (1012–1029 cm^{-1}) was able to classify (100%) between each SCV strain based on QDA based on PC1 (80.80%) and PC2 (16.60%) (Figure 9C,D). The main band highlighted within this spectral region (~1015–1017 cm^{-1}) may be assigned to tryptophan (amino acid) and C–O stretch as part of the DNA backbone (Figure 9C). Similar to region 2, region 3 (1500–1558 cm^{-1}) of the SCVs presents 100% discrimination between each SCV strain using PC1 (99.00%) and PC2 (0.60%) (Figure 9F). The main biochemical features within this spectral region include 1524 cm^{-1} (C=C stretching as part of a carotenoid molecule) and 1555 cm^{-1} (assigned as the indole ring of tryptophan) (Figure 9E). These findings indicate that Raman microspectroscopy not only can be used to identify the presence of these SCVs but also may be applied to identify other SCVs and categorize their type without the need for time-consuming detection methods.

The growth of antibiotic-resistant pathogens has motivated the creation of new antibiotics and diagnostic tests to track their development. Although molecular-based detection methods have been used extensively, novel approaches are needed that will provide rapid measurements, accurate results, and precise discrimination to identify antibiotic-resistant bacteria in various environments, aiding physicians to provide proper antibiotic treatment. Results using Raman microspectroscopy and QDA of PCA scores provided 100% accuracy in classifying *S. aureus* genetic variants and SCVs *in situ* based on the QDA boundaries. More specifically, antibiotic susceptibility and resistance based on biochemical differences

could be distinguished from WT strains. Although WT JE2 and *SAUSA300-0918* were distinguished from each other and from the other mutants, methicillin-sensitive strains (*fmtA* and *mecA*) were not discriminated from each other and neither were the nonpigmented strains (*crtM* and *ispA*). For the SCVs that were studied, each of the genetic variant strains and Newman could be distinguished from each other by utilizing two of the regions that were statistically designated (Figure 9C–F). Successful classification for each of the SCVs may be due to the highly varied modes of action to achieve the reduction of proton motive force in these strains. The proton motive force in *cyd* *gox* is reduced due to the absence of highly abundant membrane proteins; *hemB* is impacted by the absence of a heme cofactor that is not only used in cytochromes but also found in proteins such as catalase and therefore potentially impacts multiple cellular processes, and *menB* lacks the lipophilic vitamin menaquinone. These diverse biochemical changes in Newman mutants, compared to JE2 isogenic variants, may be the reason why classification was more robust in Newman strains. These pathogens were successfully discriminated on the basis of changes in their biochemical synthesis pathways, which Raman spectroscopy was able to detect directly from bacterial colonies.

In addition to bacterial detection and identification, efficacy of antibiotic treatment is critical to improving patient care. Although bacterial strains for this study were cultured for 24 h to determine the robustness of our approach, Raman spectra have been collected from microcolonies incubated for only 6 h,²⁰ which would be important for detecting bacterial growth in a shorter time. Other groups have utilized Raman spectroscopy to study vancomycin-sensitive and resistant strains of *Enterococcus faecalis*³⁸ and *Escherichia coli* (*E. coli*) that contained a plasmid with an ampicillin resistance gene.³⁹ Phenotypic profiling of the effects of antibiotic treatment on dried *E. coli* cells has also been evaluated using Raman spectroscopy, which discriminated Raman spectra on the basis of the class of antibiotic treatment using PCA and discriminant analysis.⁴⁰ With the use of aluminum coated substrates, researchers have been able to determine levels of antibiotic susceptibility and minimum concentrations of antibiotics needed to prevent bacterial growth for methicillin-susceptible *S. aureus*, wild-type *E. coli*, and clinical isolates.³⁸ While this study evaluated antibiotic susceptibility/resistance on the basis of biochemical differences only across a small set of strains (Figure 6A–C), findings from this work motivate the use of Raman spectroscopy as a diagnostic tool able to detect, identify, and discriminate clinically relevant, drug-resistant pathogens *in situ* at the species level. Future studies will evaluate additional strains with diverse genetic variants that incorporate more subtle, nonphenotypic changes that can further inform the evolution of drug resistance across other bacteria while highlighting the potential of Raman spectroscopy. While the current limitations of Raman spectroscopy and our statistical analysis approach are challenging to quantify given the low number of reported studies on this topic, they may be based on the biochemical impact of a given genetic mutation or multiple mutations and the spectral resolution of a given Raman spectroscopy system.

In conclusion, biochemical features important for identification of drug-resistant *S. aureus* strains were identified using Raman microspectroscopy in combination with spectral regions for analysis. Raman measurements were made directly on the bacterial colonies in the agar and did not require additional preparation steps after incubation. Although visual evaluation

of the *S. aureus* genetic variants' Raman spectra presented qualitative differences based on the presence or absence of carotenoid features (1159 and 1523 cm^{-1}) between two nonpigmented mutants and the rest, a statistical analysis approach is needed to discriminate other bacteria not represented by these Raman peaks. Plotting PCA correlation coefficients across the entire Raman spectrum for *S. aureus* mutants and SCVs highlights biochemical regions of interest used for subsequent QDA classification and minimizes data overfitting. Furthermore, the implementation of this classification system becomes invaluable in streamlining clinical decisions, removing the complexity of the initial spectral analysis. This approach may enhance the adoption of Raman spectroscopy as a point of care diagnostic device, especially when implemented in a hand-held or portable setup. This statistical approach of evaluating spectral regions relevant to the sample of interest may be important for identifying pathogens in environments that have a high Raman scattering background seen in the typical biological fingerprint window (700–1800 cm^{-1}).

To fully utilize this classification approach, a spectral library of *S. aureus* genetic variants and other strains will need to be developed that incorporates various biochemical features across spectral regions for discrimination. The size of the spectral library needed to have practical significance will depend highly on the function and setting of the application (e.g., research vs clinical). For example, researchers in a lab setting may be focused on evaluating strains of a small set of bacteria from a specific genus and species. Therefore, the spectral library for their case may only require the reference Raman spectra from this subset of bacteria. However, if utilized in a clinical setting, the spectral database will need to be comprehensive and include bacteria from different genera and species, especially those that are most clinically important (e.g., drug-resistant strains). Since this becomes exponentially challenging to include every single bacterial strain in this database, the bacterial identification algorithm will need to be capable of pulling out “unknown” measurements for further evaluation. Extraction of “unknown” or unrecognized spectra will be important for minimizing misclassification of spectra and recognizing the need to identify the bacterial strain behind the Raman measurement. A diverse spectral database appropriate for the respective bacterial detection/identification application will enable the scientific community to fully utilize the rich biochemical data Raman spectroscopy provides while identifying biomarkers important for classification. Furthermore, this technique and statistical analysis approach has the potential to play a major role in identifying multidrug resistant pathogens to guide care providers with accurate information for proper and timely treatment.

MATERIALS AND METHODS

Bacterial Strains

Multiple pathogens were characterized using Raman microspectroscopy, and their biochemical profile was used to determine if clinically relevant strains could be identified. The following bacteria were evaluated using Raman microspectroscopy: wild-type *Staphylococcus aureus* (WT JE2),⁴¹ *Streptococcus agalactiae* (also known as Group B *Streptococcus*, GBS), GBS 1084 (ATCC 49447) and GBS 37, and nontypeable *Haemophilus influenzae* (ATCC 49766).

S. aureus strain JE2 containing *mecA*, *ispA*, *fntA*, *crtM*, and *SAUSA300-0918* mutations⁴¹ were also investigated using Raman microspectroscopy. One of these genetic variants included *mecA*, which lacked resistance to β -lactam antibiotics and is commonly referred to as methicillin-sensitive *S. aureus* (MSSA)^{42,43} The second *S. aureus* mutant investigated was *ispA*, a geranyltransferase gene, which generates nonpigmented bacterial colonies.⁴⁴ The third *S. aureus* gene studied, *fntA*, is a member of the *S. aureus* core cell wall stimulon, and inactivation of *fntA* (*fntA*) affects the cell wall structure⁴⁵ and diminishes the ability of *S. aureus* to form biofilms.⁴⁶ The fourth *S. aureus* mutant studied included *crtM*, which encodes for dehydrosqualene synthase and disrupts biosynthesis of carotenoids, resulting in nonpigmented bacterial colonies.³³ The fifth *S. aureus* mutant is *SAUSA300-0918*, where *SAUSA300-0918* encodes for glycerolipid metabolism and is involved in the formation of membrane glycolipids. *S. aureus* small-colony variants (SCVs) were also examined to determine if Raman characterization could be used to biochemically discriminate this type of antibiotic-tolerant variant from other *S. aureus* strains. The SCVs tested included a cytochrome double knockout strain *cyd qox*⁴⁷ as well as a heme biosynthesis-deficient strain *hemB*⁴⁸ and a menaquinone biosynthesis-deficient strain *menB*.⁴⁹ The parental strain of these SCVs, Newman,³⁷ was also spectrally analyzed using Raman microspectroscopy. These strains were summarized (Table S1) and streaked on Mueller-Hinton agar plates for comparison (Figure S1A,B).

Each strain was streaked onto Mueller-Hinton (MH) agar, which was prepared by suspending 11 g of MH (BD, Franklin Lakes, NJ) powder and 7.5 g (15% agar/L) of agar (Thermo Fisher Scientific, Waltham, MA) in 500 mL of distilled water while heating (180 °F) and stirring. The mixture was then autoclaved at 121 °C for 10 min. After plating, bacteria were incubated for 24 h at 37 °C.

Raman Microspectroscopy

Acquisition of Raman spectra was performed using a Raman microscope (inVia Raman Microscope, Renishaw plc, Gloucestershire, UK) with a 785 nm laser excitation (Renishaw plc, Gloucestershire, UK). To interrogate the bacterial colonies, a 100 $\times$ (N PLAN EPI, NA = 0.85, Leica, Wetzlar, Germany) objective was used to focus a laser spot directly on the bacterial colony on the agar surface at 27 mW. The penetration depth of our Raman beam using this optical setup is on the order of tens of microns based on an intensity falloff of $1/e$ according to the Beer–Lambert law. This would hypothetically allow signal collection from tens to hundreds of cells in the beam path. Raman scattered light was detected through the same objective, then passed through a 55 μ m slit, and dispersed by a holographic grating (1200 lines/mm) onto a thermoelectrically cooled (-70 °C), deep-depleted charge-coupled device (CCD) that provided ~ 1 cm^{-1} spectral resolution. System alignment and light throughput to the sample was confirmed before and after experimental measurements with an internal silicon standard at 520 cm^{-1} and laser power at the sample.

Spectral measurements included three acquisitions per spot, three spots per colony, and three colonies per bacteria for *S. aureus* mutants for a total of 162 spectra with 917 features for each spectrum. All Raman spectra for the *S. aureus* genetic variants and SCVs were collected from different bacterial colonies of the same growth. Measurement parameters for

SCVs included three spots per colony and three colonies per bacterial strain for a total of 36 spectra with 917 features for each spectrum. First, the 785 nm laser was focused onto the bacterial colony for a 30 s photobleach of the sample to minimize fluorescence from MH agar. Subsequent spectral acquisition parameters included a 15 s exposure with 7 accumulations from 700 to 1800 cm^{-1} . Cosmic ray removal from collected Raman spectra was performed using a custom MATLAB script (Mathworks, Natick, MA, USA). Raman spectra were then processed to remove background fluorescence using a least-squares modified polynomial fitting algorithm⁵⁰ and smoothed for noise with a second-order Savitsky-Golay filter.⁵¹ To optimize background fluorescence subtraction, each raw spectrum from SCVs was divided into three segments. These segments included: (a) 700–1141 cm^{-1} , (b) 1141–1477 cm^{-1} , and (c) 1470–1700 cm^{-1} . The 7 cm^{-1} overlap of regions (b) and (c) was adjusted by using only the fitting from 1478–1700 cm^{-1} for region (c). Segment (a) used an eighth degree modified polynomial fitting compared to segments (b) and (c), which implemented a fifth degree modified polynomial fit for fluorescence subtraction. After spectral processing was performed for SCV data, spectral segments were reconstructed into one Raman spectrum for each measurement. Postprocessed spectra were mean normalized to each individual Raman spectrum for comparative analysis.

Spectral Data Analysis

Mean normalized Raman spectra of bacterial colonies were analyzed for classification. For preliminary analysis, peak ratios were calculated on the basis of distinct Raman peaks and the phenylalanine peak across all *S. aureus* mutants and SCVs. The means of the peak ratios were compared using a one-way analysis of variance (ANOVA) and corrected using a Tukey test to determine significance for multiple comparisons. To limit bias from hand-selecting peaks, a full-spectrum principle component analysis (PCA) was performed on the Raman spectra of the *S. aureus* mutants and SCVs. Since spectra from these pathogens included more spectral features (variables) compared to observations, PCA scores and correlation coefficients (loadings) were calculated using singular value decomposition (SVD).⁵² Implementation of SVD for PCA reduces the large volume of data and minimizes the loss of precision that is typically seen when using the covariance matrix approach. To use PCA via SVD, the means of the mean-normalized spectral data matrix were subtracted from each dimension to center the data. Then, the SVD of the mean-centered matrix was calculated to determine the eigenvalues and eigenvectors to interpret the scores and loadings of the original input matrix.

While the full-spectrum analysis provided a global picture of the Raman data, the model was tested to prevent over fitting the data since there are more spectral features than measurements. Therefore, loadings, which measure the correlation between the principal component score and the original variables, were used to identify spectral regions of interest for downstream analysis. The use of PCA loadings (correlation coefficients) to determine important spectral features for classification has been previously used for identifying molecular distributions in biological samples.⁵³ The approach for determining these spectral regions of interest involved the following steps. First, the two maximum (absolute value) correlation coefficients from the first two PCs were identified. If the spectral region between any of those peaks contained correlation coefficients that were at least 50% of the second

maximum correlation coefficient and the region in consideration was not greater than 15% of the total features available (wavenumbers), then that spectral region could be used for evaluation. Otherwise, the spectral region of interest would be defined by the width of the peak determined by the PC correlation coefficient.

After the spectral regions were designated for both *S. aureus* mutants and SCVs, PCA via SVD was performed on these regions. The PC scores from each spectral region were used for a discrimination analysis. A variant of Fisher's linear discriminant analysis, quadratic discriminant analysis (QDA), was applied to determine the ability of each designated spectral region to classify the various microorganisms. The use of QDA has been implemented in applications such as classifying Raman spectra of human cancer cell lines⁵⁴ and Raman imaging of naive versus activated T-cells.⁵⁵ First, a quadratic classifier was created on the basis of designated classes (each of the *S. aureus* mutants and SCVs) using the PCA scores from each spectral region as input parameters. Next, the coefficients of the respective quadratic boundaries were determined. The coefficients (*K*, constant; *L*, linear; *Q*, quadratic) were used in eq 1 to generate the curves to determine boundaries for discrimination among classes for each *S. aureus* mutant and SCV.

$$K + [x_1 x_2]L + [x_1 x_2]Q \begin{bmatrix} x_1 \\ x_2 \end{bmatrix} = 0 \quad (1)$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

S. aureus	Staphylococcus aureus
HAIs	healthcare-associated infections
SCC	staphylococcus cassette chromosome
PBP	penicillin binding protein
SCVs	small-colony variants
DNase	deoxyribonuclease
AST	antimicrobial susceptibility testing

RS	Raman spectroscopy
SVM	support vector machine
WT JE2	wild-type <i>S. aureus</i>
GBS	group B Streptococcus
<i>H. influenzae</i>	<i>Haemophilus influenza</i>
MSSA	methicillin-sensitive <i>Staphylococcus aureus</i>
crtM	gene that codes for dehydrosqualene synthase
fntA	gene that affects the cell wall structure and reduces methicillin resistance
ispA	gene that codes for geranyltransferase
mecA	gene that expresses resistance to non- β -lactam antibiotics
SAUSA300–0918	gene that affects glycerolipid metabolism
cyd qox	cytochrome genes
hemB	hemin biosynthesis gene
menB	menaquinone biosynthesis gene
CCD	charge-coupled device
PCA	principal component analysis
QDA	quadratic discriminant analysis
PC	principal component
SVD	singular value decomposition
<i>E. coli</i>	<i>Escherichia coli</i>
MH	Mueller-Hinton
ANOVA	analysis of variance

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Figure 1.

Mean $\pm$ standard deviation Raman spectra of various bacteria. Spectral signatures of bacteria shown include WT JE2, GBS 1084, GBS 37, and *H. influenzae*. Different spectral features are identified for each bacterial measurement. β , breathing; τ , twisting; ν , stretching.

Figure 2.

Mean $\pm$ standard deviation Raman spectra of WT JE2 and $\Delta crtM$, an *S. aureus* mutant that lacks pigmentation. Two major Raman bands, located at 1159 cm^{-1} $\nu(\text{C-C})$ and 1523 cm^{-1} $\nu(\text{C=C})$, are present in WT JE2 and absent in $\Delta crtM$. α , bending; β , breathing; ν , stretching.

Figure 3.

(A-C) Comparison of *S. aureus* mutants based on pigmentation and lipid features. (A) Mean $\pm$ standard deviation Raman spectra of WT JE2 and *S. aureus* mutants. (B) Mean peak ratio of 876 cm⁻¹ ν (N⁺(CH₃)₃) and 1004 cm⁻¹ β (phenyl ring) as part of phenylalanine with 95% confidence interval calculated using a one-way ANOVA performed to compare mutants vs WT JE2. (C) Mean peak ratio of 1523 cm⁻¹ ν (C=C) and 1004 cm⁻¹ β (phenyl ring) with 95% confidence interval calculated using a one-way ANOVA performed to compare mutants vs WT JE2. **** = $p < 0.0001$. β , breathing; ν , stretching.

Figure 4.

(A, B) Spectral region analysis of *S. aureus* mutants based on PC correlation coefficients.

(A) Gray bands identify regions used for analysis of WT JE2 and *S. aureus* mutants as

determined by PC correlation coefficients. (B) Spectral regions, indicated by gray bands, used for discriminant analysis of *S. aureus* mutants and WT JE2.

Figure 5.

(A–F) Spectral regions of interest and subsequent discriminant analysis for *S. aureus* mutants and WT JE2. (A) Spectral region 1 (765–934 cm^{-1}). (B) Quadratic discriminant analysis (QDA) performed on the PCA scores for spectral region 1 of *S. aureus* mutants and WT JE2. (C) Spectral region 2 (1431–1464 cm^{-1}). (D) QDA performed on the PCA scores for spectral region 2 of *S. aureus* mutants and WT JE2. (E) Spectral region 3 (1495–1544 cm^{-1}). (F) QDA performed on the PCA scores for spectral region 3 of *S. aureus* mutants and WT JE2.

Figure 6.

(A–C) Comparison of *S. aureus* methicillin-sensitive and methicillin-resistant strains and mutants. (A) Mean $\pm$ standard deviation Raman spectra of Newman (wild-type methicillin-sensitive), Δ fmtA, Δ mecA, and WT JE2 (wild-type methicillin-resistant). (B) Mean peak ratio of 1456 cm⁻¹ α (CH₂/CH₃) to 1004 cm⁻¹ β (phenyl ring) with 95% confidence interval calculated using a one-way ANOVA performed to compare mutants vs WT JE2 and Newman. (C) Mean peak ratio of 1523 cm⁻¹ ν (C=C) and 1004 cm⁻¹ β (phenyl ring) with 95% confidence interval calculated using a one-way ANOVA performed to compare mutants vs WT JE2 and Newman. **** = $p < 0.0001$. α , bending; β , breathing; ν , stretching.

Figure 7.

(A–C) Comparison of SCVs based on DNA and pigmentation features. (A) Mean $\pm$ standard deviation Raman spectra of Newman and SCVs. (B) Mean peak ratio of 781 cm^{-1} β (pyrimidine ring) and 1004 cm^{-1} β (phenyl ring) with 95% confidence interval calculated using a one-way ANOVA performed to compare mutants vs Newman. (C) Mean peak ratio of 1524 cm^{-1} ν (C=C) and 1004 cm^{-1} β (phenyl ring) with 95% confidence interval calculated using a one-way ANOVA performed to compare mutants vs Newman. **** = $p < 0.0001$. β , breathing; ν , stretching.

Figure 8.

(A, B) Spectral region analysis of SCVs based on PC correlation coefficients. (A) Gray bands identify spectral regions for analysis of Newman and SCVs as determined by PC correlation coefficients. (B) Spectral regions, indicated by gray bands, used for discriminant analysis of SCVs and Newman.

Figure 9.

(A–F) Spectral regions of interest and subsequent discriminant analysis for SCVs and Newman. (A) Spectral region 1 (772–800 cm^{-1}). (B) Quadratic discriminant analysis (QDA) performed on the PCA scores for spectral region 1 of SCVs and Newman. (C) Spectral region 2 (1012–1029 cm^{-1}). (D) QDA performed on the PCA scores for spectral region 2 of SCVs and Newman. (E) Spectral region 3 (1500–1558 cm^{-1}). (F) QDA performed on the PCA scores for spectral region 3 of SCVs and Newman.