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Leucine pools in *Escherichia coli* biofilm discovered by Raman imaging

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In structured communities of bacteria known as biofilms, a variety of biomolecules have been shown to play a unique role as signals and/or regulators in biofilm formation. Here, we report that high levels of the amino acid leucine (leucine pool) were detected, for the first time, within microcolonies in a 30-h-old *Escherichia coli* biofilm by Raman imaging. Localization of leucine revealed by multifrequency Raman images indicates leucine accumulation during the early stage of the *E. coli* biofilm formation, which may have resulted from physiological environment-specific metabolic adaptation. We demonstrate that our label-free Raman imaging method provides a useful platform for directly identifying still unknown natural products produced in biofilms as well as for visualizing heterogeneous distributions of biofilm constituents *in situ*.

Supporting information may be found in the online version of this article.

Keywords: Raman microspectroscopy; label-free imaging; biofilms; leucine

Introduction

Biofilms are structured consortia of sessile bacterial cells adherent to a surface that are encased in an extracellular matrix comprising exopolysaccharides, proteins, and DNA. While traditional microbiology has focused on planktonic bacteria, it is bacterial cells in biofilms that play a central role in many microbe-associated processes such as bacterial infections,[1,2] wastewater treatment,[3] and bioremediation.[4] In all these processes, various biomolecules serve as chemical signals, regulators, and structural components.[5] For example, in many species including *Pseudomonas aeruginosa*[6,7] and *Staphylococcus aureus*,[8] extracellular DNA has been shown to be required for initial biofilm formation. Very recently,[9] it has been reported that some D-amino acids trigger biofilm disassembly in *Bacillus subtilis* and other bacteria. These studies have been done mostly with biochemical assays and/or fluorescence imaging. However, the former often lacks space-resolved information, and the latter has only limited access to the information on molecular structures and microenvironments in biofilms.

As a first step to fully understand how the biomolecules in biofilms, regardless of whether they are already identified or not, fulfill their advanced functions, we used a label-free Raman imaging method to study the constituents of an *Escherichia coli* biofilm *in situ* and to visualize their distributions in the biofilm. We found that high levels of the amino acid leucine were localized within microcolonies in the *E. coli* biofilm.

Experimental

Space-resolved Raman spectra were recorded on a laboratory-built confocal Raman microspectrometer with 632.8 nm excitation (see the Supporting Information for details). Spatial resolutions of 300 nm in the lateral direction and 2.4 μm in the axial direction were achieved. The laser power was 3 mW at the sample point throughout this work. For the space-resolved Raman measurements, the exposure time was 60 s for the *E. coli* biofilm, 100 s for a planktonic cell, and 30 s for amino acids. For the Raman imaging measurements, the sample was translated at 0.5 μm intervals both in *X* and *Y* directions and the Raman spectrum was recorded at each point with a 1-s exposure time. To improve image contrast, we employed a numerical post-treatment based on singular value decomposition[10,11] (Supporting Information).

E. coli XL1-Blue was routinely cultured in LB medium at 37 °C. Biofilms were grown on a glass-bottomed dish at 37 °C under static conditions. After 30 h, excess LB medium was gently pipetted out from the edges of the dish. The sample was then transferred directly to the microscope stage for Raman measurements without any further pretreatment. All the amino acids were purchased from Sigma-Aldrich and recrystallized once.

Results and Discussion

Everywhere in the 30-h-old *E. coli* biofilm, microcolonies were observed as shown in Fig. 1 (inset). Figure 1(a)–(c) compares the Raman spectra recorded inside and outside the microcolony at ~3 μm above the substrate with that of a planktonic *E. coli* cell measured independently using optical trapping. The extracolonial spectrum was almost identical to the planktonic Raman spectrum with few exceptions including the Raman band at 1408 cm^{-1}. This band is due probably to the COO^- symmetric stretch

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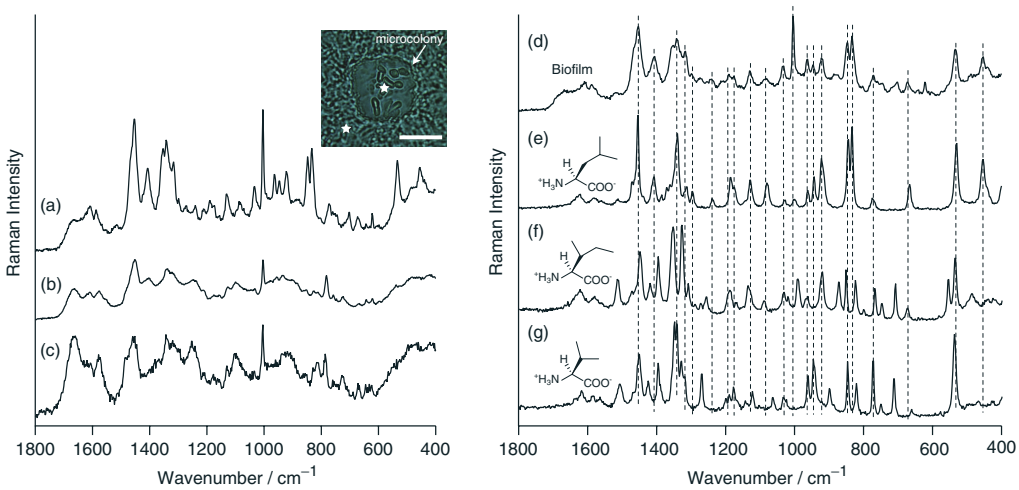

Figure 1. Left: Space-resolved Raman spectra of the 30-h-old *E. coli* biofilm and a planktonic *E. coli* cell. (a) Intracolonial and (b) extracolonial Raman spectra. (c) Raman spectrum of a planktonic *E. coli* cell (not to scale). The inset shows the optical micrograph of the microcolony on which the measurements were performed. Scale bar = 10 μm. Stars denote the positions at which (a) and (b) were recorded. Right: Comparison of (d) the intracolonial Raman spectrum (the same as (a)) with those of crystalline (e) L-leucine, (f) L-isoleucine, and (g) L-valine.

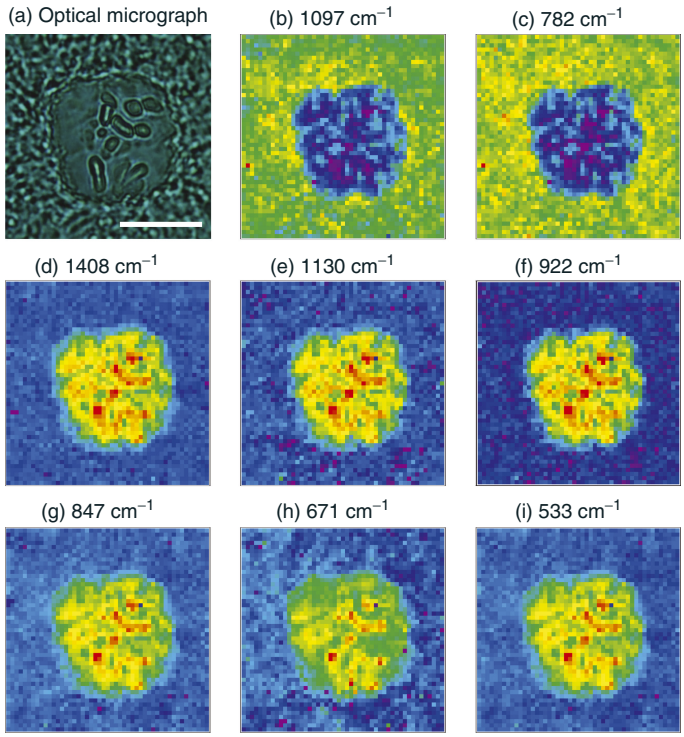

Figure 2. (a) Optical micrograph of the scanned region and Raman images of the 30-h-old *E. coli* biofilm of the Raman bands at (b) 1097, (c) 782, (d) 1408, (e) 1130, (f) 922, (g) 847, (h) 671, and (i) 533 cm^{-1}. Images (b) and (c) represent the DNA distribution, while images (d)–(i) represent the leucine distribution. Different color scale applies to each image. Scale bar = 10 μm.

of polysaccharides such as alginates,[12,13] which are a major constituent of the extracellular matrix. The extracolonial and planktonic spectra represent a typical cellular Raman spectrum dominated by proteins and DNA bands. These results, along with the optical micrograph (inset in Fig. 1), show that the intercolonial space of the biofilm was filled with a dense population of *E. coli* cells held together by the extracellular matrix.[5]

The intracolonial spectrum differs substantially from the extracolonial spectrum, indicating distinct chemical composition of the microcolony. By scrutinizing Raman spectra of biomolecules that could potentially occur in the microcolony, we found that the intracolonial Raman spectrum resembles both in wavenumber and in spectral pattern remarkably well that of crystalline L-leucine (Fig. 1(d) and (e)). We also considered other amino acids such as L-isoleucine and L-valine that are close in chemical structure to L-leucine as possible candidates, but the overall agreement of spectra was not satisfactory at all (Fig. 1(f) and (g)). Thus, we conclude that there were high concentrations of leucine (possibly with a minor contribution from phenylalanine) within the *E. coli* biofilm microcolony, forming a leucine pool.

Next we performed Raman imaging measurements. Figure 2 displays eight representative Raman images, which directly visualize the distributions of the biofilm constituents. As expected from the space-resolved Raman data, Raman images of DNA (Fig. 2(b) and (c)) show the yellow (medium to high intensity) pattern outside the microcolony, while those constructed for the bands observed in the intracolonial spectrum (Fig. 2(d)–(i)) manifest distributions localized inside the structure. Although the distribution patterns within the microcolony are highly heterogeneous, they appear to be virtually the same for all the Raman bands. This strongly supports the idea that the microcolony was composed essentially of a single molecular species, namely, leucine. Otherwise, Raman images would vary depending largely on Raman bands. Raman sectioning results shown in Fig. S1 (Supporting Information) are also consistent with this idea.

Valle *et al*.[14] found that *E. coli* biofilms secreted valine under continuous-flow conditions. In contrast to their study, our *E. coli* biofilm was grown under static conditions, giving rise to both hydrodynamically and nutritionally distinct environments for the biofilm. Given the well-known phenomena of microbial growth inhibition and its antagonism caused by amino acids,[15,16] it is plausible that our *E. coli* biofilm may have used the localization of leucine to adapt to the static environment during its early stage development. Further Raman spectroscopic and biochemical studies are under way in order to clarify the biological role of the leucine pool.

In summary, we have discovered leucine pools in a nascent *E. coli* biofilm by using Raman imaging, where high levels of leucine were accumulated. We have also demonstrated that Raman imaging is a powerful tool for detecting *in situ* and identifying key biomolecules involved in biofilms even if they are yet unknown. This study will open new avenues for developing a simple but effective chemical means that enables molecular-level elucidation of bacterial biofilms.

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Supporting information

Supporting information may be found in the online version of this article.

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