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Microbial degradation of low-density polyethylene and synthesis of polyhydroxyalkanoate polymers

Zahra Montazer, Mohammad B. Habibi Najafi, and David B. Levin

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Abstract: We have characterized the ability of eight bacterial strains to utilize powdered low-density polyethylene (LDPE) plastic (untreated and without any additives) as a sole carbon source. Cell mass production on LDPE-containing medium after 21 days of incubation varied between 0.083 ± 0.015 g/L cell dry mass (cdm) for *Micrococcus luteus* IRN20 and 0.39 ± 0.036 g/L for *Cupriavidus necator* H16. The percent decrease in LDPE mass ranged from 18.9% ± 0.72% for *M. luteus* IRN20 to 33.7% ± 1.2% for *C. necator* H16. Linear alkane hydrolysis products from LDPE degradation were detected in the culture media, and the carbon chain lengths of the hydrolysis products detected varied, depending on the species of bacteria. We also determined that *C. necator* H16 produced short-chain-length polyhydroxyalkanoate biopolymers, while *Pseudomonas putida* LS46 and *Acinetobacter pittii* IRN19 produced medium-chain-length biopolymers while growing on polyethylene powder. *Cupriavidus necator* H16 accumulated poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHB-V) polymers to 3.18% ± 0.4% of cdm. The monomer composition of the PHB-V was 94.9% ± 0.61% 3-hydroxybutyrate and 5.03% ± 0.56% 3-hydroxyvalerate. This is the first report that provides direct evidence for simultaneous bioconversion of LDPE plastic to biodegradable polyhydroxyalkanoate polymers.

Key words: low-density polyethylene, biodegradation, bioassimilation, bioconversion, polyhydroxyalkanoates.

Résumé : Les auteurs ont caractérisé la capacité de huit souches bactériennes à utiliser un plastique de polyéthylène basse densité en poudre (PEBD) (non-traité et sans aucun additif) comme seule source de carbone. La masse cellulaire produite dans du milieu contenant du PEBD après 21 jours d'incubation variait entre 0,083 ± 0,15 g/L de poids sec pour *Micrococcus luteus* IRN20 et 0,39 ± 0,036 g/L pour *Cupriavidus necator* H16. Le pourcentage de diminution de la masse de PEBD allait de 18,9 % ± 0,72 % pour *M. luteus* INR20 à 33,7 % ± 1,2 % pour *C. necator* H16. Des alcanes linéaires produits par l'hydrolyse du PEBD ont été détectés dans le milieu de culture, et la longueur de la chaîne de carbone des produits d'hydrolyse détectés variait en fonction de l'espèce bactérienne. Les auteurs ont aussi déterminé que *C. necator* H16 produisait des biopolymères de polyhydroxyalcanoate à chaîne courte alors que *Pseudomonas putida* LS46 et *Acinetobacter pittii* IRN19 produisait des biopolymères à chaîne moyenne lorsque cultivés sur une poudre de polyéthylène. *Cupriavidus necator* H16 accumulait des polymères de poly(3-hydroxybutyrate-*co*-3-hydroxyvalérate) (PHB-V) à 3,18 % ± 0,4 % de poids sec. La composition en monomères du PHB-V était de 94,9 % ± 0,61 % de 3-hydroxybutyrate et 5,03 % ± 0,56 % de 3-hydroxyvalérate. Il s'agit du premier rapport qui fournit une preuve directe de la bioconversion simultanée de plastique de PEBD en polymères de polyhydroxyalcanoate biodégradables. [Traduit par la Rédaction]

Mots-clés : polyéthylène basse densité, biodégradation, bioassimilation, bioconversion, polyhydroxyalcanoates.

Introduction

From 1950 to 2015, the total amount of synthetic plastics discarded in landfills or in the natural environment has been estimated at approximately 4900 megatonnes (1 t = 1000 kg). This amounts to about 60% of all the plastic materials ever produced. According to European data, the amount of plastic waste deposited in landfill sites varies from less than 10% of plastic waste generated (in countries where landfill bans are in effect) to nearly 80% in other countries (Geyer et al. 2017; Plastics Europe 2017; Ragaert et al. 2017). Low-density polyethylene (LDPE) is the most abundant solid plastic waste discarded in landfills, in the form of plastic bags (69.13%). Despite recycling and energy recovery efforts, a lack of proper recycling and reuse facilities has resulted in the inundation of both land and the oceans with increasing

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amounts of plastic wastes, and the harmful impacts of nonbiodegradable polyethylene (PE) waste accumulation continue to increase (Ragaert et al. 2017; Rajasekaran and Maji 2018).

Over 90 microorganisms, including bacteria and fungi, have been reported to degrade petroleum plastics (Jumaah 2017). Most studies of PE biodegradation have reported that bacteria, such as *Rhodococcus* spp. (Bonhomme et al. 2003; Gilan et al. 2004; Fontanella et al. 2010), *Pseudomonas* spp. (Rajandas et al. 2012), *Bacillus* spp. (Sudhakar et al. 2008; Abrusci et al. 2013), and *Cupriavidus necator* (also formerly known as *Ralstonia eutropha* H16) (Yoon et al. 2012), and fungi, such as *Aspergillus* and *Fusarium* (Hasan et al. 2007; Sahebnazar et al. 2010), are able to hydrolyze PE only after ultraviolet (UV) or thermal treatment or other methods of pretreatment, which render the carbon chains of polymer sensitive to biodegradation (Ammala et al. 2011).

There is no exact definition for biodegradation because it is a complex process that is dependent on many factors, such as availability of a substrate, morphology, surface characteristics, and molecular weight (Albertsson et al. 1987; Ammala et al. 2011). One key weakness of the term "biodegradable" is that it does not contain any information on the location, time scale, and extent of the decomposition process. Indeed, biodegradability is often defined in relation to the purpose or the conditions of interest, with separate standards and test methods (Harrison et al. 2018). Different locations (soil, wastewater, freshwater, or marine habitats), different conditions (in vitro or in vivo, aerobic or anaerobic), different structures and compositions of the PE substrate, and different inocula (sources of inoculum used for biodegradability tests are diverse and often nonspecific) have been applied in biodegradation experiments. Moreover, biodegradation has been quantified by a wide range of measures, including substrate weight loss, percentage of carbon dioxide emission, and changes in the mechanical properties or the chemical structure of the PE substrate used. This makes comparisons of the results from various biodegradation experiments difficult and indicates that more precise descriptions of the methods and specific parameters measured are required.

Microbial degradation of plastics is caused by certain enzymatic activities that lead to a chain cleavage of the polymer into oligomers and monomers. Biodegradation the PE plastic wastes by microbial isolates and their enzymes has become a major topic of research (Nowak et al. 2011; Kyaw et al. 2012; Gajendiran et al. 2016; Sen and Raut 2016). PE has a simple linear structure of *n*-alkanes and is extremely resistant to biodegradation. Many of the species shown to degrade PE are also able to hydrolyze and consume linear *n*-alkanes, like paraffin. Alkane hydroxylases are the key enzymes in aerobic degradation of alkanes by bacteria. Linear paraffin molecules (e.g., $C_{44}H_{90}$, Mw 618) are known to be consumed by a number of different microorganisms in 20 days (Haines and Alexander 1974). Microbial hydrolysis of paraffin is well understood, and the initial step involves hydroxylation of C–C bonds to generate primary or secondary alcohols, which are further oxidized to aldehydes or ketones, and then to carboxylic acids, which are hydrophilic (Alvarez 2003; Watanabe et al. 2003).

Carboxylated *n*-alkanes are analogous to fatty acids, which can be catabolized by bacteria via the β-oxidation pathway. These enzymatic oxidation products may be absorbed by microbial cells where they are catabolized (Usha et al. 2011). Aerobic biodegradation of PE by bacteria is thought to occur in four stages. (*i*) Biodeterioration — oxidative enzymes released by microorganisms catalyze the formation of carbonyl groups throughout the linear carbon chain. Further oxidation decreases the number of carbonyl groups due to the formation of carboxylic acids. (*ii*) Biofragmentation — results in hydrolysis and fragmentation of the polymer carbon chains and the release of intermediate materials. Surface corrosion of the plastic material occurs in this step. (*iii*) Bioassimilation — small hydrocarbon fragments are metabolized by the bacterial cells. (*iv*) Mineralization — catabolized hydrolysis products are converted to microbial biomass with the concomitant release of carbon dioxide (CO_2) and water (H_2O).

Although biodeterioration and biofragmentation have been confirmed and established (Albertsson and Karlsson 1990; Ammala et al. 2011), evidence for bioassimilation and complete mineralization are very limited. In other words, bioassimilation and mineralization of PE have not been confirmed owing to a lack of supporting evidence (Yang et al. 2014; Sen and Raut 2016). We report here on the isolation and characterization of several bacteria that are able to utilize LDPE as a sole carbon source, and provide evidence for biofragmentation and bioassimilation of petroleum-derived LDPE into biomass plus biodegradable polyhydroxyalkanoates polymers.

Capability of PE biodegradation within some isolates used in this project (*Pseudomonas putida* IRN22, *Acinetobacter pittii* IRN19, *Micrococcus luteus* IRN20) was previously reported by Montazer et al. (2018). In the present work, the percentage weight loss of LDPE and bacterial biomass production were used as indicators of biodegradation and compared among the isolates tested. However, the main object of this work was to provide evidence for microbial fragmentation and conversion (assimilation) of LDPE into polyhydroxyalkanoate polymers by the bacterial cells during biodegradation.

Materials and methods

LDPE powder

LDPE powder, with a particle size of 400 μm or less (screen retention of 50 mesh is 4.4%) and a molecular weight range between 20 000 and 150 000, was supplied by Alfa-Asar Company (USA; CAS number 9002-88-4). Ac-

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cording to the supplier, the feedstock melt index and density were 3.50 g/10 min (at 190 °C and 2.16 kg load) and 0.9227 g/cm^3, respectively. According to the supplier's data sheet, the polymer was pure and free of stabilizing agents. The PE particles were sterilized by exposure to UV light (254 nm) for 1 h while mixing.

Culture media

Bacteria were assessed for their ability to grow on LDPE as a sole carbon source using minimal salt polyethylene medium. The composition of the PE medium was as follows (per L): Na_2HPO_4 5 g, KH_2PO_4 2 g, $(NH_4)_2SO_4$ 3 g, KCl 0.15 g, NaCl 0.5 g, $CaCl_2$ 1.2 mg, $MgSO_4$ 20 mg, Fe(III)NH_4 citrate 1 mg, and trace elements solution 1 mL. Each litre of trace element solution contained the following: 0.3 g of H_3BO_3, 0.2 g of $CoCl_2{\cdot}6H_2O$, 0.1 g of $ZnSO_4{\cdot}7H_2O$, 30 mg of $MnCl_2{\cdot}4H_2O$, 30 mg of $NaMoO_4{\cdot}2H_2O$, 20 mg of $NiCl_2{\cdot}6H_2O$, and 10 mg of $CuSO_4{\cdot}5H_2O$. Also, 1 mL of Tween 80 was added as a biosurfactant to facilitate suspension of the LDPE particles in the media. The addition of surfactants such as Tween 80 and mineral or paraffin oil in the medium has been reported to enhance biodegradation of PE by microorganisms by 50% due to increased hydrophilicity of PE surface and biofilm formation (Gilan et al. 2004). The other advantage is that Tween 80 is not capable of denaturing proteins and enzymes and can detoxify some unsaturated fatty acids that would otherwise inhibit microbial growth on the PE surface (Albertsson et al. 1993). The medium was autoclaved first, and then the UV-sterilized PE powder was added to the medium in the biosafety cabinet. Ramsay's medium was used with the most active PE-degrading bacteria to assess the production of polyhydroxyalkanoates under nitrogen-limited conditions. The final pH for both media was 7.0 (Fu et al. 2015).

Bacterial strains and culture conditions

Four LDPE-degrading bacteria isolated from a plastic-dump landfill soil located in Mashhad, Iran, including *Pseudomonas putida* IRN22 (MF348181), *Acinetobacter pittii* IRN19 (MF348182), *Micrococcus luteus* IRN20 (MF348185), and *Delftia tsuruhatensis* IRN27 (MF348184), were studied in these experiments (Montazer et al. 2018). In addition, we tested four bacteria that are known to synthesize PHA polymers and for which an annotated genome is available: *Cupriavidus necator* H16, *Pseudomonas putida* LS46, *Pseudomonas chlororaphis* PA23, and *Pseudomonas monteilii* MO2. These species were available in the laboratory of the corresponding author, Dr. David Levin, at University of Manitoba, Manitoba, Canada.

These eight bacteria were cultured on paraffin minimal salt medium. Paraffin was used as the initial carbon source in minimal medium cultures to adapt the microbial physiology to LDPE degradation. The structure of LDPE is similar to paraffin, which is known to be biodegradable and can be regarded as the low molecular counterpart of synthetic polyolefins (Albertsson and Karlsson 1990). In this step, the concentration of paraffin was decreased gradually in the medium and LDPE powder added in increasing amounts in three steps: step 1, glucose (0.05%) plus paraffin (1%); step 2, paraffin (0.5%) plus LDPE (0.5%); and step 3, paraffin (0.05%) plus LDPE (1%). All bacteria were cultured on the paraffin minimal salt medium until turbidity due to increased cell density was observed. Cells from these cultures (1% *v*/*v*) were then transferred to the next medium. All media contained 0.1% Tween 80.

Controls used in these experiments

Assessment of the effect of UV irradiation on LDPE structure

The PE powder used as substrate for microbial growth in these experiments was surface-sterilized by exposure to UV light (254 nm) for 1 h. To examine the effect of the UV irradiation on LDPE structure and to determine if this exposure altered the structure of the LDPE, rendering it sensitive to microbial attack, the UV-irradiated LDPE was subjected to Fourier-transform infrared (FTIR) spectroscopy using an Agilent Cary 620 spectrophotometer equipped with a Zn-Se-Crystal ATR accessory. Analyses of the FTIR spectra were conducted to evaluate the formation of carbonyl groups, which would indicate alteration of the LDPE structure. The effect of UV irradiation on LDPE structure was assessed by measuring the ratio of adsorption at 1701.21 (which detects carbonyl-group formation) to 2914.72/cm (which detects the CH_2 asymmetric stretch) in three independent replicate experiments, both before and after exposure to 254 nm UV radiation (Abrusci et al. 2013). We also compared bacterial growth on untreated LDPE versus UV-irradiated LDPE to determine if the UV-irradiated LDPE was more susceptible to bacterial degradation.

Assessment of the effect of Tween 80 and citrate on microbial growth

Both Tween 80 and citrate in the culture medium could serve as carbon sources for bacterial growth. Therefore, three control cultures were conducted to determine if these media components could support growth: (*i*) LDPE in the culture medium containing 0.1% Tween 80 and 1 mg/L Fe(III)NH_4 citrate without inoculation of bacteria, to test for microbial contamination (i.e., to test the sterility of the irradiated LDPE); (*ii*) LDPE in the culture medium containing 0.1% Tween 80 and 1 mg/L Fe(III)NH_4 citrate inoculated with *Escherichia coli* DH5α, to determine if carbon sources other than LDPE derived from the LDPE powder may support microbial growth; (*iii*) culture medium containing 0.1% Tween 80 and 1 mg/L Fe(III)NH_4 citrate, with no LDPE, inoculated with each of the bacteria tested in this study, to determine if Tween 80 and Fe(III)NH_4 citrate could support bacterial growth as a sole carbon source.

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Degradation assays

LDPE powder (100 mg) was added to cylinder tubes containing 10 mL of the sterile basal medium. After inoculation with cultures of the eight species individually, these were shaken on a rotary shaker at 30 °C. Experiments were performed in triplicate for 3 weeks. Controls consisted of tubes containing medium with no bacterial inoculation. *Escherichia coli* DH5α was also cultured as a control to determine if any additional chemical(s) that could serve as a carbon source were in the PE powder. Bacterial growth was measured over 21 days and PE degradation was monitored by measuring the weight of the PE particles before and after incubation. Cell mass production (cell dry mass) was also measured at the end of the experiment (day 21).

Cell growth assays

The growth of bacteria on PE particles was measured according to Uchida et al. (2000) as optical density at 600 nm (OD_{600}) with a visible spectrophotometer (model Ultrospec 500 pro; Biochrom, USA). Before measurement, all tubes were shaken gently to suspend the settled biomass and then allowed to stand for 30 min to allow time for the suspended PE particles to float up and out of the path of the OD reading.

LDPE weight loss measurement

To accurately determine the dry mass of residual LDPE (after the 21 days of culturing), PE with bound cells was filtered using a single-fold tissue paper (Tork, SCA Tissue North American LLC, USA). The filter pores were small enough to capture the PE particles, yet wide enough for the mineral components and cells to be washed through. The filtered PE particles were then washed with 2% (*m*/*v*) sodium dodecyl sulfate (SDS) to lyse any remaining cells that adhered to the PE particle surface, and the cell debris was captured, dried, and weighed. The PE particles (on the filter paper) were further rinsed with distilled water and then dried overnight at 60 °C before weighing. Although some dried cells debris remained attached to the PE particles (visualized by scanning electron microscopy (SEM)), their contribution to the total PE particle mass was considered negligible. The percentage of PE particle weight loss was determined using the formula (Kyaw et al. 2012):

$$\%\,\text{Weight loss} = [(\text{Initial weight} - \text{Final weight}) / \text{Initial weight}] \times 100$$

Biomass production measurements

The residual media containing microbial biomass were centrifuged at 8000 r/min (7155*g*) in preweighed 50 mL Falcon tubes for 30 min (model IEC Multi, Electron Corporation Company, USA). The supernatants were collected, and the cell pellets were dried at 60 °C overnight. The tubes containing the dried cell mass were then weighed using a four-digit balance (model SL-114, Denver Instrument, USA).

Analysis of LDPE hydrolysis products

The presence of putative hydrolysis products (linear alkanes) in the culture supernatant generated by LDPE biodegradation were analyzed by gas chromatography – flame ionization detection (GC–FID). At the end of the experiment (day 21), triplicate tubes containing the 10 mL of bacterial cultures were extracted with 5 mL of hexane by shaking for 1 min. After the separation of the layers, the organic layer was analyzed on an Varian CP 3800 gas chromatograph (Varian, Inc., California, USA) equipped with a FID detector and fitted with a 50 m × 0.2 mm × 0.33 μm CP Sil-CB capillary column (Agilent, Canada) using a split mode (split ratio 5:1). The carrier gas was helium with volume injection of 5 mL/min and temperature of 200 °C. The oven method employed was 35 °C for 2 min, ramping at 10 °C/min to 250 °C, followed by a ramp of 20 °C/min to 320 °C and held at this temperature for 23 min. For peak identification, a solution containing C_7–C_{40} alkane standards was used (Guzik et al. 2014). To calculate the alkane concentrations, standard curves for C_{22} to C_{31} alkanes with concentrations of 1:10, 1:20, and 1:40 were used to construct the standard curve.

Scanning electron microscopy

To survey the biofilm formation, the PE particles were removed from the culture medium after 3 weeks incubation to observe the bacterial colonization of the PE particles and the extent of surface erosion. Samples were prepared according to Harshvardhan and Jha (2013). The samples were washed for 2 min in 0.01 mol/L phosphate buffer (pH 7.2) to release excess medium. In contrast, in the procedure for the examination of surface erosion, PE samples were washed with a 2% SDS solution in water followed by several rinses in warm distilled water to remove surface-adhered cells completely. Both types of PE samples were fixed in 2% glutaraldehyde in phosphate buffer (pH; 7.2) for 2 h and dehydrated in graded ethanol (50%, 70%, and 100%). After fixation, the samples were dried in a vacuum. The dehydrated samples were sputter-coated with gold at 50 mTorr (1 Torr = ~ 133.3 Pa), 45 mA, for 45 s, which resulted in a 100 Å thick gold layer (Denton Vacuum Inc., model Desk II, USA). The samples were then examined using a FEI Quanta FEG 650 Environmental SEM.

PHA production by selected LDPE-degrading bacteria

Cupriavidus necator H16, *P. putida* LS46, and *A. pittii* IRN19 (all know to be PHAs producing bacteria) were grown in 1 L Erlenmeyer flasks containing 500 mL of nitrogen-limited (1 g/L ammonium sulfate) Ramsay's media and LDPE particles at a final concentration of 1%. The flasks were incubated at 30 °C with shaking at 150 r/min. Biomass was collected by centrifugation and dried overnight at 60 °C for further analysis.

Analysis of polymer subunit composition

Accumulation of PHA polymers by *C. necator* H16, *P. putida* LS46, and *A. pittii* IRN19 biomass was analyzed by

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Table 1. Peak definition and wavelength position (/cm) at different points of the curves (baselines, edge, and peak).

	Peak position	Left baseline	Left edge	Right edge	Right baseline
CH_2 asymmetric (CHSii)	2914.72	2983.339	2929.657	2896.586	2793.48
CH_2 symmetric (CHSi)	2847.885	2999.692	2857.678	2838.224	2770.135
C=C	1462.559	1490.705	1473.66	1456.785	1425.211
Carbonyl	1701.121	1736.244	1715.731	1696.193	1669.237

Table 2. Data from control experiments.

Control	Inoculation	Culture media	Optical density*
Chemical contamination	*E. coli* BL DE3	1% LDPE, 0.1% Tween 80, plus minimal salt media	0.09±0.016
Microbial contamination	No inoculant	1% LDPE, 0.1% Tween 80, plus minimal salt media	Negative
Other carbon source consumption	Each isolate tested	No LDPE, 0.1% Tween 80, plus minimal salt media	0.05±0.008

*Average of optical density from each bacterial growth after 3 days. Maximum OD level during the incubation time.

GC. Oven-dried cell mass samples were processed by the acid-catalyzed methanolysis procedure as described by Fu et al. (2014). GC analyses were conducted by using the organic phase on an Agilent 7890A GC equipped with a split–splitless inlet (operated in split mode, split ratio 10:1), a DB23 capillary column (Agilent, 30 m × 250 μm × 0.25 μm), and a FID. Method operating parameters and peak quantification were as described by Fu et al. (2014). For peak identification, benzoic acid and DL-β-hydroxylauric acid (*n*-dodecanoic acid; purity ≥ 99% for GC analysis, a C12 fatty acid ($CH_3(CH_2)10CH_3$) were used as internal standards (Fu et al. 2014; Blunt et al. 2017).

Statistical analyses

Data for LDPE weight loss, cell growth (OD_{600}), and total hydrolysis products concentration for each bacterium were subjected to statistical analyses using the R Core Team (2017) stats package. Mean variables were compared using Duncan's test, and correlations between variables were calculated by the Pearson Square method at probability level of 5% (R Core Team 2017).

Results

FTIR assessment of UV-irradiated LDPE

The effect of UV irradiation on carbonyl-group production was assessed by FTIR spectroscopy (Table 1). The FTIR chromatograms, for both untreated LDPE and for LDPE that had been exposed to UV radiation for 1 h, showed constant ratios, suggesting that carbonyl-group formation was approximately zero (Fig. S1[1]). Thus, UV irradiation for 1 h did not have a significant effect on LDPE structure. In contrast, it is well documented that exposure of LDPE to UV radiation for 250 h or more is able to induce significant levels of carbonyl-group production, making LDPE polymers sensitive to microbial degradation (Yamada-Onodera et al. 2001; Hasan et al. 2007; Abrusci et al. 2011).

Bacterial growth on LDPE

The growth rate and final cell densities of bacteria cultured with untreated and UV-irradiated LDPE were not different statistically, confirming that 1 h of UV exposure of LDPE did not affect growth rate and cell densities (data not shown). Also, all control cultures as described in Materials and Methods were either negative for bacterial growth (no growth detected) or displayed negligible growth, which if present, was subtracted from the main data (shown in Table 2).

Culture tubes containing 10 mL of PE medium were inoculated from tubes containing 0.05% paraffin and 1% LDPE. The volume of the inoculant was 100 μL (1% of the total culture volume), which means that the amount of carry-over was small. Growth of bacteria on PE medium was rapid, with all bacteria reaching their maximum cell density in 2–3 days (Fig. 1). The experiments were carried out over 21 days because we expected microbial degradation of LDPE to be slow. Thus, the rapid initial growth of some of the tested bacteria (*C. necator* H16, *P. putida* LS46, *P. putida* IRN22, *P. chlororaphis* PA63, *P. monteilii* MO2, *A. pittii* IRN19) to high cell densities in 2–3 days was an unexpected result. Cell densities of two of the tested bacteria (*D. tsuruhatensis* IRN27 and *M. luteus* IRN20) peaked at lower levels in the first 2–3 days compared with the other bacteria.

After day 3, the bacteria can be classified in two groups. In the first group (*C. necator* H16, *P. chlororaphis* PA63, *P. monteilii* MO2, *P. putida* LS46, and *P. putida* IRN22), cell density decreased dramatically between days 3 and 10, and then stabilized (or increased very slowly) between days 10 and 21. In the second group (*M. luteus* IRN20, *D. tsuruhatensis* IRN27, and *A. pittii* IRN19), cell density also decreased sharply between days 3 and 10 but then increased significantly between days 10 and 21.

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjm-2018-0335.

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Fig. 1. Growth curves from optical density for eight low-density polyethylene degrading bacteria over 21 days.

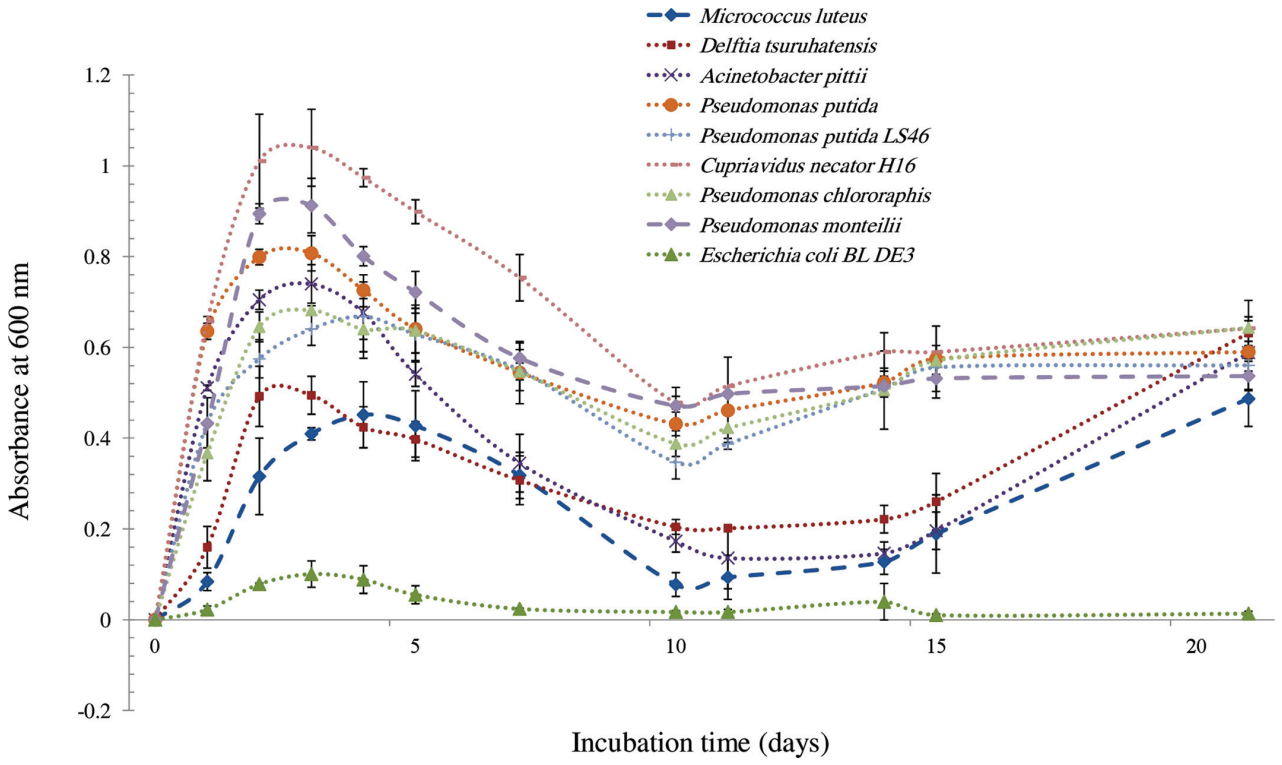
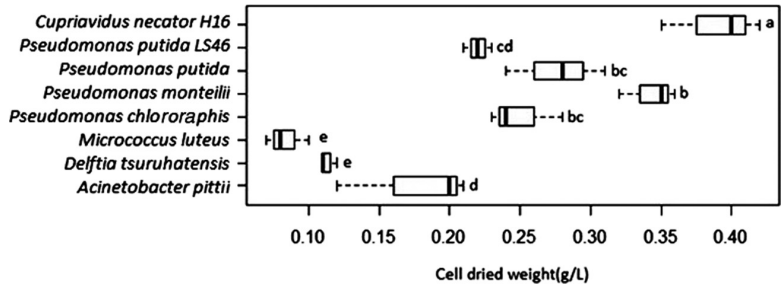


Fig. 2. Cell mass production (g/L) by different bacteria cultured with low-density polyethylene at day 21. Statistical analyses showed that each treatment sample had a skewed distribution and was nonparametric. Boxes show the upper and lower quartiles; the solid lines in the boxes represent the median of the sample; the dotted lines represent standard deviations (i.e., the largest and smallest values). Differences in means are indicated with lowercase letters. Treatments with the same letter are not statistically different ($p < 0.05$).



Biomass production and LDPE weight loss

LDPE is a carbon source that is not easily metabolized by bacteria, so large accumulations of cell mass were not expected. Cell mass production on LDPE media after 21 days of incubation varied between 0.083 ± 0.015 g/L cdm for *M. luteus* IRN20 and 0.39 ± 0.036 g/L cdm for *C. necator* H16 (Fig. 2). Degradation of LDPE also varied for each bacterium. The percent decrease in LDPE mass ranged from 18.9% ± 0.72% for *M. luteus* IRN20 to 33.7% ± 1.2% for *C. necator* H16 (Fig. 3). *Acinetobacter pittii* IRN19 also appeared to be an effective bacterium for degradation LDPE particles. Although *A. pittii* IRN19 displayed the lowest cell mass production on LDPE rather than the first bacterial group (Fig. 2), it reduced LDPE mass by approximately 30% (Fig. 3) and generated a large range of alkane hydrolysis products (Table 3). Also, although *P. putida* LS46 produced less biomass than *C. necator* H16 did (Fig. 2), there was no statistical difference in the percent decrease in LDPE mass caused by *P. putida* LS46 and *C. necator* H16 (Fig. 3).

GC analysis of material caused by LDPE biodegradation

Hydrolysis products from microbial degradation of LDPE were analyzed by GC–FID chromatography. Comparisons of GC chromatograms for two bacteria, *A. pittii* IRN19 and *D. tsuruhatensis* IRN27, with alkane standard

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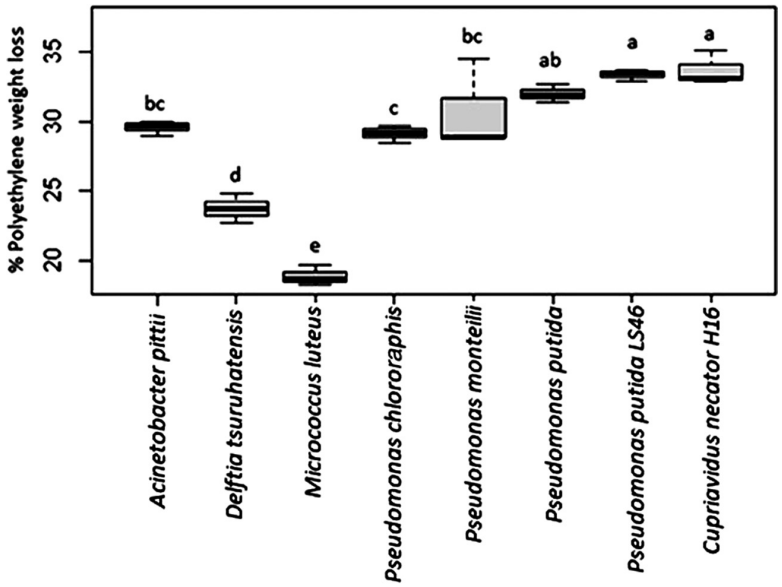
Fig. 3. Percent decrease in low-density polyethylene mass in bacterial cultures at day 21. Statistical analyses showed that each treatment sample had a skewed distribution and was nonparametric. Boxes show the upper and lower quartiles; the solid lines in the boxes represent the median of the sample; the dotted lines represent standard deviations (i.e., the largest and smallest values). Differences in means are indicated with lowercase letters. Treatments with the same letter are not statistically different ($p < 0.05$).



solution are seen in Fig. S2[1]. The concentrations of each saturated carbon chain for the eight bacteria tested are shown in Table 3. The data strongly suggest LDPE chain biodegradation by some of the bacteria tested and that different bacteria generate different hydrolysis products. The hydrolysis products were confirmed to consist of saturated linear alkanes with carbon chain lengths of C_{22} to C_{32}, because they matched perfectly with the linear alkane standards. Greater than 65% of the hydrolysis products generated by all tested bacteria were C_{25} alkanes. The greatest variation in hydrolysis product chain length (C_{24} to C_{32}) was observed in the culture supernatants of *A. pittii* IRN19 and *D. tsuruhatensis* IRN27, which both produced a wide range of alkanes during growth on LDPE.

Scanning electron microscopy: biofilm formation

All bacteria tested were observed adhering to the surface of the PE (Fig. 4). *Pseudomonas putida* LS46, *C. necator* H16, and *A. pittii* IRN19 were observed adhering to the LDPE particles and often were observed in localized clumps (Figs. 4B, 4C, and 4D). Overall, solid surfaces that are in contact with water in the environment tend to form microbial colonization, but cell surface hydrophobicity has been attributed as one of the most important factors in biofilm formation (Das and Kumar 2013), so colonization of hydrophobic PE particles by cells is to be expected.

PHA accumulation and monomer composition

Cupriavidus necator H16 is known to synthesize and accumulate the short-chain-length polyhydroxyalkanoate (scl-PHA), poly(3-hydroxybutyrate), also referred to as PHB. *Pseudomonas putida* LS46 is known to synthesize and accumulate medium-chain-length polyhydroxyalkanoates (mcl-PHAs). Both species produced these polymers after culturing with LDPE particles. *Acinetobacter pittii* IRN19 was also examined for PHA production after growing on LDPE particles. Table 4 shows the biomass production and GC analysis of biomass for these three bacteria after 21 days of culturing in Ramsay's media with 1% LDPE as the sole carbon source.

Cupriavidus necator H16 generated the highest LDPE weight loss (33.75% ± 1.2%), but only very few alkane hydrolysis products were detected compared with other bacterial cultures: only 2.64% ± 0.61% (per 100 mg LDPE powder) compared with *D. tsuruhatensis* IRN27 (8.83% ± 0.09%), *A. pittii* IRN19 (7.53% ± 0.38%), and *M. luteus* IRN20 (3.71% ± 0.11%). GC analysis of *C. necator* H16 biomass after 21 days of culture with LDPE displayed three peaks (6.33, 7.08, and 7.7 min retention time after injection), which corresponded to the methyl esters of 3-hydroxybutanoic acid (C_4), 3-hydroxyvaleric acid (C_5), and benzoic acid (the internal standard). The molar ratio of the subunit composition was 94.9% ± 0.61% C_4 and 5.03% ± 0.56% C_5 (Fig. S3[1]). No statistically significant differences in biomass production (in Ramsay's media plus 1% LDPE) were observed between *P. putida* LS46 and *A. pittii* IRN19, and both bacteria generated the same PHA composition, which consisted of 3-hydroxyhexanoic methyl ester (C_6), 3-hydroxyoctanoic ester (C_8), 3-hydroxydecanoic ester (C_{10}), 3-hydroxydodecanoic ester (C_{12}), and 3-hydroxytetradecanoic

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Table 3. Alkane hydrolysis products detected after microbial biodegradation of low-density polyethylene by different bacteria, after 21 days of incubation.

	Concn. of alkane produced											
Bacterium	C_{22}	C_{23}	C_{24}	C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	Total*
D. tsuruhatensis IRN27	0.32±0.03	0.09±0.02	0.24±0.03	7.26±0.23	0.42±0.05	0.35±0.001	0.15±0.06	0	0	0	0	8.83±0.09a
A. pittii IRN19	0	0	0.10±0.02	5.15±0.34	0.44±0.05	0.50±0.02	0.47±0.03	0.31±0.01	0.33±0.17	0.22±0.01	Nd	7.53±0.38b
M. luteus IRN20	1.11±0.02	Nd	Nd	2.44±0.13	0.10±0.02	0.07±0.02	0	0	0	0	0	3.71±0.11c
C. necator H16	0	0	Nd	2.55±0.579	0.08±0.030	0	0	0	0	0	0	2.64±0.61d
P. monteilii MO2	0.42±0.01	0	0	0	0	0	0	0	0	0	0	0.42±0.01e
P. putida LS46	0	0	0	0	0	0	0	0	0	0	0	0
P. putida IRN22	0	0	0	0	0	0	0	0	0	0	0	0
P. chlororaphis	0	0	0	0	0	0	0	0	0	0	0	0

Note: Values are the concentration of alkanes detected and are expressed in mg per 100 mg of PE powder added initially to the culture media. Nd, not detected, because of the presence of a peak that was too small to quantify.

*Different letters indicate a significant difference at $p \leq 0.05$.

ester (C_{14}), with approximately half of PHA composition consisting of the C_6 subunit.

Discussion

While most early studies of microbial degradation of PE used PE that had been subjected to some form of pretreatment, several recent studies have indicated microbial degradation of untreated PE (Kyaw et al. 2012; Yoon et al. 2012; Peixoto et al. 2017). Our results are consistent with these reports and further show that in addition to cell mass production, some bacteria were also able to synthesize and accumulate PHA polymers after growth on LDPE.

The growth curves suggest that during incubation, two types of carbon sources were consumed. LDPE is generally amorphous, with short branches (10–30 CH_3 per 1000 C atoms), consisting of one or more comonomers, such as 1-butene, 1-hexene, and 1-octene. This branching system prevents the PE molecules from stacking close together, making the LDPE chains more accessible; therefore, the tertiary carbon atoms that are present at the branch sites are more susceptible to attack. Also, some structural variations, such as unsaturated carbon–carbon double bonds, carbonyl groups, and hydroperoxide groups, formed during polymerization and subsequent processing, may also be present in the PE polymers (Ojeda et al. 2011). These short side chains may be consumed first by the bacteria, accounting for the rapid growth in the first 3 days.

Statistical analysis showed a positive relationship between bacterial biomass production and LDPE weight loss but one that varied between weak and strong among the bacteria tested, suggesting that the loss in LDPE mass may be more a function of the type and (or) amount of enzyme(s) secreted rather than total numbers of cells. We also observed greater than 65% of the hydrolysis products generated by all tested bacteria were C_{25} alkanes. Eyheraguibel et al. (2017) have shown that the molecular mass of extracted oligomers was lower than 850 Da, with a maximum chain length of 55 carbon atoms, and that a strong shift to smaller molecules (<450 Da, 25 carbon atoms) was observed, suggesting that longer molecules disappeared more rapidly than the smaller ones. This has provided a new perspective on biodegradation processes, suggesting that extracellular mechanisms leading to chain cleavage may play a significant role in PE biodegradation (Eyheraguibel et al. 2017). In our study, there were also some small peaks (Fig. S2[1]), other than alkanes, detected by GC in the culture supernatants, but these were not identified. Thus, other reaction products may have been present. However, the focus of this study was not to specifically analyze the mechanisms of LDPE biodegradation. This is the objective of a future study.

In a recent study, Guzik et al. (2014) reported growth and PHA accumulation of *Acinetobacter calcoaceticus* (0.24 ± 0.1 g/L

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Fig. 4. Scanning electron micrographs (magnification × 20 000) of (A) the low-density polyethylene surface with no microbial treatment; (B) microbial colonization by *Pseudomonas putida* LS46; (C) microbial colonization by *Cupriavidus necator* H16; and (D) microbial colonization by *Acinetobacter pittii* IRN19.

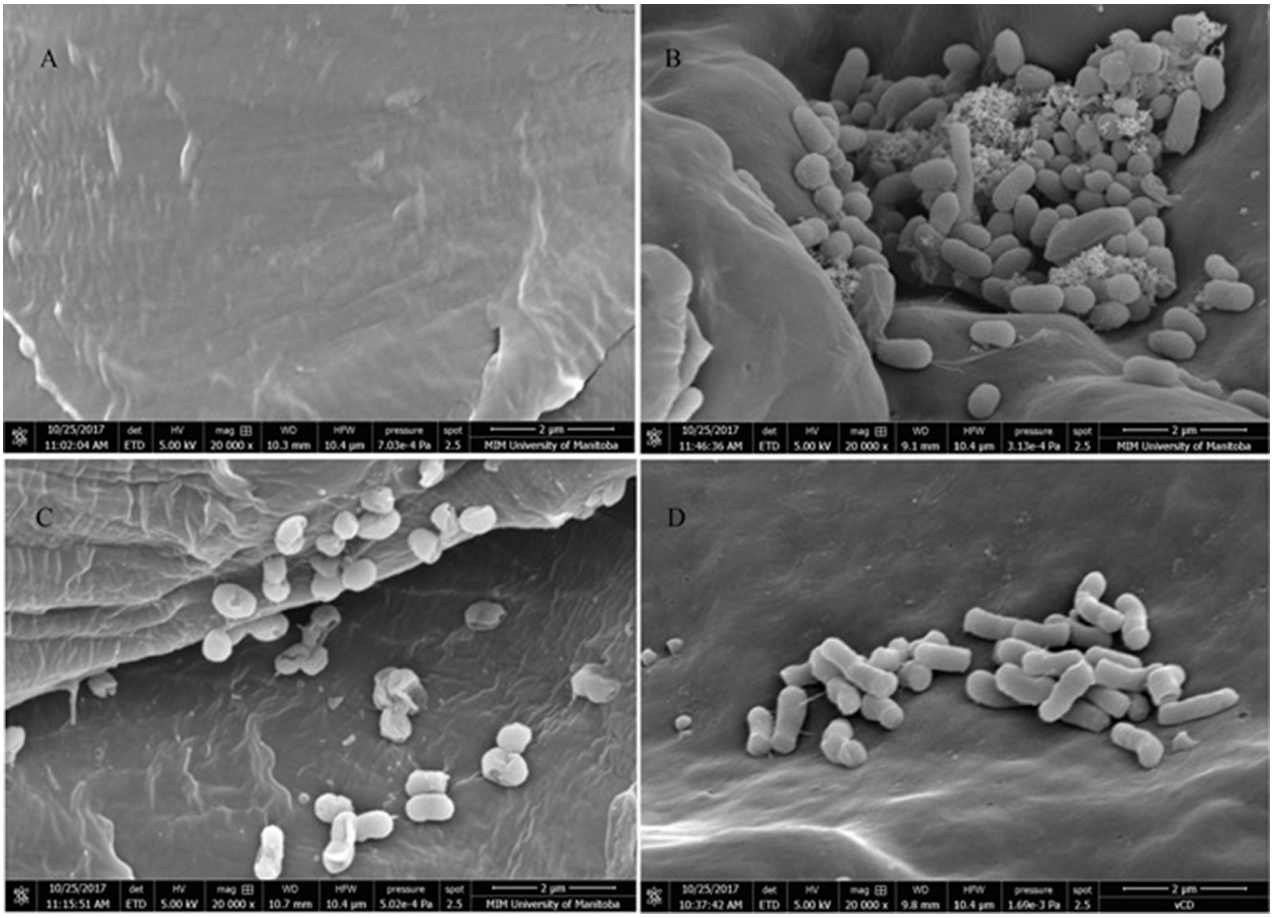

Table 4. Biomass production, polyhydroxyalkanoate (PHA) accumulation, and mol% subunit composition in *C. necator* H16, *P. putida* LS46, and *A. pittii* IRN19 after 21 days of culture in Ramsay's media with 1% low-density polyethylene.

	C. necator H16	*P. putida* LS46	*A. pittii* IRN19
Biomass (g/L cdm)	0.34±0.02a	0.24±0.01b	0.23±0.05b
PHA accumulation (% cdm)	3.18±0.4	0.54±0.037	0.49±0.019
Monomer composition (mol%)			
C_4	94.90±0.61	0	0
C_5	5.03±0.56	0	0
C_6	0	58.60±0.48	50.50±1.34
C_8	0	16.86±3.58	0.53±0.25
C_{10}	0	8.49±0.16	34.25±0.95
C_{12}	0	7.35±0.30	9.19±0.44
C_{14}	0	8.64±2.63	11.47±0.32

Note: Different lowercase letters indicate a significant difference ($p < 0.05$). cdm, cell dry mass.

cdm and 4.1% ± 0.1% cdm, respectively) and *Pseudomonas aeruginosa* (0.39 ± 0.4 g/L cdm; 18.9% ± 0.7% cdm, respectively), on 2% (*m*/*v*) PE pyrolysis wax as the sole carbon source over 48 h. In these experiments, PE was subjected to thermal pretreatment in the absence of oxygen (pyrolysis) at very high temperatures (300–500 °C), which generated a complex mixture of low molecular weight paraffins with carbon chain lengths from C_8 to C_{32} (PE pyrolysis wax) (Guzik et al. 2014). Thus, Guzik et al. (2014) demonstrated that *A. calcoaceticus* and *P. aeruginosa* were able to metabolize alkane hydrocarbons, but did not demonstrate the direct catabolism of LDPE. Our work differs

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from that of Guzik et al. (2014), as it demonstrates direct utilization of LDPE as a sole carbon and energy source and its bioconversion to PHAs polymers.

We have demonstrated that several bacteria can utilize LDPE powder as a sole carbon source for growth and that PE hydrolysis products, detected in the culture supernatants, were generated during cell growth, strongly suggesting that biofragmentation had occurred. Since LDPE was the sole carbon source, by deduction, we can state that the cell growth and PHA production was a consequence of LDPE metabolism. Although the maximum degradation of LDPE observed was 33.7%, we have demonstrated that *C. necator* H16, *P. putida* LS46, and *A. pittii* IRN19 could assimilate LDPE, could not only produce biomass but also generate alkane hydrolysis products and accumulate biopolymers in the form of scl- and mcl-PHAs. Thus, our data provide strong evidence of PE biofragmentation and bioassimilation. However, the purpose of the current manuscript was to report on our initial introduction for untreated LDPE-degrading bacteria and more detailed studies are underway and will be reported in a future publication.

The potential for degradation of PE by microorganisms is widely accepted, but our understanding of the mechanisms of microbial degradation as well as the enzymes and corresponding genes involved is very limited (Usha et al. 2011). The application of genome sciences and molecular biology may help clarify these mechanisms and improve the biodegradation of LDPE and other hydrocarbon compound contaminants in the environment. Yoon et al. (2012) investigated biodegradation of a non-oxidized low molecular weight polyethylene (LMWPE), whose molecular mass was well above the upper limit that can penetrate microbial membranes. Using *Pseudomonas* sp. strain E4, isolated from soil contaminated with crude oil, and a recombinant *Escherichia coli* BL21 that expressed the alkane hydroxylase gene (*alkB*) from *Pseudomonas* sp. strain E4, Yoon et al. (2012) showed that the AlkB enzyme played a central role in LMWPE degradation, even in the absence of the other specific enzymes like rubredoxin and rubredoxin reductase. Jeon and Kim (2016) compared the functional characterization of alkane monooxygenases for LMWPE biodegradation and showed that the AlkB2 enzyme was more effective in degrading LMWPE than the AlkB1 enzyme and that the regulation mechanism of AlkB2 was different from that of AlkB1.

Although some researchers have reported that the alkane catabolic pathway flows from β-oxidation to the tricarboxylic cycle (Yoon et al. 2012), Eyheraguibel et al. (2017) suggested that extracellular mechanisms leading to enzymatic oxidation and chain cleavage of PE polymers are also important. These insights into the mechanisms of microbial degradation of PE provide a new perspective on biodegradation processes that must be further explored.

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