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The biochemical origins of the surface enhanced Raman spectra of bacteria: metabolomics profiling by SERS

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Abstract

The dominant molecular species contributing to the 785 nm excited SERS spectra of bacteria are the metabolites of purine degradation: adenine, hypoxanthine, xanthine, guanine, uric acid and AMP. These molecules result from the starvation response of the bacterial cells in pure water washes following enrichment from nutrient rich environments. Vibrational shifts due to isotopic labeling, bacterial SERS spectral fitting, SERS and mass spectrometry analysis of bacterial supernatant, SERS spectra of defined bacterial mutants, and the enzymatic substrate dependence of SERS spectra are used to identify these molecular components. The absence or presence of different degradation/salvage enzymes in the known purine metabolism pathways of these organisms plays a central role in determining the bacterial specificity of these purine-base SERS signatures. These results provide the biochemical basis for the development of SERS as a rapid bacterial diagnostic and illustrate how SERS can be applied more generally for metabolic profiling as a probe of cellular activity.

Graphical abstract

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Compliance with Ethical Standards

The authors have no conflicts of interest.

Keywords

SERS; bacteria; nucleotide degradation; purine metabolism; metabolic profiling

Introduction

Surface enhanced Raman spectroscopy (SERS) has become a well-established analytical technique that has been exploited in a large number of biomedical applications for the identification of molecular markers of biological activity.[1–3] One recent SERS biomedical application that has generated considerable interest and activity is the use of SERS for the rapid, growth-free, detection and identification of vegetative bacterial cells for infectious disease diagnostics.[4–13] SERS offers some unique advantages for bacterial infectious disease identification: extrinsic labeling is not needed, the large signal enhancement permits the rapid, growth-free acquisition of SERS spectra, the method is less susceptible to contamination than nucleic acid amplification techniques, bacterial identification can be achieved by minimally trained personnel with relatively low cost, and portable SERS instrumentation allows for point-of-care diagnostics.

The ability to acquire SERS spectra of whole vegetative bacterial cells has been demonstrated by several research groups over the past fifteen years.[6–8, 11, 13–44] However, despite the large number of bacterial SERS observations, an interpretation of the observed vibrational signatures in terms of the chemical species and biological basis for these signals has not been achieved thus limiting the development of this technology for bacterial diagnostics and other whole cell based biomedical applications. The purpose of this paper is to unequivocally assign the molecular origins of the vibrational features that appear in SERS spectra of bacterial cells, and to identify the cellular mechanisms that account for these species specific vibrational signatures.

Reported SERS spectra of whole bacterial cells exhibit fairly consistent bacterial SERS spectra for each of the most commonly used excitation wavelengths; ~514.5 nm and ~785 nm. Normalized SERS spectra of ten representative, strain-specific bacteria excited at 785 nm are displayed in Fig. 1. Bacterial samples were grown to log phase in culture media, water washed and the whole cells were subsequently placed on an Au nanoparticle covered SERS substrate.[20] Both gram positive and gram negative species are among this set of bacteria. The spectra in Fig. 1 are very similar to those previously reported for the identical

organisms and excitation wavelength.[4, 6, 8, 11, 12, 17, 19–21, 24, 25, 29–35, 37–43] A number of vibrational bands are common to the members of this representative set of spectra.

A variety of assignments have been previously offered for the observed vibrational features in the SERS spectra of bacteria. Due to the distance dependence of the SERS enhancement mechanisms,[45] it has been generally assumed that the observed vibrational bands appearing in the SERS spectra of whole vegetative bacterial cells are dominated by the contributions of their outer cell wall components. Furthermore, the SERS vibrational features of whole bacterial cells are different than the peak frequencies seen in the normal, non-SERS Raman spectra of bacteria, consistent with cell wall molecular components accounting for SERS bacterial signals.[20, 36, 46–48] Correspondingly, the current consensus is that SERS spectra of bacteria are due to a mix of vibrational modes of cell wall components, such as peptidoglycan, lipids, lipopolysaccharides or membrane proteins and nucleic acids.[6, 7, 11, 14, 16–21, 24, 25, 29–34, 38, 39, 41–43] although bacterial SERS spectra do not correlate with cell wall architecture, i.e., gram positive vs. gram negative (Fig. 1). A few studies have also suggested the possibility that small molecules, such as adenine, may contribute to the observed bacterial SERS spectra as well.[11, 17, 34, 40]

The current understanding of these bacterial SERS spectra may be illustrated by the reported assignments for the ubiquitous and generally strongest band observed in 785 nm excited bacterial SERS spectra at $\sim 725\text{--}735\text{ cm}^{-1}$ (Fig. 1). It has been explicitly assigned to the glycosidic ring or C-N stretching mode of N-acetyl-D-glucosamine (NAG) or N-acetylmuramic acid (NAM), major components of peptidoglycan, a key bacterial cell wall biopolymer.[6, 7, 19, 24, 38, 39, 41, 43] Other proposed assignments for this band include a symmetric O–P–O vibrational mode of the phosphate group,[35] adenosine,[25] adenine[17] or adenine containing molecules such as flavin adenine dinucleotide (FAD) or nucleic acids.[7, 11, 31, 33, 34, 38–40, 43, 44] Finally, one bacterial analysis attributed these spectral features to cell culture contaminants.[37] Aside from the expectation based on the distance dependence of the plasmon enhancement mechanism, the main basis for these assignments was the similarity of the Raman band frequency in SERS spectra of model compounds and the observed bacterial spectra.

A further complication for the assignment of the chemical origins of bacterial SERS spectra is the Raman excitation wavelength dependence of these spectra. Bacterial spectra excited at 514.5 nm (or 532 nm), have been shown to be virtually identical to those of riboflavins, such as FAD, and exhibit no species/strain dependence.[13, 22, 23, 26, 27, 30, 36, 44, 49] FAD is a co-enzyme located in bacterial cell walls. These observations stand in contrast to the SERS spectra excited at 785 nm that exhibit different vibrational features and species/strain dependence. Thus, there is considerable uncertainty about the biochemical origins of these bacterial SERS signatures.

In order to maximally exploit the capabilities of SERS for bacterial identification, it is essential to understand the molecular and corresponding biochemical origins of these SERS vibrational signatures. Such information informs the design of optimal bacterial preparation and enrichment procedures from infectious human body fluids in order to provide

maximally robust and reproducible SERS signals. It also provides a basis for understanding the capabilities and limitations for diagnostics by this optical approach and is a requirement for the adoption of this novel rapid diagnostic in the medical community. Finally, a biochemical understanding of these cellular molecular markers has the potential to broaden the uses of SERS for other biomedical applications and provide an even larger chemical biology impact beyond rapid, growth-free bacterial diagnostics.

The results shown here unequivocally establish that the 785 nm excited SERS spectrum of bacteria is due to metabolites of the purine degradation pathway that result from the rapid onset of the bacterial starvation response. This analysis demonstrates the potential of SERS for monitoring real-time exogenous metabolomics and its more general use to address questions in systems biology.

Methods

Bacterial samples and preparation

The sources for most of the bacterial strains used in this study are summarized in Table 1. In addition *S. aureus* 25904 and *Bacillus cereus* 14579 were obtained from ATCC. The *E. coli* BW25113 (CGSC#7636) parent strain and its two mutants, *E. coli* JW3640-1 *ade* (CGSC#10671) and *E. coli* JW1615-1 *add* (CGSC#9376), and another *E. coli* *add* mutant SO0333 (CGSC#5937) and corresponding parent strain SO003 (CGSC#6925) were acquired from the Coli Genetic Stock Center (Yale University). *E. coli* BD11996 was donated by BD Inc. Bacterial strains were cultivated overnight before subculture in ~ 15 mL of Tryptic Soy broth (BD) or LB broth (Sigma), harvested during the log phase by centrifugation of 2mL of culture and washed four times with 2 mL of deionized Millipore or distilled water. The cell pellet was re-suspended in 0.25 mL of water and 1 μ L of the resulting $\sim 10^9$ /mL bacterial suspension was pipetted directly onto the SERS substrate for spectral acquisition. SERS measurements were made after approximately five minutes when nearly all the water on the SERS substrate had evaporated. The bacterial solution was filtered through a 0.22 micron Eppendorf filter to obtain bacterial supernatant samples. ^{15}N cell culture media and ^{15}N substituted adenine, hypoxanthine and guanine were acquired from Cambridge Isotope Laboratory Inc. (Tewksbury, MA). Sodium pyrophosphate decahydrate ($\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$) was purchase from Sigma-Aldrich and used with out further purification.

SERS substrates

All SERS spectra reported here were obtained using the *in-situ* grown, aggregated Au nanoparticle covered SiO_2 substrate developed previously in our laboratory.[20] Details concerning the production of these SERS active chips and the characterization of their performance for providing reproducible SERS spectra of bacteria have been described. These substrates are produced by a two stage reduction of an Au ion doped sol-gel and results in small (2 – 15 particles) aggregates of monodispersed ~ 80 nm Au nanoparticles covering the outer layer of $\sim 1\text{mm}^2$ SiO_2 substrate.

Spectral acquisition

The bacterial spectra were acquired with an RM-2000 Renishaw Raman microscope employing a 50× objective and excited at 785 nm. SERS spectra were obtained with incident laser powers in the 0.2 – 2 mW range in ~10 seconds of illumination time. The observed spectra typically resulted from ~10 bacterial cells within the field of view (~100 μm^2). The 520 cm^{-1} band of a silicon wafer was used for frequency calibration. Peak frequency precision is $\pm 0.5 \text{ cm}^{-1}$.

Data fitting analysis

GRAMS/AI™ Spectroscopy Software was used to manually baseline correct the experimental SERS spectra. Averaged bacterial spectra were empirically best-fit by adjusting component contributions from adenine, hypoxanthine, xanthine, guanine, uric acid and AMP. Normalized SERS spectra of 20 μM aqueous solutions of these purines were independently obtained for this analytical purpose. Due to the broad SERS spectral baseline variability and some systematic vibrational frequency shifts between the bacterial and purine only solutions, better fits to the observed bacterial spectra were achieved by this empirical fitting procedure as compared to standard automated best-fitting procedures.

Nano-electrospray ionization mass spectrometry

3–5 μL of filtered (0.22 micron filter, Eppendorf) bacterial supernatant were added to an equivalent volume of 0.2% formic acid in acetonitrile. The resulting mixture was introduced into an LTQ-Orbitrap XL mass spectrometer (Thermo-Fisher Corp., San Jose, CA) using a TriVersa NanoMate system (Advion Biosciences, Ithaca, NY). A low-flow ESI chip was used as a static emitter to deliver the sample at 20–40 nL/min. The LTQ-Orbitrap XL was operated in the nanospray positive-ion mode with acquisition over a mass range of m/z 80–1000. All mass spectra measured for ions detected in the Orbitrap were recorded with 60,000 resolution at m/z 400. Mass spectra were generated by averaging scans over a period of 1–2 min. Mass accuracy was better than 2 ppm.

Results

SERS spectra of some model compounds and bacteria

Initial evidence suggesting the molecular origins of the 785 nm excited SERS spectra of vegetative bacterial whole cells was provided by comparison of SERS spectra of bacteria and aqueous solutions of some model compounds. For example, as seen in Fig. 2a, the *S. aureus* (NCTC 8325) SERS spectrum on the Au substrate closely resembles that of a ~10 μM adenine solution, although the SERS spectra are not identical. The *S. aureus* frequencies are only slightly shifted from those in adenine. For example the most intense 739 cm^{-1} band, corresponding to the ring breathing mode transition of adenine, is slightly red-shifted to 737 cm^{-1} in the bacterial spectrum.

In contrast to *S. aureus*, the 785 nm excited SERS spectrum of *E. faecalis* ATCC 29212, (Fig. 2b) and *A. baumannii* ATCC 17978 (Fig. 2c) closely resembles the SERS spectrum of hypoxanthine and xanthine, respectively. Other vibrational bands are also evident in the SERS spectra of these bacterial samples, but the contribution of these compounds to their

respective bacterial spectra seems evident. Thus, a different purine molecule, either adenine, hypoxanthine or xanthine, appears to make the dominant molecular contribution to each of the three bacterial SERS spectra shown in Fig. 2.

Isotopic substitution

SERS spectra of *S. aureus* (ATCC 25904) and *B. anthracis* Sterne cultured in (normal) ^{14}N and isotopically labeled ^{15}N culture media are compared in Fig. 3. Red-shifted vibrational frequencies are observed for some of the bands in the SERS spectra of the ^{15}N cultured bacteria. SERS spectra of ^{14}N and ^{15}N labeled purine bases are also shown in this figure. The 14 cm^{-1} red shift of the $\sim 737\text{ cm}^{-1}$ in plane ring breathing mode and the 20 cm^{-1} red-shift of the 965 cm^{-1} ring breathing mode found for the *S. aureus* ^{15}N cultured SERS spectra are identical, within experimental precision, to the same down shifts observed for SERS spectrum of ^{15}N adenine relative to ^{14}N adenine (Fig. 3a). This is further evidence that adenine is the molecule responsible for a significant portion of the *S. aureus* SERS spectrum.

The SERS spectra of ^{14}N and ^{15}N cultured *B. anthracis* Sterne are compared with computed SERS spectra resulting from a best-fit determined (*vide infra*) linear combination of adenine, hypoxanthine and guanine SERS spectra for each nitrogen isotope in Fig. 3b. This linear combination also matches the observed red-shifts seen in the ^{15}N *B. anthracis* Sterne spectrum relative to the corresponding ^{14}N spectrum. The vertical lines in Fig. 3b highlight how the vibrational red shifts, which range from ~ 0 to 22 cm^{-1} , are captured by the purine-based simulated spectrum for bands at 668, 730, 964, 1358 and 1455 cm^{-1} in the ^{14}N spectrum. These vibrational isotopic shifts are consistent with the largest features in the *B. anthracis* Sterne SERS spectrum being assigned to purine vibrational transitions arising from a combination of adenine, hypoxanthine and guanine.

Cellular vs. supernatant SERS spectra

SERS spectra of four representative bacterial species (*E. faecalis*, *E. coli*, *B. cereus* and *S. aureus*) and their corresponding enriched supernatants are displayed in Fig. 4. The supernatant has been enriched by approximately an order of magnitude based on volume reduction as a result of lyophilization before being placed on the SERS substrate. As seen in this figure, each of the normalized supernatant spectra is nearly identical to the corresponding bacterial cell SERS spectrum when excited at 785 nm. Consequently, these pairwise comparisons reveal that these bacterial SERS signatures are *not* due to structural bacterial cell wall features and must arise from small molecules sufficiently water soluble at biological concentrations, which have been secreted from the bacterial cells and collect in the exogenous regions of these organisms. The SERS signals are larger for samples containing the cells where these molecules must be more highly concentrated (*vide infra*).

Purine fits to 785 nm excited bacterial SERS spectra

Given the above evidence suggesting that bacterial SERS spectra are due to small purine-like molecules, each of the observed bacterial SERS spectra shown in Fig. 1 was fit to a linear combination of the purine SERS spectra of adenine, hypoxanthine xanthine, guanine, uric acid and AMP. The SERS spectra of $20\text{ }\mu\text{M}$ solutions of these compounds on Au nanostructured substrates excited at 785 nm and normalized to the maximum of the adenine

spectrum are shown in Fig. 5. SERS spectra will be especially sensitive to the presence of adenine due to the large relative cross-section of its 739 cm^{-1} band (Fig. 5). The peak vibrational frequencies are shifted slightly from those observed in bulk solution Raman (non-SERS) measurements due to interactions with the Au nanoparticle surface and local solvation environment effects, as is well known for SERS.[50–52] SERS frequencies may be even slightly further perturbed relative to SERS spectra of pure aqueous solution potentially due to local pH, ionic strength or molecular complex formation in the bacterial extracellular region.

Best fits of the ten bacterial SERS spectra shown in Fig. 1 resulting from a linear combination of the six component purine spectra (Fig. 5) are displayed in Fig. 6. Excellent fits to all the observed bacterial spectra are achieved by this procedure. Nearly all vibrational features and their relative intensities seen in each of these bacterial SERS spectra are captured by this fitting procedure. Best-fits were achieved empirically due to the variable broad spectral background and the small vibrational frequency shifts observed for some bands in the bacterial spectra relative to the aqueous solution component spectra alone as mentioned above.

Since all bacterial vibrational features result from these small purine molecules and can also be seen in the SERS spectra of the corresponding cell supernatant (Fig. 4) no components of cell walls, i.e. peptidoglycan or any of its constituents, proteins, carbohydrates, or nucleic acids are required to explain the features evident in these bacterial SERS spectra and the dominant features in 785 nm SERS spectra can be assigned to a handful of purine molecules. The best-fit determined relative amounts of the purine components contributing to each of these bacterial spectra are reproducibly different from one another and summarized in Table 1. For a given purine component, the first number in a purine specific column in this table is the best-fit determined amplitude of the normalized component SERS spectrum to the observed bacterial spectrum; the second number is the relative contribution corrected by the relative SERS susceptibilities evident in Fig. 5. As seen in Table 1, adenine makes the overwhelmingly dominant contribution to the *S. pneumonia* TIGR4 and *S. aureus* NCTC8325 SERS spectra, hypoxanthine makes the largest molecular contribution in the SERS spectra of *B. anthracis* Sterne, *E. faecium* DO and *E. faecalis* ATCC 29212, and high uric acid contributions are only observed in the *P. putida* S16 and *A. baumannii* ATCC 17978 SERS spectra among this group of organisms. The subsequent distinctions, as for example, between the *S. pneumonia* TIGR4 and *S. aureus* NCTC8325 SERS spectra, result from the different relative amounts of the purines making smaller contributions to these spectra, i.e. the relative amounts of hypoxanthine, guanine, xanthine and AMP. As evident from the spectral fitting analysis summarized in Table 1, the different SERS signatures of vegetative bacterial cells is due to the unique characteristic concentration of purines in the extracellular metabolome surrounding the bacterial cells.

Biochemical origins of the 785 nm excited SERS spectra of bacteria

The SERS spectra of washed vegetative bacterial cells are reproducible vibrational signatures and have been shown for a number of species to be largely independent of typical growth media.[53] The purines contributing to these SERS signatures are the metabolites of

purine degradation.[54] The purine metabolic pathways are a highly conserved network of enzymatic reactions in all organisms and are responsible for the degradation of nucleotides and nucleic acids.[54] The end products of these degradation processes in bacteria are the set of purines that are found to dominate the SERS spectra shown here: adenine, hypoxanthine, xanthine, guanine, uric acid and AMP. The observed SERS spectra of bacteria result from these metabolites that have been secreted during sample preparation and manipulation. The concentration of these molecules is largest in regions closest to the cells they are secreted from when placed on the SERS substrates but these molecules, which range from slightly soluble to soluble in water, are found in the supernatant solution as well (Fig. 4).

The relative concentration of these purines for each organism is dependent on several factors but the most crucial appears to be the specific set of enzymes that are present for a given species (strain) in the nucleotide metabolic pathways. The genomically-determined absence or presence of specific enzymes accounts for the large range of relative concentration of these purines in the metabolome surrounding these cells and thus determining the 785 nm excited SERS spectra. The enzymes that are present or missing in the purine metabolic pathway for each bacterial species in Fig. 1 are known and catalogued in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.[54] The observed SERS spectra of these species (Fig. 1) can be rationalized in terms of these active purine metabolic pathway reactions and the resulting relative purine degradation products.

A diagrammatic representation of the active metabolic pathways are shown in Fig. 7 for six representative bacteria whose SERS spectra are given in Fig. 1. Each box corresponds to a specific nucleotide, nucleoside or purine base in the degradation of RNA, DNA or di- or tri-phosphate nucleotides. The arrows correspond to the enzymes present that convert the indicated product to reactant. In addition, some enzymes act reversibly depending on the relative concentrations, coenzymes and other allosteric regulation mechanisms and are indicated by a double-sided arrow. This reversibility also indicates how this network of enzymatic reactions functions as a purine salvage pathway in addition to a purine degradation pathway. As seen in these purine degradation pathway summaries (Fig. 7), the end products are the free purine bases that dominate the bacterial SERS spectra: adenine, hypoxanthine, xanthine, guanine and uric acid. Furthermore, this is just a subset of enzymatic reactions that impact the purine metabolic pathways but appear to be most directly linked to the appearance of the main constituents of the SERS spectra reported here. [54] The enzymatic reactions feeding into the nucleoside monophosphate (GMP, XMP, IMP, AMP) reactants at the top of this diagram are not explicitly included.

As seen in Fig. 7, different combinations of enzymes are present or absent in the metabolic degradation pathways for each of these six bacterial species. The distinct set of active purine metabolism enzymes can be used to explain or at least rationalize the observed different SERS signatures. For example, adenine dominates the *S. aureus* SERS spectrum (Table 1). Correspondingly, adenine containing nucleotides are only metabolized to adenine by adenine phosphoribosyltransferase for *S. aureus* NCTC 8325 (Fig. 7).[54] As discussed further below, this is a reversible enzymatic reaction. In contrast to the other metabolic pathways shown in Fig. 7, no enzymes are genetically coded for this organism that permit this nucleotide to be directly converted to inosine or hypoxanthine. *A. baumannii* on the other

hand, has no enzymatic pathways for adenine containing nucleotides to form adenine (Fig. 7), and hence no adenine is apparent in the SERS spectrum of this organism (Table 1). Instead, reversible enzymatic pathways are available to form guanine, xanthine, hypoxanthine and uric acid as indicated in Fig. 7, with accumulations of xanthine and uric acid dominating the observed *A. baumannii* SERS spectrum (Table 1). Enzymes are present in *A. baumannii* that can convert hypoxanthine and guanine into xanthine (Fig. 7). In contrast, *P. putida* S16 has the enzymes converting inosine and xanthosine to hypoxanthine and xanthine, and some ability to produce adenine and guanine. Correspondingly, the *P. putida* S16 SERS spectrum exhibits xanthine, guanine and uric acid and a very small amount of adenine and AMP. *B. anthracis* is missing the enzymes converting adenosine to inosine (adenosine deaminase), hypoxanthine to xanthine, and guanine to xanthine (See Fig. 7). Consequently the SERS spectrum of *B. anthracis* is due to large concentrations of hypoxanthine and guanine, and a smaller contribution from adenine (Table 1). The *E. coli* ATCC 8739 and *E. faecalis* ATCC 29212 strains have the enzymes that can interconvert adenine, hypoxanthine, xanthine and guanine but this *E. faecalis* strain is missing enzymes that convert XMP and IMP to xanthosine and inosine respectively (Fig. 7). Hypoxanthine makes the largest contribution to both of the SERS spectra of these organisms with lesser amounts due to xanthine, guanine and adenine in different proportions presumably resulting, in part, from the indicated differences in enzymes present for these two species.

Mass spectra of bacterial supernatants

Since the bacterial supernatant yields nearly the same SERS spectrum as that seen for predominantly cellular samples, we can use mass spectrometry to additionally confirm the absence or presence of molecules in these extracellular solutions and contrast this methodology with these SERS results. The mass spectrum in the 100 – 200 m/z regime of the *S. agalactiae* M781 supernatant wash is displayed in Fig. 8. The most intense features in this lower mass region are amino acids (valine, leucine, glutamate, phenylalanine) and do not correspond to the purines that dominate the bacterial SERS spectra. Masses corresponding to adenine and hypoxanthine (and xanthine and guanine, not shown here) are more than an order of magnitude weaker than these strongest signals. Furthermore, all the purines that are present (adenine, hypoxanthine, xanthine and guanine) or absent (uric acid, AMP) in the *S. agalactiae* M781 supernatant SERS spectrum (Table 1) are similarly evident or missing in the corresponding mass spectrum. Analogous results were found for the supernatants of two other bacterial species, *S. aureus* ATCC 25904 and *E. coli* ATCC 8739 (Electronic Supplementary Material (ESM), Figs. S1, S2 and Table S1) investigated by mass spectrometry. These data illustrate that the molecular susceptibilities for mass spectrometry and SERS are very different and thus these techniques are complimentary. Furthermore, the mass spectra data are consistent with the observed bacterial SERS spectra whose characteristic bands result from the different purines appearing in the bacterial exogenous metabolome.

Effects of genetic mutations on a bacterial SERS spectrum

To further confirm the molecular origins of the bacterial SERS signals and illustrate how SERS may be employed as a probe of cellular metabolic reactivity, SERS spectra of a parent *E. coli* strain BW25113 and two gene deletion mutants were acquired and are shown in Fig.

9 along with the corresponding best-fit spectra resulting from the linear combination of purine components: adenine, hypoxanthine, xanthine, guanine, uric acid and AMP. The best-fit determined relative amounts of these purines to the SERS spectra of these three *E. coli* strains are summarized in Table 2. Hypoxanthine and xanthine make the largest contributions to the observed SERS spectrum of the parent strain with adenine making a much smaller, although measureable contribution as well. Diagrammatic summaries of the purine metabolic pathways for the parent and two *E. coli* mutants are also shown in Fig. 9. The effect of deleting the gene encoding adenine deaminase (*ade*), the enzyme converting adenine to hypoxanthine, is relatively small on the SERS signature (Table 2). Interestingly, the effect of deleting adenosine deaminase (*add*), the enzyme that converts adenosine to inosine (Fig. 9), has a much larger effect on the SERS spectrum than the *ade* deletion, resulting in the appearance of a large amount of adenine in the extracellular metabolome (Table 2). Xanthine and some guanine also contribute to this spectrum, but no significant amount of hypoxanthine is evident in the SERS spectrum of this *add* mutant. In another *E. coli add* mutant (CGSC#5937) derived from a different *E. coli* parent strain (CGSC#6925), the *add* SERS spectrum is similarly dominated by adenine, in contrast to the parent SERS spectrum (See ESM Fig. S3 and Tables S2a and 2b). Thus, it appears that *add* more efficiently facilitates the formation of hypoxanthine in the adenine nucleotide degradation process as compared to the *ade* reaction pathway. In the *add* deletion mutants, adenine is secreted to the metabolome more efficiently than *ade* can convert it to hypoxanthine (Fig. 9, and ESM Fig. S3). This may be attributable to some negative feedback mechanism, allosteric interaction, lack of required co-enzyme or differential kinetic factors (e.g. K_m for *ade* is much larger than K_m for *add*[55]). Furthermore, the small effect on metabolic products resulting from the *ade* deletion is also consistent with previous observations that disruption of the *ade* gene had no obvious phenotypic consequences. [56] SERS spectra of these set of mutants further confirm that the 785 nm excited SERS spectra of bacteria are not from any cell wall components but due to the purine end products of nucleotide degradation.

Purine metabolism kinetics

Most observed SERS spectra appear promptly after the water washed bacterial cells are placed on the SERS substrate and the ~1 μ L of supernatant water surrounding the suspended cells has evaporated. Signal acquisition has been described as prompt, although the four cycles of washing, sample placement and ~1 μ L water evaporation takes about 15 minutes. However for *Staphylococcus* and *Streptococcus* species, the maximum signal is not observed until 30 to 60 minutes after completion of the washing procedure. A SERS spectrum of *S. aureus* ATCC 25904, observed promptly, designated as $t = 0$, and a spectrum of the same bacterial sample after a 60 minute delay and maintained at room temperature, are plotted on the same relative intensity scale in Fig. 10. A best-fit of the $t = 60$ spectrum reveals that the SERS spectrum of this *S. aureus* strain is predominantly due to adenine (95%) and a very small contribution from guanine (5%) (ESM, Fig. S4, Table S3). More significantly, however, the $t = 60$ minute spectrum is ~40 times more intense than the $t = 0$ spectrum judging by the relative intensities of the bacterial 736 cm^{-1} adenine band. Adenine phosphoribosyltransferase catalyzes the reversible reaction of AMP to adenine,[57] as well as GMP to guanine, in the well-established purine metabolic degradation/salvage network by the following reaction: [54]

The catalyzed transformation of AMP via this degradation step requires diphosphate (diphosphoric acid) forming 5-phospho-alpha-D-ribose 1-diphosphate, (PRPP) and adenine (Scheme 1). When the *S. aureus* cells were washed with a 1 mM solution of sodium pyrophosphate, forming diphosphate by hydrolysis, an intense prompt SERS spectrum identical to the water washed $t = 60$ minute spectrum was observed as soon as the cells were placed on the SERS substrate and the surrounding water evaporated (Fig. 10). These data are consistent with the source of the SERS spectrum of *S. aureus* predominantly resulting from the metabolic degradation of AMP. The enzymatic conversion of AMP (and GMP) to adenine (and guanine) has been accelerated by the cellular uptake of the excess diphosphate and thus accounts for the faster rate of adenine appearance in the cell's exogenous metabolome.

Discussion

All of the evidence cited above unequivocally establishes that the 785 nm excited SERS spectra of bacterial cells are dominated by the reproducible SERS contributions of the free purine nucleobases: adenine, hypoxanthine, xanthine, guanine and uric acid, and AMP. We attribute these observed molecular signals to result from the bacterial cell stress response to the no-nutrient, water only environment they are placed in during sample washing and signal acquisition. In these starvation conditions, it is known that the stringent response of bacterial cells is stimulated.[58, 59] Metabolic rates can change rapidly as the bacterial cell adopts a survival strategy. Cell growth is suspended, and most energy dependent *de novo* biosynthesis pathways are turned off. The cell adopts this survival strategy in order to enable bacteria to persist in stressful environments, reorganizing metabolic activity for both maintenance and survival until conditions potentially become favorable again for the resumption of growth. [60][61] Nucleobases are not normally present as free bases in the intra- or extracellular regions but are almost exclusively found as nucleotides under normal nutrient growth conditions.[60] Recent studies have reported increased concentrations of various purines when bacteria are subjected to starvation conditions and their immediate uptake once nutrients are supplied. For example, in an HPLC based study endogenous and exogenous accumulation of nucleobases (xanthine, hypoxanthine and uracil) was reported in the metabolome of *E. coli* when cells entered the stationary phase.[60] LC-MS measurements of *E. coli* metabolites showed dramatic increases of adenine, hypoxanthine and AMP concentrations following carbon starvation.[62] Most recently, a real-time ESI-TOF-MS study of bacterial cultures injected directly into the MS sampling loop revealed purine base (xanthine and hypoxanthine) accumulation during bacterial starvation in the intra and extracellular metabolome. These concentrations immediately diminished with the addition of glucose to the nutrient environment.[61] These observations reveal the transient accumulation of exogenous nucleobases during conditions causing declining growth rates, such as nutrient starvation conditions, and are consistent with the SERS results reported here where spectra are obtained after cells have been enriched from nutrient rich environments and repeatedly washed in pure water prior to signal acquisition.

Furthermore, the enzymes responsible for the conversion of the purine XMP nucleotides to the base X, such as adenine, guanine and hypoxanthine phosphoribosyl-transferases, are located at the cell membrane and not only facilitate the $X \rightleftharpoons XMP$ conversion but also

transport these reactants and products across the cell membrane.[57, 63, 64] Hence, the SERS intensities are generally the most intense for excitation of spatial regions closest to the bacterial cell wall regions. Although the exact details of why secretion of these purines occurs as part of the starvation response is not clear, it is recognized that nucleotide degradation during starvation is necessary for cell viability[65] and this process has been attributed at least to the degradation of rRNA as part of the metabolic transformation in response to starvation. [60, 65] It may be speculated that this degradation process provides a low cost energy source for cell maintenance and survival during the low nutrient conditions, as well as serving as an instant nitrogen source for the bacterial community once a carbon source or other required nutrients are available. It has been well established that glucose stimulates purine uptake by orders of magnitude in low nutrient conditions[63] and their uptake provides a metabolically cheap one-step salvaging pathway for nucleotide production over the more costly *de novo* biosynthesis during growth resumption. [61]

Conclusions

Conclusive evidence is presented here that clarifies the molecular and biochemical origins of the SERS spectra of bacterial cells excited at 785 nm on Au substrates. The SERS spectra of vegetative bacterial cells harvested from culture media and washed in water result from the purine bases adenine, xanthine, hypoxanthine and guanine, as well as uric acid and AMP. These compounds appear at the outer layer of bacterial cells and in the extracellular metabolome, and result from the degradation of nucleotides that occurs as part of the bacterial starvation response. The characteristic SERS spectra are due to the different exogenous concentrations of these six compounds that contribute to each of the bacteria-specific SERS spectra. The observed ^{15}N isotopic vibrational frequency shifts, the near equivalence of SERS spectra of the enriched supernatant and bacterial cells, and the excellent fits of the observed bacterial SERS spectra to linear combinations of these six molecular purine SERS spectra, unequivocally identify these molecular species as the major constituents that account for the 785 nm excited SERS spectra of bacteria. These molecular species are secreted from the cells and appear most heavily concentrated in the extracellular regions near the outer cell walls where purine phosphoribosyltransferases, enzymes that convert purine mononucleotides to purine bases and transport the products across cell membranes, are located. These purines are the end products of the metabolic degradation of nucleic acids and nucleotides, such as RNA, ATP, GTP and other nucleotide containing molecules, which rapidly accumulate when bacteria are placed in a nutrient-free environment. Experiments with defined bacterial mutants and substrate perturbation of a purine catalytic pathway further confirm the purine metabolic origin of these bacterial SERS spectra. The rapid exogenous increase in nucleobase concentration is part of the characteristic stringent or starvation response of bacterial cells. The SERS spectra of the bacterial species analyzed here are each unique due to the different amounts of these purine components in this extracellular region and can be understood in terms of the different enzymes that are present or functional for a given organism. This result is in contrast to the long held assumption that structural cell wall components, more specifically the peptidoglycan layer components, NAG, NAM, lipids and proteins make the dominant

contribution to the observed SERS spectra at 785 nm and account for their unique characteristic vibrational signature.

Although the bacterial SERS spectra excited at 514.5/532 nm are strikingly similar to each other, they do not resemble the 785 nm SERS spectra of bacteria as has been noted previously.[13, 30, 44] The 514.5/532 nm excited bacterial spectra are nearly equivalent to SERS spectra of FAD.[27, 49] FAD has an electronic absorption in the 500 – 350 nm region, [66] hence at 514.5/532 nm this molecule will be electronically as well as plasmonically enhanced due to this near resonance.[44] Purine absorptions are further in the UV, hence the 514.5 nm signature of bacterial SERS is dominated by this single molecule, which provides little basis for diagnostic purposes.

The results described here provide part of the justification for the success of a 785 nm SERS-based methodology to distinguish different bacterial cell types enriched from culture media or human body fluids for rapid, growth free infectious disease diagnostics. Whereas many of the observed relative intensities of the bacterial SERS spectral features can be qualitatively rationalized in terms of the absence or presence of specific purine degradation enzymes (Figs. 7, 9), it remains for future studies to demonstrate the extent that strain specific diagnostic identification may be robustly accomplished by this SERS-based metabolic profiling approach. Preliminary data acquired in our lab point to the ability of these SERS to provide strain specificity classification within some well-defined bacterial classes.[32, 67, 68]

The bacterial exogenous metabolome is a complex mixture that contains many more compounds[60–62] than the purines that identify these bacterial species via SERS. Since purines dominate the bacterial SERS spectra, the SERS susceptibilities of these compounds must be much larger than those of other molecular components in these mixtures such as leucine, glutamic acid, phenylalanine, valine, arginine, succinic acid, and pyroglutamic acid, which generally appeared with larger signal strength than the purines in mass spectra of bacterial supernatants (Fig. 8 and ESM, Figs. S1 and S2). Furthermore, despite the apparent close proximity of the bacterial cell wall, lipids, polysaccharides, peptidoglycan or protein components of the cell wall structure do not contribute to these bacterial signatures as well. The gold nanostructured surface apparently has a strong affinity and preferentially interacts with these multiple nitrogen containing purines despite the large number of compounds in the cellular environment. It is well-known that SERS intensity enhancements are strongly dependent on the details of the surface-analyte interactions[69, 70] and thus the Au nanoparticle structures are selectively enhancing for these purine compounds. The nanostructured Au surface not only acts a local Raman amplifier but is also a highly selective filter for a preferential class of small molecules resulting in the observed purine dominated SERS spectra of bacteria with 785 nm excitation.

The time-resolved SERS measurement described above (Fig. 10) illustrate how real time SERS measurements can be exploited to monitor the rate of the appearance of this fundamental class of metabolites in the extracellular region and how the kinetics of purine metabolic pathway reactions may be manipulated for studies of factors that affect these metabolic processes and their relationship to other biosynthetic mechanisms and

metabolites. As demonstrated here, SERS offers the potential advantages of speed, ease-of-use and simpler sample handling protocols with high sensitivity relative to chromatography or mass spectrometry based techniques for metabolomics profiling, at least for some metabolites. Future SERS applications may include examination of the effect of defined bacterial genetic mutants, turnover rates, co-enzyme dependence, and the effect of therapeutics on bacterial or cell types either entering or leaving stress environments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

785 nm excited SERS spectra of ten bacterial species on Au nanoparticle substrates. Displayed spectra are averages of 4 – 6 individual spectra and normalized to the intensity of the strongest feature in each spectrum. Gram positive (+) and negative (–) types are indicated for each.

Figure 2.

Comparison of SERS spectra of three bacterial species and some model compounds. The SERS spectra of *S. aureus* (NCTC 8325), *E. faecalis* (ATCC 29212) and *A. baumannii* (ATCC 17978) are compared with adenine, hypoxanthine and xanthine SERS spectra, respectively. The SERS spectra of the model compounds are shown in red; the SERS spectra of the bacteria are in black and have been raised by 0.25 units for viewing convenience. Contributions from these compounds appear dominant in each of these bacterial SERS spectra.

Figure 3.

SERS spectra of *S. aureus* (ATCC 25904) and *B. anthracis* Sterne grown on both ¹⁴N and ¹⁵N growth media compared to ¹⁴N and ¹⁵N labeled purines. In 3a, experimental ¹⁵N isotopic red-shifts of *S. aureus* spectra match those observed for ¹⁵N labeled adenine. In 3b, observed isotopic red-shifts of *B. anthracis* Sterne match those observed for best fit determined linear combination of ¹⁵N labeled adenine, hypoxanthine and guanine. See text for more details.

Figure 4.

Comparison of the SERS spectra of four bacterial strains (blue) and their corresponding enriched supernatant (red). The supernatant has been enriched by approximately an order of magnitude before analysis. Each displayed spectrum is normalized by its band maximum, and enriched supernatant spectra are offset for better viewing.

Figure 5.

SERS spectra of 20 μM aqueous solutions of the indicated purine components of bacterial SERS spectra. Spectra have been offset for viewing and are normalized to the maximum peak intensity of the 20 μM adenine solution.

Figure 6.

Empirically determined best-fits (red) of the bacterial spectra (black) shown in Fig. 1 to a linear combinations of purine (adenine, hypoxanthine, xanthine, guanine, uric acid and AMP) SERS spectra shown in Fig. 5. Excellent fits are obtained for all bacterial SERS spectra.

Figure 7.

A subset of the purine metabolic pathways given by the KEGG database[54] for six of the ten bacterial species shown in Fig. 7 that result in the formation of the free nucleobases: adenine, hypoxanthine, xanthine, and guanine, and uric acid and AMP. Single and double-sided arrows correspond to enzymes present for each of the indicated reactions.

Figure 8.

Positive-ion nESI mass spectrum of the *S. agalactiae* M781 supernatant plotted over the 120 – 160 m/z range. Expanded (×20) intensity scale shows m/z bands corresponding to adenine and hypoxanthine. Xanthine and guanine were also identified in this mass spectrum. Uric acid and AMP, however, were not evident in this mass spectrum but could be detected in the mass spectra of other samples recorded under the same experimental conditions.. The results are in qualitative agreement with the SERS results (Table 1 and ESM Table S1).

Figure 9.
The SERS spectrum of an *E. coli* parent strain BW25113 and two mutants, JW3640-1 (Δade) and JW1615-1 (Δadd) and their corresponding purine metabolism pathway diagrams.

Figure 10. SERS spectra of *S. aureus* ATCC 25904 as a function of time and as a function of diphosphate exposure. The SERS spectrum appears immediately when the *S. aureus* cells are washed with a 1 mM sodium pyrophosphate solution.

Scheme 1.

AMP – adenine conversion catalyzed by adenine phosphoribosyltransferase.

Table 1

Best-fit determined relative (%) purine components to bacterial spectra of 10 species in Figure 1.

Organism	Source or Reference	Adenine ^a	Hypoxanthine ^a	Xanthine ^a	Guanine ^a	Uric acid ^a	AMP ^a
<i>S. agalactiae</i> M781	ATCC	8/2	39/56	47/38	6/4	0	0
<i>S. pneumonia</i> TIGR4	Reference[71]	65/62	0	3/8	0	0	32/30
<i>B. anthracis</i> Sterne	Colorado Serum Co.	27/9	45/71	0	28/20	0	0
<i>S. aureus</i> NCTC8325	Reference[72]	75/69	0	0	8/16	0	17/15
<i>P. putida</i> S16	Reference[73]	2/6	0	12/11	19/14	54/71	12/4
<i>P. aeruginosa</i> PA14	Reference[74]	31/13	17/34	11/13	36/32	5/8	0
<i>E. faecium</i> DO	Reference[75]	41/14	40/70	5/5	14/11	0	0
<i>E. faecalis</i> 29212	ATCC	21/5	61/80	10/8	8/7	0	0
<i>E. coli</i> 8739	ATCC	14/3	49/54	25/28	13/14	0	0
<i>A. baumannii</i> 17978	ATCC	0	0	60/55	10/7	30/38	0

^aThe first number is the % contribution of the purine component SERS spectrum normalized by the spectrum maximum, the second number is the % contribution scaled by the relative cross section of the purine component and is thus the relative number of purine component molecules accounting for the SERS signature.

Table 2

Best-fit determined relative (%) purine components to an *E. coli* parent strain and two mutants.

Organism	adenine	hypoxanthine	xanthine	guanine	uric acid	AMP
<i>E. coli</i> BW25113 (parent)	7/2	37/53	56/45	0	0	0
<i>E. coli</i> JW3640-1 (<i>ade</i>)	0	48/62	52/38	0	0	0
<i>E. coli</i> W1615-1 (<i>add</i>)	75/53	0	17/34	8/13	0	0

^aThe first number is the % contribution of the purine component SERS spectrum normalized by the spectrum maximum, the second number is the % contribution scaled by the relative cross section of the purine component and is thus the relative number of purine component molecules accounting for the SERS signature.