Published in final edited form as:

Biopolymers. 2010 February ; 93(2): 171–177. doi:10.1002/bip.21315.

Probing Nanostructures of Bacterial Extracellular Polymeric Substances (EPS) versus Culture Time by Raman Microspectroscopy and Atomic Force Microscopy

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Abstract

The structure of a bacterial cell wall may alter during bacterial reproduction. Moreover, these cell wall variations, on a nanoscale resolution, have not yet fully been elucidated. In the present work, Raman spectroscopy and atomic force microscopy (AFM) technique are applied to evaluate the culture time-dependent cell wall structure variations of *Pseudomonas putida* KT2440 at a quorum and single cell level. The Raman spectra indicate that the appearance of DNA/RNA, protein, lipid, and carbohydrates occurs till six hours of cultivation time under our experimental conditions. AFM characterization reveals the changes of the cellular surface ultrastructures over the culture time period, which is a gradual increase in surface roughness during the time between the first two and eight hours cultivation time. This work demonstrates the feasibility of utilizing a combined Raman spectroscopy and AFM technique to investigate the cultivation time dependence of bacterial cellular surface biopolymers at single cell level.

Keywords

Pseudomonas putida KT2440; extracellular polymeric substances; Raman spectroscopy; atomic force microscopy

Introduction

Raman microspectroscopy has been utilized to study the vibrational properties of macromolecules/polymers. Raman spectral frequencies are recorded in wavenumber units which are proportional to vibrational energies based on molecular polarizability [1]. Since discovery in 1928 by C.V. Raman [2] until the early 1980's Raman microspectroscopy literature focused mainly on physical and structural investigations [3]. During recent years, however, this technique has increased in popularity in the field of microbiology as a tool to identify microorganisms, such as bacteria [4;5;6;7;8;9]. Raman microspectroscopy is a nondestructive vibrational/structural characterization method that can be utilized to identify characteristic spectroscopic fingerprints of bacteria based on chemical molecular compositions [7]. More recently, a few applications of Raman microspectroscopy have been reported to monitor the appearance of surface biopolymers (including DNA/RNA, lipids, proteins, and carbohydrates) in growth and antibiotic use [10], during the cultivation of *Staphylococcus* [11], and in metabolism characterization of *Staphylococcus Epidermidis* [12].

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Atomic force microscopy (AFM), which developed from scanning tunnel microscopy (STM) in the 1980's [13], works differently from other types of microscopy such as optical and electron microscopy. The AFM technique obtains sample topography by detecting dynamic variation of interaction force between the AFM tip and the sample surface as the AFM tip scans across the sample surface [14]. AFM possesses unique advantages over other types of microscopies [14;15], for example, AFM measurement can be performed *in situ* or in near-physiological conditions [16;17], sample preparation is simplified, and the capability to achieve the nanoscale resolution [14;15]; therefore, it is a valuable nanotechnology tool that can provide new opportunity and insights into nano-biology [18]. Over the past decades, AFM had been widely applied to achieve the nanostructures and nano-mechanics of biological materials [14;15;18;19;20;21;22]. Even though, to the best of our knowledge, very few applications of AFM have been utilized to visualize dynamic changes of cellular surface ultra-structures during cell culture and growth.

The Gram-negative bacteria cell wall (Fig. 1) is compiled of an outer membrane, the periplasmic space, and an inner membrane. The outer membrane, which is made up of lipopolysaccharides (LPS) and an inner leaflet, is the first line of defense for Gram-negative bacteria and aids in evading phagocytosis. The outer membrane also serves as a protection barrier for antibiotics, enzymes, detergents, heavy metals, bile salts, and certain dyes [23] and also maintain the cell rigidity and shape [24]. The LPS at the inner leaflet, is made up of the endotoxin lipid A that is anchored to an inner core then followed by an outer core where O polysaccharides function as antigens (termed O antigen) at the terminal end [24;25]. The outer membrane structures, from lipid A to the O antigen, are the hydrophobic portion that is exposed to the outside physiological environment [24]. Previous study has shown the length of the LPS ranges between <0.5 nm and ~2-3 nm, yet the length of the O antigen portion can be up to 40 nm [24]. Next the inner leaflet that is compiled of phospholipids and lipoproteins, possesses covalently bonded lipoproteins which are bonded to the peptidoglycan layers. As for the periplasmic space, it accommodates a multitude of enzymes that range from degradation to transport proteins [23].

Pseudomonas putida KT2440 is a Gram-negative bacterium derivative of *P. putida* strain mt-2 (the toluene-degrading isolate) and was developed as the first biosafety strain to be considered by the Recombinant DNA Advisory Committee [26;27;28]. KT2440 is utilized as a model soil bacterium in bioremediation due to diverse metabolism [27], the complete genome sequence [27;29], and an ability to survive in the general environment. Also, KT2440 has been exploited for other applications such as agriculture and biotransformation. Studies have shown that bacterial EPS play a major role in bioremediation through the biosorption of heavy metals [30], especially in activated sludge [31] and within the marine food chain [32]. Another study focused on the structural analysis of extracted EPS from *Pseudomonas putida* G7 to the mechanisms of toxic metal binding [33]. By utilization of Raman microspectroscopy and AFM techniques, this work presents the new insightful views of EPS growth and nanostructural features of KT 2440 cell wall at different culture times.

Materials and methods

Bacterial culture

For the purpose of tracking analysis using Raman spectroscopy, *Pseudomonas putida* KT2440 was cultured on Luria Broth (LB) agar composed of yeast extract, peptone from casein, sodium chloride and agar (mass ratio, 1:2:1:3) at room temperature in air. The agar plate containing KT2440 was inoculated, transferred onto the stage of Raman microscope, and observed for 30 hours.

For the purpose of atomic force microscopy (AFM) evaluation, KT2440 cells were cultured in LB medium composed of yeast extract, peptone from casein and sodium chloride (mass ratio, 1:2:1) at room temperature by shaker flask. The bacteria at growth periods of 0, 2, 4, 6, and 8 hours were isolated by centrifugation for 2 min at 2000 rpm (Eppendorf Minispan), then 5 μ l of bacteria were dropped onto a clean cover glass substrate and allowed to expose in air for less than 1 min before imaging (*P. putida* KT2440 did not undergo any additional fixation treatment).

Raman microspectroscopy

Raman spectra from *Pseudomonas putida* KT2440 on agar were recorded over a 30 hour culture period using a Renishaw inVia Raman microscope (controlled by WiRE 3.0 software) equipped with a 785 nm near-IR laser. Laser light was focused through a Leica 50 $\times$ 0.75 N PLAN optical glass microscope objective. Rayleigh scattering was eliminated by the use of filters. Within the spectrometer, Raman scattering passed through a 50 μ m slit, then was collected and dispersed by a holographic diffraction grating (1200 l/mm), and finally the entire Raman shift was recorded as a spectrum by a thermoelectrically cooled charge coupled device (CCD). The instrument was wavelength calibrated with silicon at a static spectrum centered at 520.5 cm^{-1} for 1 s. The KT2440 agar plate was mounted on a standard stage (Leica 11501200) for an Olympus microscope, focused under the collection assembly, and spectra were collected in static mode of 3 accumulations at 10 sec laser exposure over a wavenumber range of 500 – 1840 cm^{-1} .

Atomic force microscopy

The Picoplus AFM controlled by Picoscan 5.4 (Agilent Technologies, USA) was used to measure bacterial cells at room temperature in air by contact mode. The spring constant of the cantilevers (Veeco Inc. USA) was 0.12 N/m, and the curvature radius of Si_3N_4 tip was approximately 10 nm (offered by manufacturer Veeco Inc). The roughness, including root-mean-square roughness (Rms) and average roughness (Ra), of the nanostructures acquired on the bacterial cell surface were obtained utilizing the software Gwyddion 2.5 (a free SPM data analysis framework, <http://gwyddion.net/>). During measurements conducted by contact mode, the tip makes gentle contact with the sample surface. When the tip pulls away from the sample surface, because of the hydrophobic property of cell surface, the adhesion force between the tip and sample surface, needs to be overwhelmed, and such adhesion force can be measured from the retracting branch of deflection (nm) vs. distance (nm) curves (see Fig. 3A-3). In our experiments, the adhesion force was extracted from deflection (nm) vs. distance (nm) curves via the software Scanning Probe Image Processor (SPIP) (Image Metrology, Denmark). All statistical data (roughness and adhesion force) were reported as mean $\pm$ SD (standard deviation), and the histograms were created using OriginPro 7.5 (OriginLab Corp., USA).

Results and Discussion

Fig. 2 is a stacked overlay of Raman spectra for KT2440 recorded at incremental time periods during the first 30 hours of culture. All spectra during this experiment were collected from the same sample location under identical acquisition conditions. A background spectrum for the agar (0 hr not shown) was acquired after KT2440 inoculation for background subtraction of peaks associated with the LB agar composition. Prior to background subtraction, all spectra were normalized at the 1006 cm^{-1} peak and after background subtraction Savitsky-Golay smoothing [34] was applied to each spectrum. The KT2440 spectrum at 2 hours contains a few very weak peaks in the 700 – 1200 cm^{-1} range. The spectrum at 4 hours of culture contains some weak peaks in the 700 – 1200 cm^{-1} range; respectively 785 cm^{-1} , 851 cm^{-1} , 874 cm^{-1} , and 1006 cm^{-1} . At 6 hours of culture, there are

moderate peaks in the 700 – 1200 cm^{-1} range; 785 cm^{-1} , 815 cm^{-1} , 1006 cm^{-1} , 1098 cm^{-1} , and 1127 cm^{-1} . Also, the signal for the 1200 – 1700 cm^{-1} range is more pronounced with broad peaks at 1314 cm^{-1} , 1342 cm^{-1} , 1454 cm^{-1} , 1574 cm^{-1} , and 1660 cm^{-1} . For the spectrum at 8 hours of culture, the peak intensities increase in the 700 – 1200 cm^{-1} range and the 1200 – 1700 cm^{-1} range peaks become better resolved. Finally at 10 and 30 hours of culture, the peak intensity weakens in the 700 – 1200 cm^{-1} range along with the broad peaks in the 1200 – 1700 cm^{-1} range.

It is interesting to note that there are characteristic peaks for KT2440 that appear as the culture time increases. For example at 4 hours of culture, observed peaks are very weak; however, all peaks in the shaded regions are not fully evident until the 6 hour culture period and these appear to change in intensity by 30 hours of culture. The changes in peak intensity appear to be dynamic and could be attributed to organism response to environmental changes such as the increase in cell population [35], changes in EPS expression [36], or perhaps a combination of these and other proliferation/aging events, such as utilization of stored lipids [37] as environmental nutrients are depleted.

To further understand which chemical compositions are present in the quorum population as culture time increases, tentative peak assignments are made based upon the characteristic fingerprint spectra obtained during the dynamic cultivation. Table 1 is a list of characteristic peak assignments for KT2440 according to the Raman spectra of other Gram-negative microorganisms [8;38;39;40;11]. There are two dominant peaks observed at 785 cm^{-1} and 1006 cm^{-1} and four broad peaks at 1127 cm^{-1} , 1342 cm^{-1} , 1454 cm^{-1} , and 1660 cm^{-1} . DNA/RNA breathing modes of cytosine, thymine, and uracil bases can be assigned to the band at 785 cm^{-1} [8;39]. The peak at 1006 cm^{-1} could be assigned to phenylalanine of proteins or δ (ring) deformation [38;39;40]. For the peaks in the broad bands, 1127 cm^{-1} can be assigned to C-C stretching in saturated fatty acids [8], while 1342 cm^{-1} can be assigned to guanine in DNA or δ (CH_2) deformation in proteins and carbohydrates [38;39]. The major peak at 1454 cm^{-1} could correspond to a ν (CH_2) stretching or a δ (CH_3) deformation associated with lipids, proteins, and carbohydrates [38;39;11]. The 1660 cm^{-1} peak can be assigned to the amide I band [39].

These tentative characteristic peaks assignments (as related to chemical compositions) could be beneficial to evaluate the presence or absence of particular EPS components developed on KT2440 cell surface during different culture times. For example, the 785 cm^{-1} peak intensity increases in the first 8 hours, but decreases over 10 – 30 hours. That could imply that an increase in DNA molecules within the EPS [36] and quorum biofilm [41], in the first 8 hours of culture period or a general increase in DNA as quorum population increases. On the other hand, the lipid, protein, and carbohydrates are gradually produced up to the 10 hour culture, evidenced by the presence of 1454 cm^{-1} and accompanied by the presence of 1660 cm^{-1} (amide I). The Gram-negative outer core contains carbohydrates and the inner core and lipid A region incorporates amines, phosphates, and carboxylates [25]. Both carbohydrates were tentatively identified at 1342 cm^{-1} and 1454 cm^{-1} and an amide I band was at 1660 cm^{-1} . It is not surprising to observe weak characteristic peaks for (un)saturated fatty acids during the measured culture period at 1098 cm^{-1} and 1127 cm^{-1} . The brightfield light microscopy images acquired over the time course of the cell cultivation are shown in Fig. s1 (see Supplementary Material).

Based upon these tentative peak assignments (Table 1), we are able to gain more insight into the dynamic changes of the chemical compositions of the quorum and perhaps the EPS compositions of KT2440 during the growth on the agar substrate. Raman microspectroscopy provides the possibility of non-destructive, *in situ*, measurement in real-time to track the changes of the bacterial cell surface components at the single cell level or on bacterial

colonies. Also, by coupling Raman spectroscopy with other *in situ* high resolution imaging techniques such as atomic force microscopy (AFM), one would be able to quantify biophysical properties (ultrastructures and cell biomechanics) of a single bacterial cell surface, or cell colonies that developed in the dynamic cultivation process.

Next, culture time-dependent surface nanostructural variations of single KT2440 cells that are collected at the first incremental hours of growth time are also detected by contact mode AFM in air, as seen in Fig. 3. The topographical and biomechanical changes of the cellular surface are presented in different columns: 3-D topographical images (column 1), surface ultrastructures (column 2), representative deflection-distance curves (column 3), and histograms of the adhesion force distribution (column 4). Surface topographic features acquired at 6 hours and 8 hours exhibit strap-like structures or crumpled structures (D2, E2), which are visibly different from those observed at 0, 2, and 4 hours (A2, B2, C2, respectively). To quantitatively analyze the time-dependent variations in surface ultrastructure roughness, including Rms and Ra, of each ultrastructure image acquired in Fig. 3 are given in Table 2. The results reveal that the roughness (Rms and Ra) of the surface ultrastructures decreased at the initial 2 hours of culture time. However, both Rms and Ra actually increase after the first 2 hours (e.g., from 2 to 8 hours) (Fig. 4a, Table 2). It appears that more EPSs would have been developed after first 2 hours of culturing. The culture time-dependent changes of the cell surface roughness imply the development of cell wall components on the KT2440 cell surface during the observed culture period. Moreover, such cell surface roughness changes may involve the appearance/disappearance of biochemical constituents of the cell wall during bacterial growth and reproduction. This assumption is supported by the time course Raman spectra shown in Fig. 2. On the other hand, as the ultrastructures of cell wall alter with the increased culture time, it is reasonable to hypothesize that the surface adhesion property could also be different for the cells grown during different time stages. As expected, there is variation in the adhesion force measured on the single cells collected at different culture time periods (see column 4 of Fig. 3). This comparison further indicates that the adhesion force dramatically increases during the initial culture stage (0-2 hours); and then gradually decreases over the following 2~8 hours of culture time (Fig. 4b), which appears opposite to the trend of roughness over the culture time. The variation in adhesion property may be due to the fluctuation of biochemical constituents and/or physiochemical properties of the cell wall polymers in the process of growth and reproduction.

In conclusion, from the prospective of chemical composition and topographical visibility, Raman microspectroscopy and AFM each are powerful tools with individual advantages for analyzing nanostructures of bacterial EPS during culture and growth over time. Raman micro-spectroscopy was able to identify biochemical structural components of bacterial EPS versus culture time. Especially, Surface-enhanced Raman scattering (SERS) would provide higher Raman signals that could be potentially applied for chemical structural identifications of these cell surface biocomponents produced during the cell culture. AFM evaluation revealed the culture time-dependent variations of the cell wall surface nanostructures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the financial support from the National Institute of Health (ES013688-01A1), and the Huntsman Environmental Research Center at Utah State University. The Raman microscope was purchased from a Department of Defense DURIP grant (#W911NF-06-01-0139). G.D.M is also thankful for supports from the USU

VPR Graduate Research Fellowship and the USU Center for Integrated Biosystems Graduate Research Support Program (CIB-R).

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Figure 1.
Schematic representation of typical Gram-negative bacterium outer and inner membrane.

Figure 2.

Representative bulk Raman spectra from *Pseudomonas putida* KT2440 on agar were recorded over a 30 hour culture period. The spectra were collected in static mode of 3 accumulations at 10 second laser exposure over a wavenumber range of 500 – 1840 cm^{-1} . Shaded regions indicate peaks that appear/increase with time. a.u.: arbitrary units.

Figure 3. Measurements of *P. putida* KT2440 at growth periods of 0, 2, 4, 6, and 8 hours (rows) acquired by contact mode AFM in air. Three dimension (3-D) topographical images (Column 1); cellular surface nanostructures (Column 2); examples of deflection-distance curves (Column 3); adhesion force distribution (Column 4). The scanning range of each image in Column 2 is 500×500 nm and the resolution of images is 256×256 pixels.

Figure 4. (A) Comparison of surface roughness (Rms and Ra) variations at the different growth period (0, 2, 4, 6, and 8 hours) (the data were obtained from multiple cells). (B) Comparison of adhesion forces measured from different growth period.

Table 1Tentative assignments of *P. putida* KT2440 extracellular polymeric substances

Wavenumber	Description	Assignment
785	i, vs	ρ (ring) DNA/RNA U, T, C
814	m	γ (C-N)
851	w	Tyrosine (Fermi resonance of ring fundamental and overtone)
874	w	γ (C-C)
1006	i, vs	δ (ring) Phenylalanine
1098	i, m, b	γ (C-N)
1127	i, m, b	γ (C-N) and ν (C-C)
1252	i, w, b	G, C (NH ₂)
1314	i, w, b	t (CH ₃ -CH ₂), collagen/lipid
1342	i, w, b	δ (CH ₂)
1454	i, b	ν (CH ₂)/asymmetric δ (CH ₃)
1574	i, w, b	DNA/RNA G, U
1665	i, b	amide I

Abbreviations: vs, very strong; m, medium; w, weak; vw, very weak; b, broad; i, peak intensity increases over time; ν , stretching; δ , deformation; t , twisting; ρ , breathing.

Table 2

Comparison of roughness including Rms and Ra of cell wall nanostructures acquired from the respective image shown in column 2 of Fig. 3.

	0 hr	2 hr	4 hr	6 hr	8 hr
Rms (nm)	21.5	16.38	26.7	40.9	45.9
Ra (nm)	17.6	13.58	22.1	33.7	38.1