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Low-temperature Degradation Mechanism Analysis of Petroleum Hydrocarbon-degrading Antarctic Psychrophilic Strains

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Laser tweezers Raman spectroscopy (LTRS) can help with observing and studying individual cells or organelles in a natural state for a relatively long period. In this study, LTRS and GC-MS were used to report physiological metabolism of active psychrophilic petroleum hydrocarbon-degrading strains isolated from the Antarctic Ocean. Based on GC-MS analysis and analysis of the Raman spectrum of degradation productions, the results showed *Planococcus* sp.NJ41 and *Shewanella* sp.NJ49 degraded diesel, n-hexadecane, polycyclic aromatic hydrocarbons, and other petroleum hydrocarbons with high efficiency at low temperature (0°C -10°C). GC-MS showed the long straight-chain hydrocarbons were decomposed into short straight-chain hydrocarbons. The Raman spectrum showed there were more proteins and carbohydrate than lipids were produced during the *Planococcus* sp.NJ41 and *Shewanella* sp.NJ49 growth and degradation. Finally, the Raman intensity at 1512 cm^{-1} to 1514cm^{-1} represented β-carotene that was involved in the regulation of membrane fluidity in Antarctic bacteria, allowing it to adapt to the extreme low-temperature environment of the Antarctic.

Keywords: Antarctic microorganisms, Laser tweezers Raman spectroscopy (LTRS), psychrophile, low-temperature biodegradation.

Laser tweezers Raman spectroscopy (LTRS) is a new technique that uses optical confinement technology to provide useful information about molecular composition and structure in a biological sample. It uses a beam of light to stimulate the Raman spectroscopy of cells. Using this method, the physiological activity of cells is not affected, allowing the researcher to observe the cell in its natural physiological state to monitor biological cells, tissues, or organelles (Xie *et al*., 2002; 2003; 2005). The greatest advantage of Raman spectroscopy is its high sensitivity and capability for non-invasive sensing (Edwards *et al*., 2005).

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Now, LTRS has been widely used as a novel bio-analytical technique in the field of life science research in recent years, leading to advances in the understanding of cell structure, function, and growth. This technique has become a powerful analytical tool for studying DNA, RNA, protein, lipid, and carbohydrate, and it has been developed to identify the differences between cancer tissue and normal tissue (Xie *et al*., 2002; Edwards *et al*., 2005; Choo-Smith *et al*., 2002).

Until recently, the Raman spectroscopy technique has not been applied to biodegradation of aromatic hydrocarbons, petroleum hydrocarbons, and other organic pollutants by polar psychrophilic microorganisms. The problems of large-scale petroleum hydrocarbon spills and petroleum hydrocarbon pollutants in the ocean have been increasingly severe. Since biodegradation could be the key to cleaning up

petroleum hydrocarbon pollutants, marine microorganism biodegradation has been the focus of recent research. The low-temperature habitat of the Antarctic region is rich in natural resources, and the cold-adapted bacteria in the polar ocean have shown the potential to remediate pollution by petroleum hydrocarbons and polycyclic aromatic hydrocarbons in the area. Those bacteria could provide an effective means for bioremediation of pollution by oil and polycyclic aromatic hydrocarbon pollution at low temperatures. Therefore, isolating the strains that could degrade the petroleum hydrocarbons and polycyclic aromatic hydrocarbons is becoming an important research topic. Some scholars have isolated different species of petroleum hydrocarbons-degrading microbial from the Antarctic Sea, such as *Halomonas* (Milva *et al*., 2005), *Rhodococcus* (Whyte *et al*., 2006), and *Sphingomonas* (Yakimov *et al*., 2007). Some microorganisms can degrade petroleum hydrocarbons and alkanes at 4°C (Michaud *et al*., 2004; Brakstad *et al*., 2006) and can degrade polycyclic aromatic hydrocarbons with high efficiency at a lower temperature (Siren *et al*. 1995). Thus, biodegradation as an oil attenuation process in cold environments was well documented.

In this paper, we applied GC-MS and LTRS to the study of the biodegradation processes of polycyclic aromatic hydrocarbons and petroleum hydrocarbons at low temperature by strains of *Planococcus* sp.NJ41 and *Shewanella* sp.NJ49 isolated from the Antarctic Ocean. After GC-MS analysis of degradation productions, collection and analysis of the Raman spectrum, we discussed the relationships among Raman spectrum, cell growth, and the degradation processes. This study increases the knowledge of Antarctic microorganisms' degradation of polycyclic aromatic hydrocarbons and petroleum hydrocarbons at low-temperature by a single cell and a colony cells.

MATERIAL AND METHODS

Bacterial strains

Bacteria strains were isolated and purified from Antarctic seawater and sea-ice, which were collected during the Chinese 19th Antarctic Science Exploration in 2002-2003. Named *Planococcus* sp.NJ41 and *Shewanella* sp.NJ49, these strains can grow in a minimal medium with PAHs such as naphthalene as the sole source of carbon and energy, and they can degrade petroleum hydrocarbon and PAHs with high efficiency at low-temperatures (0°C-15°C) (Liu *et al*., 2010; Wang *et al*., 2011). They were deposited in the low-temperature strain library of Key Laboratory of Marine Bioactive Substances, SOA.

Reagents, culture media, and methods

Screening media (MMC): NaCl 24 g/L, $MgSO_4 \cdot 7H_2O$ 7.0 g/L, NH_4NO_3 1.0 g/L, KCl 0.7 g/L, KH_2PO_4 2.0 g/L, Na_2HPO_4 3.0 g/L, pH 7.4. Microelements media: $CaCl_2$ 0.02 mg/L, $FeCl_3 \cdot 6H_2O$ 0.5 mg/L, $CuSO_4$ 0.005 mg/L, $MnCl_2 \cdot 4H_2O$ 0.005 mg/L, $ZnSO_4 \cdot 7H_2O$ 0.1 mg/L. The screening media were supplemented by some microelements and diesel oil, naphthalene, or n-hexadecane. Diesel oil, naphthalene, n-hexadecane, and microelements were sterilized using 0.2 µm microfiltration membranes (Wang *et al*., 2011).

The bacteria cultures were incubated at 10°C, in a shaker-flask culture, 120 r/min and operated 24 h. Bacteria cultures were washed twice with sterilized MMC. 1.0 g pre-sterilized diesel oil (or 200 mg naphthalene) was added to 100 mL pre-sterilized medium. These media were inoculated with 1.0 mL bacterial suspension and incubated at 10 °C, 120 r/min, and shaker-flask culture, operated for 15 days.

GC-MS analysis

Diesel oil (or naphthalene, or n-hexadecane) was extracted from the culture medium and was conserved at 4°C.

6890N GC-5973N MS (Agilent, U.S.): HP-5 MS, 30 m×0.25 mmID×0.25µm, FID, and NIST.

Raman spectroscopy experimental setup and Acquisition of Raman spectra

The elliptical-shaped beam from a laser diode near 785 nm was made circular with a pair of anamorphic prisms, spatially filtered and introduced in an inverted microscope (Nikon TE 2000) equipped with a high numerical objective (100×, NA=1.30) to form a single-beam optical trap. The Raman-scattering light of the trapped particles was collected with the same objective, passed through a holographic notch filter and a pinhole, and was then focused onto the entrance slit of an imaging spectrograph (a green-filtered illumination lamp and a video camera system are used to observe the image of the trapped cell) equipped with a liquid-

nitrogen-cooled charged-coupled detector (CCD) (Xie *et al*., 2002, 2003; Tang *et al*., 2007).

A single cell in media was randomly trapped by the laser beam after loading the sample, and Raman spectra of the trapped cell were acquired with a 20 mW laser power and 30 s exposure time. Recorded the trapped cell, and then released the sample from the beam focus and the background spectrum of isolation media without the cell was also obtained with the same acquisition time and power. At last, we recorded more than 30 single cell and 10 media background which were randomly selected, then obtained the average of Raman spectra of them. The spectral resolution of our Raman system was about 6 cm^{-1} and the Raman spectra was recorded in the "finger print" range from 600 cm^{-1} to 1800 cm^{-1}.

RESULTS

Both strains NJ41 and NJ49 grew well from 0°C to 20°C, but did not survive above 25°C, the optimum temperature for growth was 10°C. They grew in inorganic salt nutrient medium with n-hexadecane, polycyclic aromatic hydrocarbons, and other petroleum hydrocarbons as the only carbon source. The bacteria degraded them with high efficiency at low temperatures (0°C -10°C) (Liu *et al*., 2010; Wang *et al*., 2011).

GC-MS analysis of degradation productions

After degradation, diesel oil and n-hexadecane of the MMC substrates were analyzed by GC-MS. The results showed that the MMC without bacteria included 18 kinds of alkanes from C_9 to C_{26}. Seven kinds of alkanes from C_{15} to C_{21} were detected in the diesel oil components degraded by NJ49, and the kinds and contents of alkanes decreased significantly.

The GC-MS analysis (Fig.1, and Fig.2) showed that: *Planococcus* sp.NJ41 and *Shewanella* sp.NJ49 degraded diesel, n-hexadecane, polycyclic aromatic hydrocarbons, and other petroleum hydrocarbons with high efficiency at low temperature. The bacteria decomposed long straight-chain hydrocarbons into short straight-chain hydrocarbons.

Raman spectrum of Antarctic bacteria

After LTRS has been used, useful information about the composition, secondary structure, and interactions of DNA-protein complexes inside the living cells could be yielded from the positions, intensities, and line widths of the Raman peaks in the spectra (Xie *et al*., 2003, 2005; Jess *et al*., 2006). The Raman spectra of single Antarctic bacteria were recorded, and an average result was calculated for over 30 individual bacteria.

Table 1. Raman bands of single Antarctic bacteria cells and tentative assignation

Bands (cm^{-1})	Assigned to
724	Adenine
782	RNA/DNA
812	RNA
1004	Phenylalanine
1091	Lipids
1153~1156	Carbohydrate and Protein
1290	Lipids
1336	Polynucleotide chain(DNA)
1437~1442	CH_2 bending modes and CH_3 deformation of lipids and proteins
1512~1514	β-carotene
1569	Guanine and Adenine
1650~1655	Amide I

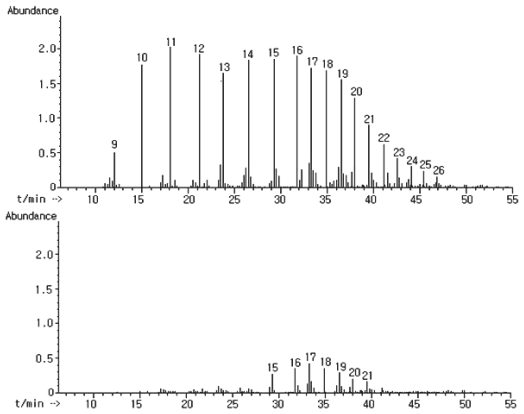

Fig. 1. GC-MS of diesel oil blank and after degradation by *Shewanella* sp.NJ49

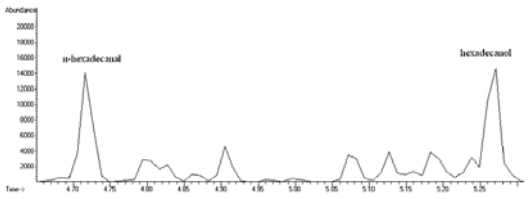

Fig. 2. Chromatogram of GC-MS analysis of *n*-Hexadecane degradation of *Planococcus* sp.NJ41

Fig.3 presents the growth Raman spectra of *Planococcus* sp.NJ41, from cell growth procession, we observed signal peaks at 1004 cm^{-1}, 1153 cm^{-1}, 1442 cm^{-1}, and 1512 cm^{-1}, and the Raman intensity gradually increased.

Fig.4 presents Raman spectra of the Antarctic psychrophile *Planococcus* sp.NJ41, and Fig.5 presents Raman spectra of Antarctic psychrophile *Shewanella* sp.NJ49. The Raman spectra that we obtained from NJ41 and NJ49 were similar. Observing the average Raman spectra of *Planococcus* sp.NJ41 and *Shewanella* sp.NJ49, we found that there were prominent spectral peaks at 812 cm^{-1}, 1004 cm^{-1}, 1156 cm^{-1}, 1442 cm^{-1}, 1514 cm^{-1}, and 1650 cm^{-1}, and the bands at 1156 cm^{-1} and 1514 cm^{-1} were stronger than the others. These spectra bands were assigned to the physiological metabolism productions during the *Planococcus* sp.NJ41 and *Shewanella* sp.NJ49 growth and degradation, such as the lipids, proteins, nucleic acids, and carbohydrates (Tang *et al*., 2007; Jess *et al*., 2006).

The band at 1004 cm^{-1} was commonly found in biological samples, and this band was assigned to the ring-breathing mode of phenylalanine. The bands at 1091 cm^{-1} and 1290 cm^{-1} were assigned to lipids. The signal at the 1153 cm^{-1} to 1156 cm^{-1} band was assigned to carbohydrate and protein, this band was the most prominent spectral peak during NJ41 and NJ49 growth and degradation. The band at 1336 cm^{-1} was assigned to the polynucleotide chain, which comes from DNA. The broad band at 1437 cm^{-1} to 1442 cm^{-1} was assigned to the CH_2-bending modes and CH_3 deformation of lipids and proteins (Xie *et al*., 2003; Tang *et al*., 2007; Wang *et al*., 2008; Tao *et al*., 2009; Jess *et al*. 2006). The strong band at 1512 cm^{-1} to 1514 cm^{-1} was assigned to β-carotene that was involved in the regulation of membrane fluidity in Antarctic bacteria, which allowed them to survive in low temperature (Jagannadham *et al*., 2000). The signal at 1650 cm^{-1} to 1655 cm^{-1} band was assigned to amide of proteins (Xie *et al*., 2003; Tang *et al*., 2007; Wang *et al*., 2008; Tao *et al*., 2009; Jess *et al*. 2006). Amide vibrations, such as the amide band (due to C=O stretching) and amide III band (due to C-N stretching and N-H bending) in proteins were easily identifiable. Table1 shows the tentative assignment for the observed Raman signal of NJ41 and NJ49.

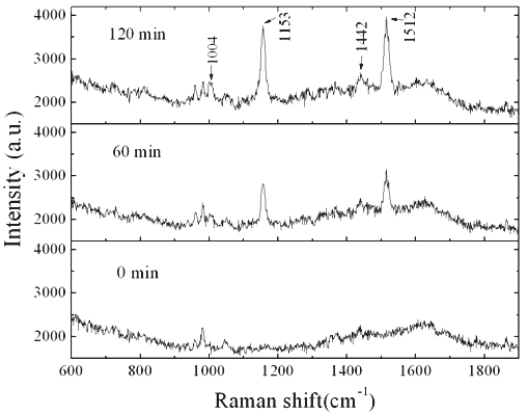

Fig. 3. Raman spectra of single Antarctic bacteria *Planococcus* sp.NJ41 growth

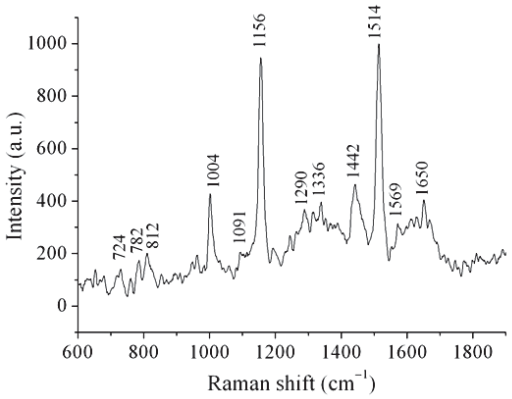

Fig. 4. Average Raman spectra of single Antarctic bacteria *Planococcus* sp.NJ41

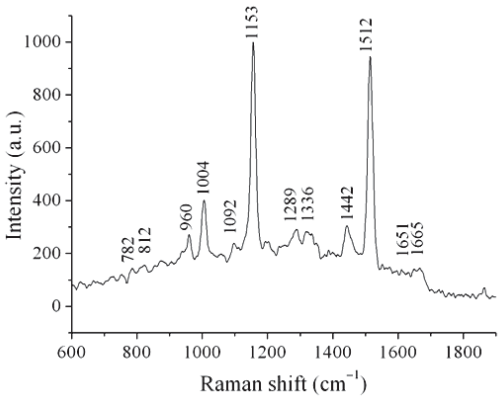

Fig. 5. Average Raman spectra of single Antarctic bacteria *Shewanella* sp.NJ49

DISCUSSIONS

The research of localization of degradation enzyme of *Planococcus* sp.NJ41 indicated that micro-biological degradation of alkane needed the enzymatic reaction of the oxidase system. The extracellular enzyme played a key role in the process of n-hexadecane degradation of *Planococcus* sp.NJ41, and accomplishing the preliminary degradation of n-hexadecane by the terminal oxidation. The intracellular enzyme played little role in the process of n-hexadecane degradation. This conclusion was in accordance with the result of GC-MS analysis.

Analyzing the Raman spectra of *Planococcus* sp. NJ41 and *Shewanella* sp.NJ49, we found that more proteins and carbohydrates than lipids were produced during growth and degradation. The amount of protein which produced by the strains corresponded with the production of degradation rate-limiting enzyme and was also related to the capacity of low-temperature degradation of aromatic hydrocarbons and diesel oil. Many researchers have characterized the various pathways of diesel oil and aromatic hydrocarbon biodegradation. Some enzymes had been studied such as monooxygenase and dioxygenase (Merimaa *et al*., 2006; Gibson *et al*., 2000), and we have had some enzymes from NJ41 and NJ49, and determined their basic enzymatic properties (these relevant results will be presented in the following paper).

In both NJ41 and NJ49, the Raman spectra bands assigned to β-carotene were strong, and that was involved in regulation of membrane fluidity in Antarctic bacteria to adapt to extreme low-temperature environments of the Antarctic (Jagannadham *et al*., 2000). The Antarctic bacteria produced carotenoids which can increase the fluidity of the cell membrane with the decrease of temperature, so that the cells could survive at the low temperatures. Then, we saw a large increase about the band which peaks carotene in the Raman spectra.

To our knowledge, this is the first study involving Raman spectroscopy of a single cell of an Antarctic microorganism and the first study of the bio-degradation of aromatic hydrocarbons, petroleum hydrocarbons, and other organic pollutants of individual polar microorganisms using Raman spectroscopy in an optical trap. In this paper, we reported physiological metabolism, such as cell growth and degradation, and described the relationship between degradation production GC-MS analysis, Raman spectra, and degrading enzyme exudation. Still unanswered, though, are questions about the characteristics of the degrading enzyme, we have already finished cloning the coding sequence of the enzyme. Further, more research is needed to explain the associations among degradation production GC-MS analysis, Raman spectra, and degrading enzyme exudation. Finally, the Laser tweezers Raman spectroscopy can be used to provide molecular information of single biological samples in real time and may be a useful tool for understanding fundamental cell processes, and it may have very broad application to research on polar microorganisms.

CONCLUSIONS

We screened two bacteria from the Antarctic region (NJ41 and NJ49) that could degrade PAHs and petroleum hydrocarbons with high efficiency at low temperatures (0°C -10°C). After we applied the LTRS system to monitor real-time changes of structure and bioactivity of single Antarctic bacteria, analyzed the Raman spectra changes during the cells growth and degradation, and combined that with GC-MS analysis of degradation productions, we found that petroleum hydrocarbons and long straight-chain hydrocarbons can be decomposed into short straight-chain hydrocarbons by NJ41 and NJ49. Furthermore, Raman spectra showed there were more proteins and carbohydrates than lipids produced during *Planococcus* sp.NJ41 and *Shewanella* sp.NJ49 growth and degradation. The Raman spectra band at 1512 cm^{-1} to 1514 cm^{-1} was assigned to β-carotene that was involved in the regulation of membrane fluidity in Antarctic bacteria, even in low-temperature Antarctic environments.

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