Use of Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy To Identify Microbial Metabolic Products on Carbonate Mineral Surfaces^{∇†}

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This paper demonstrates the use of attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy to detect microbial metabolic products on carbonate mineral surfaces. By creating an ATR-FTIR spectral database for specific organic acids using ATR-FTIR spectroscopy we were able to distinguish metabolic acids on calcite surfaces following *Escherichia coli* growth. The production of these acids by *E. coli* was verified using high-performance liquid chromatography with refractive index detection. The development of this technique has allowed us to identify microbial metabolic products on carbonate surfaces in nutrient-limited cave environments.

Molecular phylogenetic analysis using the 16S rRNA gene sequence has revolutionized microbiology, allowing investigators to create comparative and evolutionary phylogenies for microbial species (49, 50). Phylogenetic placement also allows metabolic inference for the activity of previously uncultivated species within the environment, dramatically changing our understanding of microbial ecology and the role of microbial species within the biosphere (3, 36, 41). While metabolic inference is a valuable tool for estimating metabolic activity, specific metabolic responses to environmental conditions are harder to estimate, even for closely related species (1, 24). To overcome such limitations, investigators can employ a number of different techniques to relate identified microbial phylotypes to environmental metabolic activities, including the use of isotopic probing and quantitative mRNA and metagenomic analyses (43). Such techniques are technically challenging and often difficult in the context of low-biomass environments (12). In this paper we evaluated the utility of attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy to examine microbial metabolic products on carbonate rock surfaces in low-biomass environments. ATR-FTIR spectroscopy is a powerful technique to characterize solid/gas and solid/solution interfaces and has been applied to monitor adsorption of chemical reactions at surfaces in situ (14, 16, 22, 25, 33, 53). Our results demonstrate that ATR-FTIR spectroscopy may represent a technique that will enable investigators to correlate inferred metabolic activity, based on molecular phylogenetics, with geochemical measurement (10).

We have been studying microbial adaptation to starvation within cave environments (7–10). This research has been geared toward understanding what carbon and energy sources drive microbial community subsistence and growth during ex-

treme starvation, allowing us to identify the ecological mechanisms that drive high species diversity in cave environments (7). Past work suggests that soil-derived humic and fulvic acids can percolate significant distances (>300 m) into the subsurface and provide a source of nutrients and energy (10, 11). It is our hypothesis that these aromatic and polyaromatic soil-derived compounds contribute to the overall energy budget of these systems. Metabolic breakdown of such reduced compounds by heterotrophic microbial species can lead to the release of partially oxidized, secondary metabolic products (7), such as formic and acetic acids (17, 46–48). Such organic acids may then permit the growth of additional microbial species, which can use them as a source of carbon and energy (46–48, 51). The production of these organic acids may therefore promote resource sharing and allow the establishment of more-complex microbial communities, similar to those we have identified in extremely starved cave environments (<0.5 mg/liter total organic carbon) (10, 11). If these organic acids are available for microbial growth, then it should be possible to detect the presence of these compounds within the environment. To measure the presence of metabolic acids on rock surfaces, we aimed to determine whether spectroscopic techniques such as ATR-FTIR spectroscopy could detect organic acids on mineral surfaces. While ATR-FTIR spectroscopy would not make it possible to infer the carbon and energy sources for individual species, the presence of organic acids may explain the presence of certain members of the community, such as acetate-utilizing species (44), or the mineral changes observed following microbial growth (6, 35).

ATR-FTIR spectroscopy has been previously applied to evaluate carboxylic acid complexation on mineral surfaces; however, these studies were conducted in situ in the gas phase (2, 15, 52) or in aqueous solutions to assess surface coordination of adsorbates (18, 19, 21, 23, 32). To our knowledge, no one has determined if a profile of adsorbed organic acids can generate unique signatures on a surface that are stable over time and that could be used as a reference to monitor microbial metabolic activity. As our studies are carried out within cave environments, we determined whether the ATR-FTIR

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spectroscopy profile of eight common microbial metabolic products (acetic, butyric, citric, formic, glyoxylic, propionic, pyruvic, and succinic acids) could be detected on calcium carbonate, the primary mineral comprising the limestone in which most caves are formed (37). In our preliminary studies we used pure calcite ($>99\%$ CaCO_3) crystals. All chemicals, including the calcite crystals, were obtained from Sigma-Aldrich (Atlanta, GA) unless otherwise indicated. Either the organic acids were diluted to 1 M and dropped onto the crystal, in the case of highly reactive acids (as determined by rapid CO_2 liberation) such as acetic acid (pH 2.38) [$2\text{CH}_3\text{COOH} + \text{CaCO}_3 \rightarrow (\text{CH}_3\text{COO})_2\text{Ca} + \text{CO}_2 + \text{H}_2\text{O}$], or the crystal was soaked in the acid overnight at room temperature, as in the case of succinic acid (pH 4.92). ATR-FTIR spectroscopy measurements were made with a Nicolet Nexus 670 FTIR spectrometer (Waltham, MA) equipped with a DTGS detector and a MIRacle ATR accessory (Pike Technologies, Madison, WI) with a 45° Si/ZnSe crystal. Data collections and spectral calculations were performed using OMNIC software. Spectra were taken at 64 scans and with a resolution of 4 cm^{-1} . Pure calcite crystals were used because of the ability to form a tight interaction with the ATR crystal and the simplicity of the chemical reaction, compared with the complex chemistry of a cave rock (limestone) reaction.

The results (Fig. 1 and 2) demonstrate a recognizable signature for each organic acid. Infrared (IR) adsorption bands of reacted calcite correlate with IR spectra of calcium salts for the respective organic acids (i.e., calcium acetate, etc.) and are consistent with literature values (2, 5, 15, 18, 19, 21, 23, 32, 39, 40, 42, 45, 52). For example, in the case of acetic acid adsorption (Fig. 1C), the spectral features are consistent with the presence of acetate (CH_3COO^-) on the surface. The COO^- asymmetric ($1,550\text{ cm}^{-1}$) and COO^- symmetric ($1,412\text{ cm}^{-1}$) stretches indicate a Ca-acetate complex (15, 19, 39, 52). Similarly, in Fig. 1B the C=O carbonyl stretch ($1,708\text{ cm}^{-1}$) along with other bands (COO^- asymmetric stretch, $1,627\text{ cm}^{-1}$; COO^- symmetric stretch, $1,405\text{ cm}^{-1}$; CH_3 bend, $1,350\text{ cm}^{-1}$) correlates with pyruvate salts and pyruvate adsorbed on TiO_2 (19, 40). Sensitivity was evaluated by correlating IR band intensity ratios for acids (acetic acid, $1,020\text{ cm}^{-1}$; pyruvic acid, $1,708\text{ cm}^{-1}$) to the calcite peak ($1,795\text{ cm}^{-1}$) as a function of acid concentration ($1.0 \times 10^{-6}\text{ M}$ to 1.0 M). The organic acids demonstrate a Langmuir adsorption isotherm on calcite, consistent with adsorption of other organics onto metal oxide surfaces (4, 28), with a detection limit of $1.0 \times 10^{-4}\text{ M}$ (see Fig. S1 in the supplemental material). It should be noted that direct quantification is difficult with IR spectroscopy as the surface coverage by molecules may vary across the crystal surface.

It is likely that organic acids produced by microbial activity within the soil will react with the calcium carbonate (limestone) of the epikarst horizon, defined as the interface zone between soil and rock in karst landscapes, preventing percolation deep into the subsurface. Nonetheless, to rule out the possibility that any acids detected in this study were percolating through this zone, we set up a 15-mm-diameter, 200-mm-long column packed with calcium carbonate powder. A 25-ml aliquot of 0.1 M acetic acid (pH 2.88) was passed through this column. The aliquots collected showed undetectable amounts of acetic acid, analyzed using gas chromatography-mass spectrometry with ion trap detection (Varian Inc., Palo Alto, CA).

FIG. 1. Normalized ATR-FTIR spectra of (A) calcite, (B) calcite reacted with 1 M pyruvic acid, and (C) calcite reacted with 1 M acetic acid. The influence of acid adsorption coverage on IR spectra is shown in panel B. Variations in acid coverage on the calcite crystal can lead to IR sampling of both the calcite substrate and the deposited salt under low surface coverage (i) or only the calcium salt under high surface coverage (ii). The asterisk indicates poor subtraction of gas phase CO_2 adsorption on the surface.

Indeed, the ability of calcium carbonate to react with these acids was shown by mixing 20 mg of CaCO_3 with 1 ml 0.1 M acetic acid on an orbital shaker for 1 h. Following mixing, acetic acid could be detected only in trace amounts ($\sim 1 \times$

FIG. 2. Normalized ATR-FTIR spectra of acids reacted with calcite: (A) formic acid, (B) succinic acid, (C) butyric acid, (D) citric acid, (E) propionic acid, and (F) glyoxylic acid. The asterisk indicates poor subtraction of gas phase CO_2 adsorption on the surface.

10^{-5} M) in the remaining solution. We are therefore confident that reactive acids detected within the cave environment, often separated from the surface by tens or hundreds of meters of carbonate rock, are not percolating in from soil detritus.

The ATR-FTIR spectroscopy analysis of pure organic acids reacting with a calcite crystal demonstrated that this technique could detect and differentiate metabolic acids on these sur-

faces; however, this did not imply that such acids could be detected at the concentrations produced during microbial metabolism. To determine if organic acids produced at metabolic levels can be detected by ATR-FTIR spectroscopy, we placed calcite crystals in cultures of actively growing *Escherichia coli* K-12 cells. In this experiment, three small (~ 4 -mm) sterile calcite crystals were placed into 3 ml of lactose broth (15 g/liter

FIG. 3. HPLC-RI chromatograms of metabolic products produced by *E. coli* during growth in lactose broth under anaerobic conditions. Samples were removed from the same sample following incubation at 12, 24, and 48 h and subjected to chromatography as described in the text. The peaks correspond to lactose (1), pyruvic acid (2), glyoxylic acid (3), succinic acid (4), lactic acid (5), formic acid (6), and acetic acid (7), methanol (9), and ethanol (10). The unidentified eighth peak was also present in the uninoculated media. The methanol peak (9) represents the void volume from the needle wash in the HPLC autosampler.

with methyl red as a pH indicator) and inoculated with 0.1 ml of an overnight culture of *E. coli*. These cultures were incubated at 20°C under three different growth conditions: with shaking (aerobic), without shaking (microaerobic), and without shaking in a Bactron I anaerobic/environmental chamber (Sheldon Manufacturing, Inc., Cornelius, OR) containing 5% CO₂, 5% H₂, and the balance N₂ (anaerobic). Samples of broth (0.1 ml) and a calcite crystal were aseptically removed at 0, 12, 24, and 48 h. The organic acids produced during *E. coli* growth were measured in the growth medium at each time point using high-performance liquid chromatography (HPLC) with refractive index (RI) detection (HPLC-RI) on a Waters Alliance 2695 system with a 2414 RI detector (Waters Corporation, Milford, MA). The method employed was similar to that developed for fermentation broths (54) using an ion exclusion column (IC-Pak; 7.8 by 300 mm; 50 Å; 7 µm; Waters Corporation) at a flow rate of 0.6 ml/min and a mobile phase of 5 mM sulfuric acid. The chromatograms for each incubation revealed the production of numerous organic acids, such as formic and pyruvic acids, which correlated well with growth conditions, overflow metabolism, and the availability of oxygen to act as a terminal electron acceptor (Fig. 3; see Fig. S2 in the supplemental material) (47, 48).

As the calcite crystals would act as a buffer in the media during growth, controls were also prepared without calcite. We then monitored the effect of calcite on the production of organic acids using HPLC-RI. These data (not shown) did suggest that there was a subtle change in the production of individual acids in the presence of the calcite crystal, but we did not see a drastic change in the profile of organic acids produced by *E. coli* in the presence of this mineral (data not shown).

The calcite crystals removed from each *E. coli* culture condition were dried under ambient conditions and examined by ATR-FTIR spectroscopy as described above. The results (Fig. 4; see Fig. S3 in the supplemental material) demonstrated that it was

possible to detect organic acids produced at the levels of microbial metabolic activity on calcite surfaces using ATR-FTIR spectra. For example, ATR-FTIR spectroscopy of the anaerobic crystal (Fig. 4) indicates the presence of pyruvate, demonstrated by the unique C=O carbonyl stretch (1,710 cm⁻¹) along with other bands associated with the Ca-pyruvate complex (19, 40). In addition, distinct band signatures associated with acetate adsorption, at 2,918 cm⁻¹ and 2,847 cm⁻¹ (CH₃ asymmetric stretch/symmetric stretch vibrations) (34), along with bands at 1,050 cm⁻¹ and 1,021 cm⁻¹, point to the presence of acetate (Fig. 4A) (2, 15, 32, 39). Interestingly, it was possible to detect the presence of pyruvic acid in the spectra for the sample grown under anaerobic conditions (Fig. 4), even after the majority of pyruvic acid had been reassimilated from the medium (Fig. 3) (51). This demonstrates that this metabolic product remains on the surface of the rock as calcium pyruvate, presumably as a result of changing solubility or a structural change that limits transporter uptake. As a result a residual amount of organic material remained detectable on the calcite surface long after the soluble acid had been removed from the media (51). Over longer periods of incubation, the accumulation of *E. coli* cells growing on the surface of the rock began to obscure the ability to obtain a clear ATR-FTIR signature for many of the acids analyzed; adsorption bands associated with bacterial functional groups (i.e., phosphates and carbohydrates in the region of 950 to 1,150 cm⁻¹) become more evident during the incubation (Fig. 4) (26, 27, 38).

The ATR-FTIR spectroscopy results as a function of incubation time were correlated with an increase in the number of bacterial cells (measured by 4',6-diamidino-2-phenylindole [DAPI] staining and direct counts) covering the surface of each crystal from 2.15×10^5 to 3.80×10^5 cells cm⁻² at 12 and 24 h, respectively, under anaerobic conditions (Fig. 4A and B). The number of cells covering the surface of the crystal did not significantly increase at 48 h (3.82×10^5 cells cm⁻²), but the substantial extracellular matrix that covered the crystal may be

FIG. 4. Normalized ATR-FTIR spectra of calcite crystals exposed to *E. coli* growth in lactose broth under anaerobic conditions. Calcite crystals were removed at different incubation times: (A) 12 h, (B) 24 h, and (C) 48 h. ▼, bands associated with acetate; ◇, bands associated with pyruvate. The asterisk indicates poor subtraction of gas phase CO_2 adsorption on the surface.

responsible for obscuring the ATR-FTIR signal (Fig. 4C). Similar results were seen on the crystals grown under the other environmental conditions (data not shown). Nonetheless, such high levels of biomass are not normally associated with surfaces under nutrient-limited conditions, suggesting that such

FIG. 5. Normalized ATR-FTIR spectrum of a calcite crystal (A) placed within Lake Louise, Lechuguilla Cave, for 4 months and a polished limestone slide (B; host rock), placed within the Deep Secrets (LC27Z) Lake for 13 years. ▼, bands associated with acetate; ◇, bands associated with pyruvate.

accumulation may be less of an issue for environmental measurements (8, 11, 12).

The preliminary work testing ATR-FTIR spectroscopy as an analytical tool for metabolic products under these conditions was carried out on pure calcite (>99% CaCO_3) crystals using relatively high concentrations of organic acid; however, these results did not demonstrate that such a technique would work under the complex geochemical conditions and low metabolic activity normally associated with cave samples (8, 11, 37). We therefore tested the ability of this technique to analyze microbial metabolic activity on calcite crystals and a limestone slide that were placed in a low-nutrient cave environment. The calcite crystals were placed in two lakes within Lechuguilla Cave: (i) Deep Secrets Lake (LC27Z) at 297.5 m below ground and (ii) Lake Louise at 300.0 m below ground. The calcite crystals were placed just below the surfaces of these lakes and incubated in situ for 4 months. Following recovery, the crystals were examined by ATR-FTIR spectroscopy, as described above. The resultant IR spectroscopy profile clearly demonstrated peaks corresponding to the presence of both acetic and pyruvic acids on the surfaces of the crystals (Fig. 5A). Likewise,

a portion of a limestone slide made from polished host rock placed in Deep Secrets Lake in January 1994 was recovered and subjected to ATR-FTIR analysis. Again, the IR profile demonstrated the accumulation of acetic and pyruvic acids (Fig. 5B) on this more chemically complex geologic sample.

Our results (Fig. 5) demonstrate that the accumulation of both acetic and pyruvic acids in situ could be detected on mineral surfaces using ATR-FTIR spectroscopy. The presence of acetic acid supports previous observations (10) and our hypothesis on the role of reduced compounds entering the cave system; bacterial species may remove excess reducing equivalents through the production of organic acids (51). These acids in turn degrade the rock surface on which microbial species grow, leading to many of the structural changes on surfaces seen in these subterranean environments (6, 35). The presence of pyruvic acid is more difficult to explain, as bacteria generally excrete this acid only under conditions of overflow metabolism (47, 48). While the low levels of total organic carbon (<0.05 mg/liter) normally seen in this environment would hardly be considered excess energy, it is likely that the microorganisms living in this static subterranean environment have very slow metabolic processes (31). As we have demonstrated that pyruvic acid may remain on surfaces, even in the presence of a microbial species capable of assimilating this compound, it is possible that we are observing a result of transient environmental change within the system. Alternatively, given the large variety of species in cave environments and their likely complex metabolic interactions (10, 20, 29, 30, 35), the detection of pyruvic acid opens up a new line of investigation into microbial metabolic interactions in such systems. In addition, by placing calcite crystals over specific time periods in these lakes and correlating bacterial molecular phylogenetic profiles with the appearance of such acids using ATR-FTIR spectroscopy, it may be possible to correlate the major metabolic players and rates of metabolic activity in these extremely nutrient-limited environments.

Given the low biomass and amount of measurable metabolic activity in geomicrobial systems, ATR-FTIR spectroscopy represents a new technique to analyze bacterial interactions on exposed mineral surfaces. While the high reactivity of calcite with many acids can be viewed as a model for monitoring subtle microbial activity on a mineral surface, the ability of organic acids to adsorb to other surfaces may allow ATR-FTIR spectroscopy to detect geochemical activity on less-reactive minerals, such as those composing granite or quartzite. Given the increasing interest in examining the relationship between microbes and mineral surfaces, ATR-FTIR spectroscopy may prove to be a valuable technique for remote sensing of microbial metabolic products and nonvolatile organic acids (13).

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