Microbial Transformation of Multiwalled Carbon Nanotubes by *Mycobacterium vanbaalenii* PYR-1

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*Supporting Information

**ABSTRACT:** Carbonaceous nanomaterials are widely used in industry and consumer products, but concerns have been raised regarding their release into the environment and subsequent impacts on ecosystems and human health. Although many efforts have been devoted to understanding the environmental fate of carbonaceous nanomaterials, information about their microbial transformation is still rare. In this study, we found that within 1 month a polycyclic aromatic hydrocarbon-degrading bacterium, *Mycobacterium vanbaalenii* PYR-1, was able to degrade both pristine and carboxyl-functionalized multiwalled carbon nanotubes (p-MWCNT and c-MWCNT), as demonstrated by consistent results from high resolution transmission electron microscopy, Raman spectroscopy, and confocal Raman microspectroscopy. Statistical analysis of Raman spectra identified a significant increase in the density of disordered or amorphous carbon in p-MWCNT and c-MWCNT after biodegradation. Microbial respiration further suggested potential mineralization of MWCNTs within about 1 month. All of our analyses consistently showed higher degradation or mineralization of c-MWCNT compared to p-MWCNT. These results highlight the potential of using bacteria in engineered systems to remove residual carbonaceous nanomaterials and reduce risk of human exposure and environmental impact. Meanwhile, our finding suggests possible transformation of carbonaceous nanomaterials by polycyclic aromatic hydrocarbon-degrading bacteria in the natural environment, which should be accounted for in predicting the environmental fate of these emerging contaminants and in nanotechnology risk regulation.

**INTRODUCTION**

Because of their unique physicochemical properties, carbon nanotubes (CNTs) have attracted worldwide commercial interest in diverse applications for both industry and consumer products.¹,² Current global production capacity of CNTs has surpassed 5,000,000 kg per year, and is increasing by about 500,000 kg annually.³ Such rapid growth in production and widespread use of CNTs has raised concerns about their impact on human and environmental health following their release into the environment.³–⁶

Although many efforts have been made to understand their environmental fate, information about biotransformation of CNTs in the environment is still scarce.⁷ Several studies have reported in vitro transformation of single-walled carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs), catalyzed by plant-derived horseradish peroxidase,⁸–¹⁰ manganese peroxidase from white-rot fungus,¹¹ and human myeloperoxidase and eosinophil peroxidase.¹²,¹³ Given the extremely high abundance and diversity of microbes in the environment and their versatile metabolic potentials, microbial degradation of CNTs is very likely the major biotransformation process in the environment and deserves more investigation.¹⁴ Recently, Zhang et al.¹⁵ reported mineralization of as much as 6.8% of MWCNTs within 7 days by a bacterial community.
through cometabolism with extra carbon sources and identified *Burkholderia kuruvensis*, *Delftia acidovorans*, and *Stenotrophomonas malophilia* as three potential key players. Parks et al. reported that after 6 months less than 0.1% of SWCNTs were mineralized by the white-rot fungus *Trametes versicolor* or by microbiota from polychlorinated biphenyl-contaminated sediments or aerated wastewater treatment plant sludge. These dissimilar degradation rates warrant more efforts toward a better understanding of microbial transformation of CNTs.

A promising group of candidate microbes for CNT biodegradation are bacteria capable of degrading polyyclic aromatic hydrocarbons (PAHs), which share similar structural skeletons with CNTs. Previous studies have unambiguously demonstrated that species of *Mycobacterium* can utilize PAHs as the sole source of carbon and energy. It is possible that those bacteria can also transform CNTs and even utilize CNTs as their carbon and energy source. To address this question, we utilized a model PAH-degrading bacterium, *Mycobacterium vanbaalenii* PYR-1, and studied its interaction with pristine (p-MWCNT) or carboxyl-functionalized MWCNT (c-MWCNT) under various incubation conditions. Multiple analytical methods, including transmission electron microscopy (TEM), Raman spectroscopy, and confocal Raman microspectroscopy, were applied to characterize physicochemical changes in MWCNTs after reaction with this bacterium. Potential mineralization of MWCNTs was investigated using a variety of conditions. By comparing results from p-MWCNT and c-MWCNT, we explored the role of surface functionalization in biodegradation of MWCNTs by PYR-1.

**MATERIALS AND METHODS**

**MWCNTs Preparation.** Research-grade MWCNTs were used in this study: (1) pristine MWCNT (p-MWCNT) (Nanocyl NC3150) and (2) carboxyl-functionalized MWCNT (c-MWCNT) (Nanocyl NC3151). According to the manufacturer, both MWCNTs have an average diameter of 9.5 nm and an average length of <1.0 μm. The p-MWCNT has >95.0% carbon and <5.0% metal oxide, while the c-MWCNT has >80.0% carbon, <5.0% metal oxide, and <8.0% COOH functional group.

The MWCNTs’ surface functional groups were characterized using X-ray photoelectron spectroscopy analysis. Thermal stability was examined through thermogravimetric analysis. Detailed information can be found in the Supporting Information (SI).

MWCNT stock solution (50 ppm) was prepared by suspending MWCNTs in autoclaved Milli-Q water and sonicating the suspension at low power for 12 h (Branson Ultrasonic 2510, output power of 100 W at 40 kHz). c-MWCNT was fully dispersed by this approach, while p-MWCNT was not (visualization shown in Figure S1A). Sonication did not affect the structural integrity of either MWCNT, as demonstrated by high-resolution TEM (HRTEM) (Figure S1B), nor did sonication significantly influence MWCNTs’ thermal stability (Figure S1C). Physicochemical characteristics of as-received and sonication-dispersed MWCNTs are listed in Table S1.

**Incubation of MWCNTs with Bacterial Cultures.** We used a PAH-degrading bacterium *M. vanbaalenii* PYR-1 (= DSM 7251), originally isolated from oil-contaminated sediments, as the model microbe in this study. The strain was kindly provided by Dr. Carl E. Cerniglia at the National Center for Toxicological Research, Food and Drug Administration. Bacterial cells were grown aerobically at 30 °C on Middlebrook 7H11 agar (Difco). After 12 days, a single colony was inoculated into the minimal salt medium (MSM) (pH 7.0) supplemented with 1% glucose and 5 mM pyrene, following previous studies.20,21 Bacterial cultures were incubated at 30 °C in the dark for 10–12 days by shaking at 200 rpm. Cell growth was monitored by measuring optical density at 600 nm (OD600).

After bacterial density reached ~10⁶ cell/mL, a small amount of the culture (10⁻³ dilution) was inoculated into MSM amended with (1) p- or c-MWCNT at different concentrations; (2) p- or c-MWCNT at different concentrations plus 1% glucose; or (3) p- or c-MWCNT at different concentrations plus 1% glucose and 5 mM pyrene. These setups represented three different carbon conditions besides the presence or absence of MWCNTs: no extra carbon source, glucose as the extra carbon source, and both glucose and pyrene as the extra carbon source, respectively. In all three setups, the final concentrations of MWCNTs were 0, 1, 5, or 25 ppm. In addition, abiotic controls consisting of the same chemicals but no bacteria were included for comparison. All cultures and abiotic controls were incubated at 30 °C in the dark for up to 25 days by shaking at 200 rpm. To monitor bacterial growth kinetics in the presence or absence of MWCNTs, OD600 was continuously measured as before.22,23 While OD600 may not accurately reflect cell numbers, particularly when particles coexist, an increase in OD600 indicates an active growth of bacterial populations. For each medium condition and each concentration of either MWCNT, duplicate growth curves from independent batches were monitored. More details on bacterial growth, OD600 measurement, and kinetic analysis are described in the SI.

**Electron Microscopy.** TEM was used to examine structural changes in MWCNTs. Suspensions of initial MWCNTs, MWCNTs after incubation with bacteria, as well as MWCNTs after abiotic incubation were collected and centrifuged at 15000 rpm for 1 min. Pellets were washed twice with Milli-Q water and resuspended in 100% ethanol (0.22 μm filtered).

An aliquot (25 μL) of each sample was loaded on a holey or lacey carbon-coated copper grid (400 mesh, Pacific-Grid Tech). Grids were dried under ambient conditions before observation under TEM (JEOL 2100F operating at 200 kV). In conjunction with TEM, electron diffraction was recorded for the area under beamline, an evidence for crystal lattices of MWCNTs. TEM and scanning electron microscopy (SEM) (detailed in the SI) were also used to examine bacterial cell morphology and biofilms formed under different conditions.

**Raman Spectroscopy and 2-Dimensional (2-D) Confocal Raman Microspectroscopy.** Raman spectroscopy was employed to study changes in MWCNTs during biodegradation. To remove bacterial cells and biomaterials, suspensions of initial MWCNTs (newly mixed with bacterial enrichment cultures), MWCNTs after biodegradation, and MWCNTs from abiotic controls were thoroughly mixed by vortex and subjected to sequential washes by water and acetone, followed by protease K digestion using a published protocol.24 A flowchart of detailed sample preparation is shown in Figure S2. These procedures have been proven effective to remove most cell contents from residual MWCNTs24 and served this purpose well in our study. Suspensions of 25 μL were then loaded by drop-casting on 70% ethanol-cleaned microscope slides and dried overnight under ambient conditions. A DXR Raman microscope (Thermo Scientific) with an excitation wavelength of 532 nm and high-resolution grating (2 cm⁻¹ fwhm) was used for collecting spectra over the range of 50 to 1870 cm⁻¹, with a 50X objective, 10 mW laser power, and 8 s exposure time. For each sample, >6 areas (10–50 μm × 10–50 μm) were analyzed, and the relative
intensity ratio of the D band and G band \(I_D/I_G\) associated with MWCNTs was determined from >30 spectra.

A point-scan confocal Raman system\(^5,26\) was utilized to construct Raman maps of D and G bands, from which the distribution of \(I_D/I_G\) ratios was calculated. Samples were prepared as mentioned above, and 20 \(\mu L\) aliquots were drop-casted on 70% ethanol-cleaned quartz coverslips (0.2 mm thick, Technical Glass Products) and air-dried overnight. A 785 nm diode laser beam (0.3 mW power) was used for excitation, with an acquisition time of 1 s per spectrum. Each map was generated by scanning a 6 \(\mu m \times 6 \mu m\) region, which resulted in 400 Raman spectra (1 spectrum per 0.3 \(\mu m \times 0.3 \mu m\)). For each sample, three to six regions were examined.

**Measurement of \(CO_2\) from Different Incubations.** We measured \(CO_2\) evolved from bacteria incubated with different external carbon sources. After *M. vanbaalenii* PYR-1 cultures (MSM + 1% glucose + 5 mM pyrene) reached a density of \(10^9\) cell/mL, they were harvested, washed three times with phosphate-buffered saline (pH 7.0), and resuspended in autoclaved Milli-Q water for respiration measurement. Respiration experiments were set up in duplicate or triplicate in 473 mL polystyrene jars, with each jar containing approximately \(4 \times 10^9\) bacterial cells and \(111.17\) mL one of the following media: (1) MSM (pH 7.0), (2) MSM + 10 mg of p-MWCNT, (3) MSM + 10 mg of c-MWCNT, (4) MSM + 1% glucose and 5 mM pyrene + 1 mg of p-MWCNT, (5) MSM + 1% glucose and 5 mM pyrene + 1 mg of c-MWCNT. Medium 1 served as a negative control without external carbon source; medium 2 or 3 represented a condition with MWCNTs being the only external carbon source; medium 4 or 5 represented a positive control containing both energetic carbon sources and low-dose MWCNTs. Respiration jars were wrapped with aluminum foil and incubated aerobically at 20–25 °C for about 32 days. The jar lids were then tightened and sealed with parafilm to keep gastight; headspace \(CO_2\) level was continuously monitored for 3 days using a Vaisala GMP343 probe that was inserted through the lid. \(CO_2\)-based estimation of mineralization is detailed in the SI.

**Data Analyses and Statistics.** Bacterial growth kinetics was analyzed by fitting experimental data to the modified Logistic or Gompertz equations\(^27\) using R (3.2.4). Raman spectra were analyzed using R and MATLAB (more details in the SI). Statistical analyses were performed with R. Levene’s test was conducted to check the homogeneity of variance, after which data were compared using ANOVA or the Welch t-test as well as the Mann–Whitney U test.

## RESULTS AND DISCUSSION

*M. vanbaalenii* PYR-1 Growth. For each medium condition and each concentration of both p- and c-MWCNT, growth trends from two independent batches were consistent (Figure S3). In contrast to other reports on CNT’s concentration-dependent cytotoxicity on microorganisms,\(^5\) the presence of either p-MWCNT or c-MWCNT stimulated *M. vanbaalenii* PYR-1 growth when glucose alone or both glucose and pyrene served as the primary carbon source. For instance, after 312-h growth, bacterial cultures in the glucose medium containing 25 ppm p-MWCNT or c-MWCNT reached an OD\(_{600}\) reading 3.0 or 1.7 times that of cultures grown in the same medium without MWCNTs, respectively (Figure S3, A1 and B1). Similarly, after 312 h growth, bacterial cultures in the glucose plus pyrene medium containing 5 ppm p-MWCNT or c-MWCNT reached an OD\(_{600}\) reading 1.4 or 1.6 times that of cultures grown in the same medium without MWCNTs, respectively (Figure S3, A1 and B1).

The addition of MWCNTs triggered earlier exponential growth of bacterial cultures. Growth kinetics was fitted with the Logistic model (eq 1) or the modified Logistic model incorporating population decline (eq 2) that are commonly used for proliferating populations\(^27\)

\[
y = \frac{A}{1 + \exp\left(\frac{-\mu}{\lambda}(\lambda - t) + 2\right)}
\]

\[
y = \frac{A \exp(-d t)}{1 + \exp\left(\frac{-\mu}{\lambda}(\lambda - t) + 2\right)}
\]

where \(A (-)\) is the maximal population size, \(\mu_m\) (h\(^{-1}\)) is the maximum specific growth rate, \(\lambda\) (h) is the lag time, and \(d\) (h\(^{-1}\)) is the decline rate (fitted parameters shown in Table S2). Using cultures grown in each medium without MWCNTs as reference, we assessed the impact of MWCNTs on PYR-1’s lag time. As shown in Figure 1, the addition of 25 ppm p-MWCNT or c-MWCNT to the glucose medium resulted in a 46.20% or 39.05% decrease in the lag time, respectively.
bacterial growth through promoting cell attachment, proliferation and biofilm formation.\(^{11}\) EPS as a protective barrier was also reported for *Shewanella oneidensis* under the stress of metal nanoparticles.\(^{23}\) In this study, *M. vanbaalenii* PYR-1 cells aggregated with MWCNTs (Figures S4 and S5) and formed biofilms encapsulating MWCNTs (Figure S6), a phenomenon previously observed for *Salmonella* exposed to SWCNT.\(^{22}\) Mycobacterial strains are known to attach and form biofilms on PAH particles with their extremely hydrophobic rigid cell envelope and can modify their cell wall composition in response to carbon sources.\(^{32}\) We thus speculate that MWCNTs may induce biofilm formation by PYR-1 (SEM images shown in Figure S6) and consequently shorten the lag time of its exponential growth, although more research is needed. The small amount of amorphous carbon on the surface of MWCNTs (Table S1), although insufficient to serve as the sole carbon/energy source, might also contribute to the observed growth stimulation. Alternatively, MWCNTs can potentially up-regulate key genes involved in this bacterium’s growth. For example, MWCNTs are known to promote plant growth through up-regulating genes for cell division/cell wall formation and water transport.\(^{33}\) Similar mechanisms could have enhanced *M. vanbaalenii* PYR-1 growth (e.g., up-regulation of DNA metabolism shown in Figure S7), but to determine the exact mechanism requires further investigation. In any case, a majority of PYR-1 cells attached on MWCNTs remained alive with intact cellular integrity (green cells in Figure S5) and cell morphology (Figure S6), whereas losing cellular integrity is

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**Figure 2.** HRTEM images of (A) p-MWCNT and (B) c-MWCNT before (A1, A4, B1, B4) and after 25-day incubation with *M. vanbaalenii* PYR-1 in the glucose medium (A2, B2) or the glucose plus pyrene medium (A5, B5), in comparison with abiotic controls (A3, A6, B3, B6). Scale bars indicate 5 nm. White arrows indicate disordered graphene layers.
believed to be one major mechanism for CNT’s bacterial cytotoxicity.\textsuperscript{7,34}

**Biotransformation of MWCNTs.** TEM imaging of initial p- and c-MWCNT revealed sharp-edge tubular structures and crystal lattices (10 layers on average according to HRTEM; Table S1), without substantial amorphous materials on the surface (Figure 2, Figure S8, and Table S1). The same morphology was observed for MWCNTs after abiotic incubation (Figure 2 and Figure S8). In contrast, a variety of drastic morphological changes were detected in both p-MWCNT and c-MWCNT incubated with *M. vanbaalenii* PYR-1 for 25 days, regardless of the growth medium (containing glucose or both glucose and pyrene). We observed shorter and thinner nanotubes and highly disordered tubular structure with kinks and bends (Figure S8, A3–D3), as well as nanotubes with broken ends (Figure S9). Moreover, we observed exfoliated graphitic flakes with substantial amorphous materials on the surface, likely residues from incomplete degradation of graphite lattices (Figure 2, A2, A5, B2, B5, and Figure S8, A4–D4). These TEM observations indicated degradation of MWCNTs by PYR-1, likely through oxidization of crystal lattices and subsequent exfoliation of graphitic walls. Similar structural transformations were observed in the degradation of CNTs by horseradish peroxidase, microglial cells, and a microbial consortium, where CTNs were broken down to shorter nanotubes or deformed through a layer-by-layer mode.\textsuperscript{9,15,35}

As another line of evidence for degradation, Raman spectra were analyzed for MWCNTs incubated with *M. vanbaalenii* PYR-1 and compared to initial and abiotically incubated MWCNTs. Incubations using the glucose medium (Figures 3 and 4) or the glucose plus pyrene medium (Figures S10 and S11) yielded similar results. As shown in Figure 3, Raman spectra were similar for initial p- and c-MWCNT, displaying two fingerprint peaks as the tangential-mode G band (\( \sim 1580 \text{ cm}^{-1} \)) and the disorder-induced D band (\( \sim 1340 \text{ cm}^{-1} \)).\textsuperscript{36} The intensity ratio of D band to G band (\( I_D/I_G \)) for p- and c-MWCNT was measured as 1.07 \( \pm \) 0.05 (\( n = 67 \)) and 1.16 \( \pm \) 0.11 (\( n = 48 \)) (Figure 4), consistent with other reports on the same MWCNTs.\textsuperscript{37} Surface functionalization of p-MWCNT with carboxylic functional groups (Table S1) slightly increases the \( I_D/I_G \) value (\( p < 0.001 \)), a phenomenon caused by formation of defect sites on MWCNTs.\textsuperscript{38} For p-MWCNT incubated with PYR-1 for 25 days, the G band and D band were still detectable (Figure 3). However, the \( I_D/I_G \) ratio changed to 1.27 \( \pm \) 0.11 (\( n = 157 \)), significantly higher than initial p-MWCNT (\( p < 0.001 \)) (Figure 4). Meanwhile, incubation with PYR-1 introduced three carotenoid peaks (1004–1008, 1160, and 1520–1538 cm\(^{-1}\)) and two minor lipid peaks (1260 and 1440 cm\(^{-1}\)),\textsuperscript{39} which were also seen for PYR-1 grown in the same medium without MWCNTs (Figure S12A). Mycobacteria can

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**Figure 3.** Raman spectra (532 nm excitation) of (A) p-MWCNT and (B) c-MWCNT before (A1, B1) and after (A2, B2) 25-day incubation with *M. vanbaalenii* PYR-1 in the glucose medium, in comparison with abiotic controls (A3, B3). Each spectrum represents average of \( \geq 10 \) individual spectra from a sampling area.

**Figure 4.** \( I_D/I_G \) ratios (532 nm excitation) of (A) p-MWCNT and (B) c-MWCNT after 25-day incubation with *M. vanbaalenii* PYR-1 in the glucose medium (A1, B1), in comparison with abiotic controls (A2, B2) and initial MWCNTs (A3, B3) (\( p < 0.001 \) in both comparisons). Box-and-whisker plot shows minimum and maximum (whisker bottom and top), first and third quartile (box bottom and top), and median (line inside box) of \( I_D/I_G \) values, along with sample sizes.
produce diverse pigments, of which a vast majority are characterized as carotenoids. Carotenoids are efficient antioxidants scavenging singlet molecular oxygen and peroxyl radicals and may have thereby protected M. vanbaalenii PYR-1 from MWCNT-induced oxidative stress. As a comparison, the $I_D/I_G$ ratio was 1.15 ± 0.05 for abiotically incubated p-MWCNT ($n = 32$), only slightly different from initial p-MWCNT ($p < 0.001$) and much lower than p-MWCNT incubated with PYR-1 ($p < 0.001$) (Figure 4). The slight $I_D/I_G$ increase in abiotic p-MWCNT could be attributed to better dispersion after 25-day continuous shaking. Generally, the G band of CNTs originates from in-plane tangential vibration of pairs of C sp² atoms within graphene sheets, while the D band arises from amorphous carbon or disorders in the graphene structure, such as finite or nanosized graphitic planes, rings with defects, and heptagon–pentagon pairs and kinks. The relative intensity ratio of these two bands is therefore an indicator for CNT’s defect density. An increase in $I_D/I_G$ for p-MWCNT incubated with PYR-1 suggests more amorphous or disorder carbon from biodegradation, consistent with the aforementioned TEM observations.

Raman spectra from c-MWCNT incubated with PYR-1 changed more significantly compared to controls. For 43 out of 89 analyzed spectra, two MWCNT fingerprint peaks were no longer prominent, while the carotenoid and lipid peaks dominated (e.g., Figure S12C). For 46 of the 89 spectra that still displayed detectable D and G bands (Figure 3), the $I_D/I_G$ ratio was measured as 1.34 ± 0.21 ($n = 46$), significantly higher than initial c-MWCNT ($p < 0.001$) (Figure 4). In comparison, the Raman spectra from abiotically incubated c-MWCNT still had prominent D and G bands (Figure 3), with a $I_D/I_G$ value of 1.14 ± 0.10 ($n = 40$), similar to initial c-MWCNT ($p > 0.3$) and much lower than c-MWCNT incubated with PYR-1 ($p < 0.001$) (Figure 4). The fact that nearly half of the spectra (43/89) from biodegraded c-MWCNT showed indistinct fingerprint peaks, but all the spectra (157/157) from biodegraded p-MWCNT still had prominent fingerprint peaks and the fact that biodegraded c-MWCNT had a more dramatic $I_D/I_G$ increase than biodegraded p-MWCNT (Figure 4) suggest greater biodegradation of c-MWCNT than p-MWCNT. This might be due to c-MWCNT’s better dispersion and therefore higher bioaccessibility. Furthermore, studies on SWCNTs indicate that surface carboxylation-induced structural defects and the closer orientation of carboxylated SWCNTs to the active sites of enzymes could facilitate their biodegradation as compared to pristine SWCNTs, which might also apply to MWCNTs in our case.

A significant increase in $I_D/I_G$ was also observed for MWCNTs incubated with PYR-1 in the glucose plus pyrene medium, as compared to initial or abiotically incubated MWCNTs ($p < 0.001$ in both comparisons) (Figures S10 and Figure S11), whereas there was no significant difference between initial MWCNTs and abiotic controls in that medium ($p > 0.004$). Moreover, regardless of the incubation medium, biodegraded MWCNTs generally displayed greater $I_D/I_G$ variance than initial MWCNTs or abiotic controls (Figure 4 and Figure S11), which may suggest larger heterogeneity of biodegraded MWCNTs in defect density.

Along with the Raman spectra analysis for bulk samples, we further applied confocal Raman microspectroscopy to residual MWCNTs after different treatments (Figure S and Figure S13). The 785 nm excitation wavelength allowed us to focus on signals only from MWCNTs, and a space resolution as fine as 300 nm per pixel was achieved. For initial MWCNTs and abiotic controls in the glucose medium (Figure S), Raman signals of the D band and G band coincided in regions of ~6 μm × ~6 μm, consistent with the sizes of MWCNT aggregates (bright-field images in Figure S14). $I_D/I_G$ ratios ranged from 1.02 to 1.09 in the scanned areas, being 1.04 ± 0.01 for initial p-MWCNT and its abiotic control and 1.07 ± 0.02 for initial c-MWCNT and its abiotic control. After 25-day incubation with PYR-1, $I_D/I_G$ values increased to 1.09 ± 0.04 for degraded p-MWCNT and 1.13 ± 0.00 for degraded c-MWCNT ($p < 0.001$ when compared to initial or abiotic samples) (Figure 5). Similar increases in $I_D/I_G$ over the entire scanned regions were identified in MWCNTs incubated with PYR-1 in the glucose and pyrene medium (Figure S13). Excitation wavelength and laser power could influence peak intensity, so the exact $I_D/I_G$ values measured here are not comparable to those obtained in regular Raman analysis. But the 2-D confocal Raman hyperspectral maps reflect a similar trend of increased $I_D/I_G$ in MWCNTs after incubation with PYR-1 as revealed by statistical analysis on regular Raman spectra and further suggest biodegradation of MWCNTs, which became more amorphous and/or disordered afterwards.

**Mineralization of MWCNTs.** During microbial degradation, it is possible that a proportion of MWCNTs were mineralized by M. vanbaalenii PYR-1. To test and quantify potential mineralization of MWCNTs, we measured PYR-1 respiration (≈4 × 10⁶ cells initially) in the presence of different carbon sources. Without any external carbon source (i.e., bacterial cells incubated in the MSM alone), CO₂ in the headspace of the measurement chamber slightly increased from 392.7 ± 9.4 to 509.5 ± 11.5 ppm during 72 h (Figure S15), partially contributed by endogenous metabolism. As a positive control, with 10 g/L glucose, 1 g/L pyrene, and 9 mg/L MWCNTs (either p- or c-MWCNT) added to the MSM, respiration of PYR-1 increased CO₂ in the headspace to 2883.5 ± 480.5 ppm after 72 h, without statistical difference between p-MWCNT and c-MWCNT (Figure S16), suggesting that at low concentrations both MWCNTs had similar influences on bacterial respiration. When 89.4 mg/L c-MWCNT but no other carbon source was added to the MSM, CO₂ concentration in the headspace increased to 1003.7 ± 243.7 ppm after 72 h, much higher than the MSM control, suggesting partial mineralization of c-MWCNT by PYR-1. Assuming all CO₂ had evolved from MWCNTs or endogenous metabolism, the 72 h mineralization fraction was estimated as 0.55 ± 0.21% for c-MWCNT (i.e., 55.2 ± 21.0 μg carbon from 10 mg of c-MWCNT) (calculations in the SI). Consistently, 2-D confocal Raman spectroscopic analysis of the residual c-MWCNT identified an increase in $I_D/I_G$, suggesting more defects resulting from biodegradation (Figure S17). In comparison, the increase in CO₂ concentration in the headspace was much lower when 89.4 mg/L p-MWCNT was added to the MSM, reaching 86.0 ± 49.7 ppm after 72 h. By estimation, 0.07 ± 0.04% of p-MWCNT (7.4 ± 4.3 μg carbon from 10 mg of p-MWCNT) was mineralized. Moreover, incubation with ¹³C-enriched p-MWCNT resulted in a slight shift in CO₂ ¹³C/¹²C, further suggesting potential mineralization (see Figure S18). Currently, it is unclear whether additional CO₂ yield from the c-MWCNT samples, as compared to the p-MWCNT samples, was generated from mineralization of amorphous carbon or carboxyl groups on c-MWCNT surface wall. The addition of MWCNTs in the MSM might have also stimulated PYR-1’s endogenous respiration, resulting in higher CO₂ yield than the MSM control, and c-MWCNT might have exerted stronger stimulation than p-MWCNT. Currently, there is no report on such stimulation effects, which requires further investigation. Also limited are quantitative studies of microbial degradation and/or mineralization of CNTs. Zhang et al. reported that a bacterial community...
could mineralize as much as 6.8% of $^{14}$C-labeled MWCNT within 7 days through cometabolism with additional carbon sources. In contrast, Parks et al.\textsuperscript{16} reported that with additional carbon sources, less than 0.1% of $^{14}$C-SWCNT was mineralized within 6 months, either by the white-rot fungus \textit{T. versicolor} or by microbiota from polychlorinated biphenyl-contaminated

![Figure 5. 2-D confocal Raman hyperspectral maps (785 nm excitation) of biodegraded p-MWCNT (panel A) and c-MWCNT (panel B). Shown are MWCNTs before (A1−A3, B1−B3) and after (A4−A6, B4−B6) incubation with \textit{M. vanbaalenii} PYR-1 in the glucose medium for 25 days as well as abiotic controls (A7−A9, B7−B9) incubated in the same medium for 25 days. Color bars indicate Raman signal intensity (in arbitrary units) or $I_D/I_G$ range.](image-url)
sediments. Transformation and degradation of CNTs could be facilitated by utilizing bacterial consortia consisting of multiple bacterial species, which might have a limitation in terms of complete degradation and mineralization. Further studies are required to explore the underlying mechanisms and to optimize relevant reactions. It is also possible that by utilizing bacterial consortia consisting of multiple PAH-degrading species, a higher extent of MWCNT degradation and mineralization could be achieved.

**Environmental Implications.** During the past decade, CNTs have received increasing attention due to their release into the environment and subsequent potential harmful effects on ecosystems and human health. Model estimations suggest that CNTs accumulate in the environment could have reached concentrations of 0.01–3 µg/kg in soils and 30–1000 µg/kg in sediments. Transformation and degradation of CNTs could significantly affect their transport, bioavailability, and ecotoxicity in the environment. Through analyses using TEM and Raman/confocal Raman, as well as respiration measurements, we found that the PAH-degrading bacterium *M. vanbaalenii* PYR-1 can partially transform and potentially mineralize both p-MWCNT and c-MWCNT, in spite of a low mineralization rate of 8%–0.25% (carbon mass) per day. Surface functionalization was found to facilitate PYR-1-mediated biodegradation. To our knowledge, this is the first report of a single bacterium capable of degrading MWCNTs in various growth media. Given a vast diversity of genetic and metabolic potentials of microbes in the environment, it is possible that microbial communities could achieve considerable biodegradation of CNTs through metabolic networks, which deserves more research in future. Overall, our findings add to the understanding of microbial degradation of MWCNTs in the environment and highlight the importance of surface functionality in the degradability of MWCNTs.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b04523.

Detailed methods; MWCNT characterization; growth analysis; microscopy for cell/MWCNT aggregates, live/dead stained cells, biofilms, and cell morphology; gene regulation; TEM and Raman analyses on MWCNT biodegradation; mineralization assessment (PDF)

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**Notes**

The authors declare no competing financial interest.

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