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Gaining insights in the microbial degradation of polyethylene plastics

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Abstract

Plastics production, use and degradation are hot topics that have come to the forefront over recent years. Polyethylene (PE) represents more than 60% of all petroleum-derived plastics worldwide and is accumulating at rates of several millions of tons per year because of its strong recalcitrance to biotic and abiotic degradation. Microbial degradation has been proposed as a possible alternative way to reduce plastic wastes.

The general aim of this work was the identification of bacterial strains able to metabolize PE and to identify the biochemical pathways of this biodegradation process.

In an abandoned landfill we collected different plastic samples; using a metagenomic approach, we found a strong relationship between the plastic properties (including the presence of colorants) and the microbial community. Also, a possible correlation between the level of plastic degradation and the bacterial community structure was detected.

By screening the natural microbial community exposed to PE in environment, we isolated 10 bacteria which revealed the ability to grow on PE as only energy and carbon source.

A bacterium, *Pseudomonas aeruginosa* UC4003, showed the highest growth rate in minimal salt medium and polyethylene. When grown on PE, this strain produced an extracellular enzyme, protein-like activator for *n*-alkane oxidation (PA), involved in the first step of polyethylene degradation. The

role of PA was investigated by proteomic and expression analysis. To confirm its involvement in PE degradation, an overexpression of *pra* gene was carried out. The comparison from wild type and overexpression strain (::*pra*) showed the higher capability of ::*pra* strain to grow on PE.

Chapter 1

1. Introduction

1.1 Plastics rate production

In today's world, a life without plastics seems unimaginable. Plastic is used in many aspects of our everyday lives. Our society is strongly dependent on petroleum-derived plastic polymers, which are used in an increasing number of applications, from consumers goods to medical and safety areas, due of their lightness, durability and cheapness (Gewert et al., 2015). Therefore, plastics can be considered as one of the dominant materials of the modern economy**.** Its usage has grown inexorably from humble beginnings to a position where humanity finds it essential.

The extraordinary global expansion of this now indispensable material can be seen in the severe rise of produced plastics, from the less than 2 million tonnes manufactured in 1950 to the 335 million tonnes made annually today, as summarised in Figure 1, (PlasticsEurope, 2017; Zalasiewicz et al., 2016).

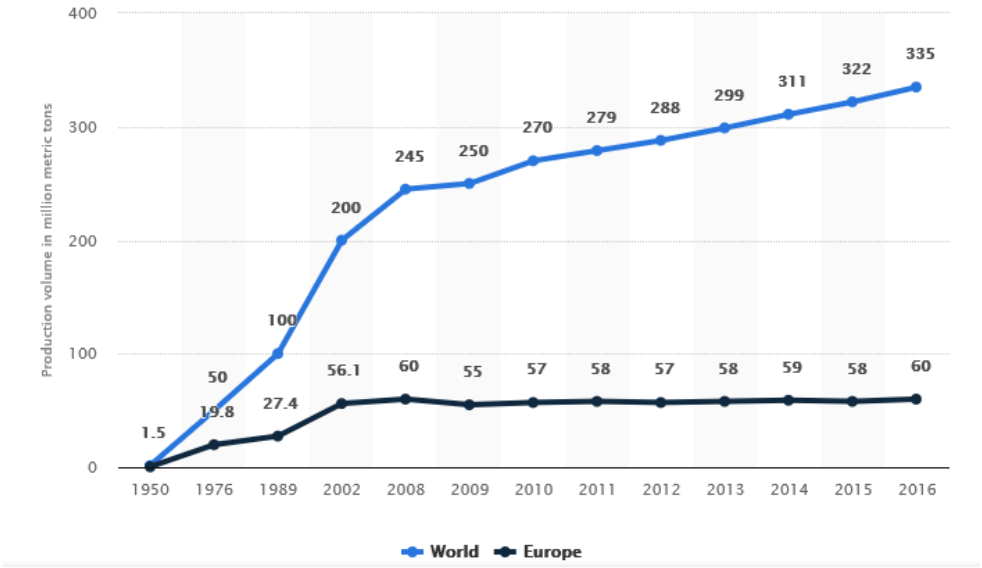

Figure 1. Global plastic production from 1950 to 2016 (in million metric tons). Source PlasticsEurope 2017 and Statista 2018.

About 80% of the synthetic plastic is constituted by polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polyurethane (PU) and polyethylene terephthalate (PET) (PlasticsEurope, 2017). Approximately 42 % of all polymer resins are used in the packaging sector, which manly works with materials composed of PE, PP and PET (Geyer et al., 2017). Normally, the lifetime estimation of packaging products is less than 1 year. Such a time-limited life expectancy leads to a strong reduction of their usage possibilities in terms of time and therefore, to their early discard.

As a result, they accumulate, instead of decomposing, in landfills or in the natural environment (Figure 2) (Geyer et al., 2017), thereby increasing the dangers and side effects that plastics can wreak on the wildlife.

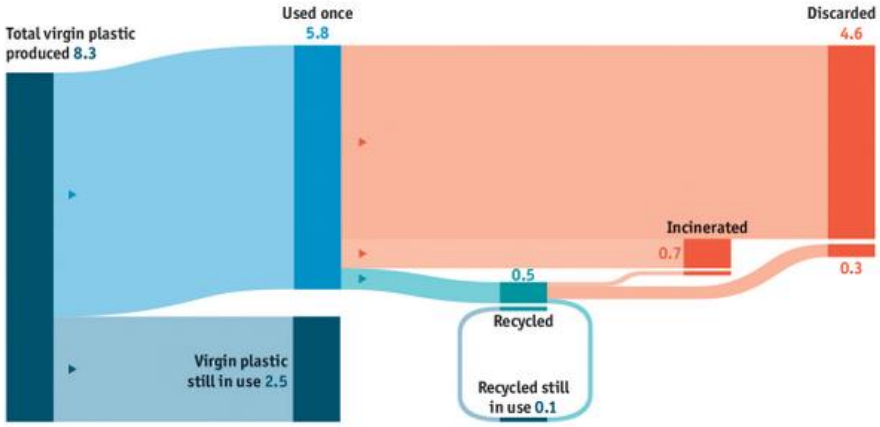

Figure 2. Global plastic production and use, 1950-2015, tonnes, bn. (Source: "Production, use and fate of all plastics ever made" (Geyer et al., 2017), adapted by Economist.com).

A study of Thompson and colleagues reported a significative status of the effects of plastics debris in the environment, stressing that plastics could offer considerable benefits for the future, but equally clearly stating that our current approaches to plastics production, use and disposal are not sustainable and thus representing an actual concern for both wildlife and human health. In this scenario, the degradation rates and proposing recycling strategies are becoming more and more relevant.

1.2 Microbial degradation of polyethylene

Polyethylene (PE) is the synthetic plastic mostly produced worldwide, and it is constantly accumulating in the environment because of its recalcitrance to environmental degradation: it consists in long hydrocarbon source chains with varying degrees of branching depending on its desired properties. Polyethylene is deemed as highly resistant to degradation and utilization by microorganism, because of its unreactive C-C and C-H bonds, its hydrophobic nature, its high molecular weight and the lack of functional groups (Arutchelvi et al., 2008; Gautam et al., 2007; Puglisi et al., 2017).

PE is mainly distinguished in two classes, low-density (LDPE) and high-density (HDPE), with the first including a large share of the packaging industry materials. The degradation of PE can follow two courses: abiotic degradation, which is determined by environmental factors such as UV irradiation, oxygen, temperature air, rain or dust, and biotic degradation, primarily caused by the action of microorganisms that modify and partly consume the polymer, leading to changes in its properties. The two processes are usually acting cooperatively in the environment (Hakkarainen & Albertsson, 2004), but the process under normal conditions is extremely slow, with degradation rates that after years remain below 15% of the initial mass (Arutchelvi et al., 2008; Eubeler et al., 2010; Restrepo-Flórez et al., 2014). Although linear alkenes are usually easy to be degraded, for PE there is an inverse relationship between molecular weight and degradability (Hadad et al., 2005).

In the last 30 years, the biodegradation of polyethylene was investigated and several bacterial strains were identified, despite of PE recalcitrance to degradation.

Aquatic environments were studied more than the terrestrial due to the large diffusion of microplastics in the oceans (Amaral-Zettler et al., 2015; Kettner et al., 2017), while there is still scarcity of information on the ecology of microorganism able to colonize plastic debris in soils, which are however an important sink for plastic pollutants (Nizzetto et al., 2016). Several studies showed evidences proving biodegradation of polyethylene. Usually the techniques used to estimate the biodegradation of the polymer are: surface topography, crystallinity, the presence of functional groups, mass balance, molecular weight distribution, hydrophobicity and mechanical properties (Restrepo-Flórez et al., 2014). After the first identification of *Streptomyces* species able to attack pro-oxidized PE (Pometto et al., 1992), many reports were published along the years, with *Arthrobacter* (Albertsson et al., 1998; Balasubramanian et al., 2010), *Bacillus* (Roy et al., 2008; Watanabe et al., 2009), *Pseudomonas* (Koutny et al., 2009; Rajandas et al., 2012), *Rhodococcus* (Gilan et al., 2004; Gravouil et al., 2017) and *Staphylococcus* appearing as the most represented genera (Nowak et al., 2011; Restrepo-Flórez et al., 2014).

Researches produced until today only partially showed the mechanism involved in the microbial attack on PE materials.

The colonization of polyethylene is the first step in its degradation process. Biofilm production increases the metabolic activity of the microbial populations (Kirchman & Mitchell, 1982). However, polyethylene, due to its

hydrophobic surface, can interfere with colonization. The production of surfactants helps the microorganism in the colonization process of the hydrophobic surface (Karlsson et al., 1988).

The second necessary step in polyethylene biodegradation is a reduction of its molecular weight. In fact, molecular weight reduction is essential to allow the transport of molecules through the cellular membrane. To perform the reduction of molecular weight, the release of extracellular enzymes was proposed. Wasserbauer and colleagues (Wasserbauer et al., 1990) firstly suggested that the degradation of PE from a homogenate of bacterial and liver cells was due to extracellular hydroxylase system; afterwards other groups confirmed the presence of extracellular enzymes in three *Streptomyces* species capable of attacking a pre-heated PE sample blended with starch and pro-oxidants (Pometto et al., 1992), while Chatterjee and his group (Chatterjee et al., 2010) reported that extracellular enzymes of approximately 55 and 35 kDa were involved in the degradation of LDPE by a *Staphylococcus epidermidis*.

After the process of reduction of molecular weight, oxidation is crucial to provide a functional group such as alcohol or carbonyl group to support the microorganism in the process of degradation (Wilkes & Aristilde, 2017). Santo et al. (Santo et al., 2013) proved the role of laccase in the PE degradation of a strain of *Rhodococcus ruber*, demonstrating that the addition of copper increased the mRNA levels of the genes and accordingly the degradation of the polymer, while Yoon and colleagues (Yoon et al., 2012) identified an alkane hydroxylase (*alkB*) gene in a *Pseudomonas* strain able to

degrade low-molecular-weight PE, and confirmed its role by cloning and expressing it in an *Escherichia coli* host.

A transcriptomic approach used in *Rhodococcus ruber* strain, incubated with oxidized and non-oxidized PE, revealed a strong induction of the pathways related to alkane degradation and β-oxidation of fatty acids (Gravouil et al., 2017). Furthermore, in the same work, the role of laccases was investigated; in *R. ruber* transcriptome, Gravouil et. al., identified three homologous sequences related to laccases/multicopper oxidases, but none of them showed up or down- regulation in presence of PE (Gravouil et al., 2017)

At this point, it is possible conclude that the additions of functional groups led to a transformation of the alkane into a carboxylic acid that was metabolized by β-oxidation and the Krebs cycle, as shown in Figure 3 (Albertsson et al., 1987; Restrepo-Flórez et al., 2014).

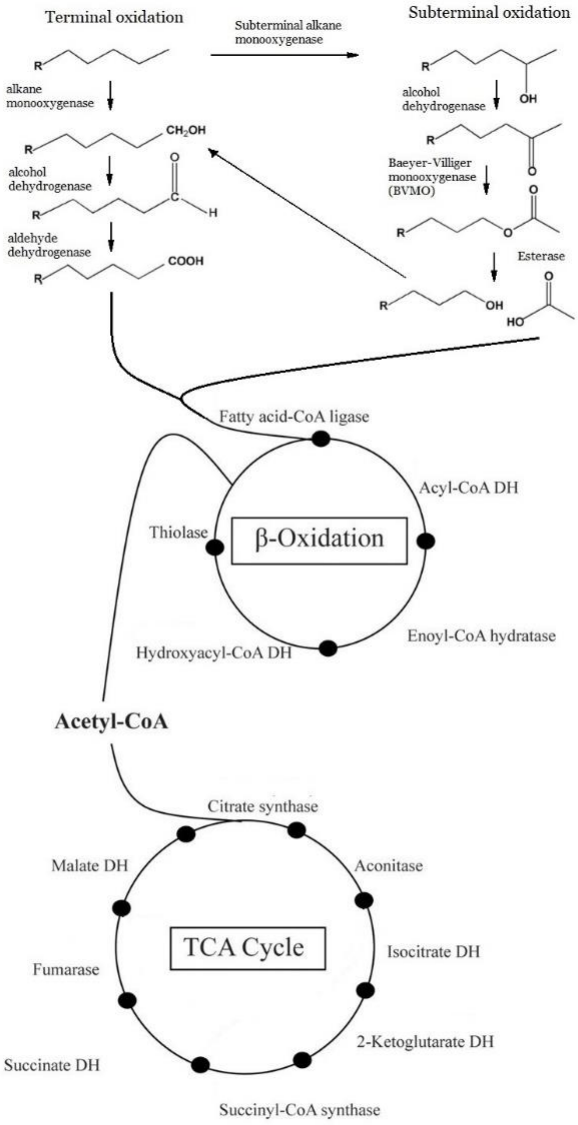

Figure 3. Proposed biodegradation pathway of polyethylene.

Nonetheless, there is still a lack of knowledge on the complete metabolic pathways involved in the process as well as in the structure and identity of all the enzymes implicated. In particular, the biotic mechanism involved in the breaking down of polyethylene results unknown as yet. The gaps in the scientific knowledge outlined above can help to understand why, despite more than 20 years of research on the topic, bioremediation technologies for recalcitrant polymers such as PE are still missing, research is mainly confined to lab-scale studies, and degradation rates are still very limited.

Aim of this Thesis

The general state of the art drawn in the introduction, reveals several gaps in the scientific knowledge of polyethylene microbial degradation. The first one to be filled is indeed the identification of bacterial strains able to metabolize PE and to identify the genes and the biochemical pathways of this biodegradation process. These tasks have been the main purposes of this research project.

To do so, the work has been divided in three main activities.
In the very first part of the study, a fine scale assessment of microorganisms growing on plastics from a landfill site was performed.
In the second part, the attention was focused on the characterization and the isolation of soil bacterial strains from PE waste in an abandoned landfill. A research of the best bacteria strains able to grow on polyethylene was carried out.
In the third section of the work, the ability of *Pseudomonas aeruginosa* to use polyethylene as only energy and carbon source was investigated. A proteomic and a genetic approach were used to shed light on the potential metabolic pathways involved in PE degradation.

The different steps of the research led to results that will be explained in detail within each chapter.

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Chapter 2

2. Selective colonization processes of polyethylene-loving bacteria in an abandoned landfill site

2.1 Introduction

The accumulation and fragmentation of plastics, almost due to a restricted recovery of discarded materials, is one of the most pervasive and long-lasting recent changes to the surface of our planet (Barnes et al., 2009; Rillig, 2012). This persistence makes plastic ubiquitous as an environmental pollutant and also supports the current worries about the possible ecological impacts of the growing rate of plastic materials in ecosystems (Horton et al., 2017).

Polyethylene is one of the mainly commonly-used polymers, and is extremely resistant to biodegradation due to their physical and chemical properties (Gautam et al., 2007).

Biodiversity and presence of polymer degrading microorganisms differ according to the environment, such as soil, sea, compost, activated sludge, etc.. Thus, the investigation of the distribution and population of polymer-degrading microorganisms in different ecosystems is essential. Commonly, the major mechanism implicated in the microbial degradation of plastics is the adherence of microorganisms on the surface of plastics, followed by the colonization of the exposed surface (Tokiwa et al., 2009)

Nevertheless, some studies showed that polyethylene degradation by microorganisms can occur, although this kind of process actually happens extremely slowly in nature (Restrepo-Flórez et al., 2014).

So far, the total amount of microorganisms able to degrade polyethylene is limited to 17 genera of bacteria. Nonetheless, these numbers are expected to

increase following the more sensitive isolation and characterization techniques settled on rDNA sequencing. Such a technology allows to a more expanded approach to assess the composition of a community, including the non-culturable fraction of microorganisms, invisible by traditional microbiology methods yet, but constituting up to the 90% of the real biodiversity in an ecosystem (Hugenholtz et al., 1998; Restrepo-Flórez et al., 2014).

The purpose of this part of the work is to study the microbial community involved in polyethylene colonization.

2.2 Materials and Methods

2.2.1 Site description and samples collection

Sampling was carried out in an abandoned dumpsite in Northern Italy (Località Tavernelle, Fiorenzuola d'Arda). The dumpsite had an area of 10,600 m^2, and received an average of 12,000 m^2 each year of municipal solid wastes between 1972 and 1982. In total, approximately 42,000 m^2 of wastes were grounded in 10 years up to a depth up to 4 m. While most organic materials and pollutants were either degraded or leached over years, several persistent plastic materials were found at the surface of the site during the sampling. Plastic sampling was carried out with aseptic techniques, retrieving from different part of the field 5 plastic films characterized by different colors: black (B), white (W), red (R), green (G) and yellow (Y). Three replicates per each plastic type were sampled, for a total of 15 replicates.

Furthermore, soil samples from the dumpsite and from a neighboring uncontaminated site (cultivated with *Medicago sativa* L.) were also collected in triplicates.

2.2.2 Recovery of biofilms from plastic samples

In order to retrieve the biofilms growing on the surface of the plastic samples, the films were firstly aseptically cleaned from soil debris, cut in squares of 10 x 10 cm, and homogenized with 90 mL of sterile physiological solution using a Stomacher (400 Circulator; International PBI, Milan, Italy) for 1 minute at maximum speed for 5 minutes. The procedure was repeated three times. After extraction, the water phase containing the biofilms was centrifuged at 4000 rpm for 5 minutes at 4 °C and resuspended in 5 mL of physiological solution. An aliquot of the resuspension was then used for DNA extraction and concomitant molecular analyses, and another one for culture-based analyses as explained below.

2.2.3 Plastic samples characterization

All analyses on plastic samples were carried out after the biofilm retrieval process above described. Attenuated Total Reflectance (ATR) spectra were obtained using a diamond crystal on a Perkin Elmer Frontier instrument, with 32 scans per replicate in a scanning range of 4000-500 cm^{-1} and scan rate of 4 cm^{-1}. Differential Scanning Calorimetric (DSC) analyses were performed

on 5-6 mg samples using a TA Q20 instrument in hermetic aluminium pans, under nitrogen flow (50 mL/min) at 10º C/min heating rate from 0 to 200 ºC.

Scanning Electronic Microscopy (SEM) analyses were performed on 0.5 cm^2 plastic samples stepwise dehydrated in ethanol 75%, 85%, 95% and 100% for 1 h at room temperature. Critical point drying was performed in a BalTEC CPD030 critical point dryer. Samples were then gold-coated (Palumbo et al., 2004) and observed with a Philips XL30 ESEM (Environmental Scanning Electron Microscope)

2.2.4 Isolation and characterization of cultivable bacteria

The biofilm retrieved from the plastic surfaces were plated by serial dilution on minimal salt medium (SM) containing (per L of distilled water): 4.0 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 1.0 g K_2HPO_4, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.15 g KCl, and 1.0 mg of each of the following microelements: $FeSO_4 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$ and $MnSO_4$ (Gilan et al., 2004). Paraffin oil (2%) was added to the plates as carbon source in order to isolate strains with potential degradation abilities. Plates were incubated at 30 °C in aerobic conditions.

DNA from isolates purified colonies was extracted with Microlysis kit (Microzone, Haywards Heath, UK) according to the manufacturer's protocol. Randomly Amplified Polimorphic DNA-Polymerase Chain Reaction (RAPD-PCR) was performed on each isolate using single stranded oligonucleotide primers RAPD2 (5'-AGC AGG GTC G-3') (Cocconcelli et al.,

1995) and M13 (5′-GAG GGT GGC GGT TCT-3′) (Huey & Hall, 1989). Amplification reactions with both primers were conducted in a programmable T100 thermocycler (BioRad) in volumes of 25 μL containing 1 μM of primer, 3 mM of $MgCl_2$, 0.2 mM of DNTPs, 2.5 U of Taq DNA polymerase (Fermentas, Selangor, Malaysia). The PCR fragments electrophoretic profiles were captured, and pattern analysis was performed with the Fingerprinting II software (BioRad) in order to obtain similary profiles of bands based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and select representative unique isolates. The latter were identified by PCR amplification using the primers P0 5′-GAG AGT TTG ATC CTG GCT- 3′) and P6 (5′-CTA CGG CTA CCT TGT TAC-3′) as previously described(Di Cello & Fani, 1996). The PCR products were purified using the Wizard SV Gel and PCR Clean-Up system, according to the package insert (Promega Corpo- ration, Madison, WI) and sequenced at the BMR Genomics of Padova, Italy. The identification of sequences was performed by alignment against the Ribosomal Database Project (RDP) database using the Naïve Bayesian Classifier (Wang et al., 2007).

2.2.5 Bacterial communities: molecular analyses

Microbial DNA extraction was carried out from 0.5 mL of biofilm suspensions using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's protocol, and the concentration of double-stranded DNA in the extracts was determined using the Quant-iT

dsDNA HS assay kit and the Qubit fluorometer (Invitrogen, Carlsbad, CA, USA).

PCR amplification of the bacterial V3-V4 16S rRNA hypervariable regions was carried out with the Phusion Flash High-Fidelity Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the primer pairs 343F (5'-TACGGRAGGCAGCAG-3') and 802R (5'-TACNVGGGTWTCTAATCC-3'), as previously detailed (Fontana et al., 2016). Amplifications were carried out in 25 µl of volume reactions, containing 1 ng of DNA and 0.25 µM of each primer, using a two-step PCR approach (Berry et al., 2011). The first step consisted in a 5 min step at 94°C of initial template denaturation and polymerase activation, 25 cycles parted by 30 sec of denaturation (94°C), 30 sec of primers annealing (50°C) and 30 sec of primers elongation (72°C), followed by a final elongation step (72°C) of 10 min. The same conditions were applied in the second step PCR, where 1 µL of first step products served as a template was used and the same PCR conditions described were applied, using 8 cycles instead of 25. In order to allow simultaneous analyses of several samples in a single sequencing run, the second PCR step was performed using indexed forward primers throughout a 9 nucleic acids base extension at their 5' end, with the first seven bases served as a sample index sequence for multiplexing, while the two bases next to the original primer served as linker bases, not matching any bacterial sequence at this position according to Ribosomal Database Project (RDP) entries.

High-throughput sequencing data filtering, multiplexing and preparation for concomitant statistical analyses were carried out as previously detailed

(Połka et al., 2015; Vasileiadis et al., 2013). In summary, paired-reads were assembled to reconstruct the full V3-V4 amplicons with the "pandaseq" script (Masella et al., 2012) allowing a maximum of 2 mismatches and at least 30 bp of overlap between the read pairs. Samples demultiplexing was then carried out with the Fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/).

Mothur v.1.32.1 (Schloss et al., 2009) was applied in order to remove sequences with large homopolymers (≥10), sequences that did not align within the targeted V3-V4 region, chimeric sequences (Edgar et al., 2011) and sequences that were not classified as bacterial after alignment against the Mothur version of the RDP training data set. The resulting high-quality sequences were analysed with Mothur and R (Team, 2012) following two main approaches: the operational taxonomic unit (OTU) and the taxonomy-based approach. For the OTU approach, sequences were first aligned against the SILVA reference aligned database for bacteria (Pruesse et al., 2007) using the NAST algorithm and a kmer approach (DeSantis et al., 2006; Schloss, 2010), and then clustered at the 3% distance using the average linkage algorithm. OTUs having a sum of their abundances across all samples of than 0.1% of the total were grouped into a single "rare OTUs" group. For the taxonomy based analyses, sequences were classified into taxa using an amended version of the Greengenes database (McDonald et al., 2011).

Statistical analyses on OTU and taxonomy matrixes were performed in Mothur and R, and included hierarchical clustering with the average linkage algorithm at different taxonomic levels, Principal component analysis (PCA) to assess the unconstrained samples grouping, Canonical correspondence

analyses (CCA) to assess the significance of different treatments on the analysed diversity. Metastats (Paulson et al., 2011) was applied to identify features that were significantly different between treatments. Functional genes were inferred from 16S rRNA data using PICRUSt (Langille et al., 2013) and afterwards analyzed and visualized with STAMP (Parks et al., 2014).

2.3 Results and discussions

2.3.1 Plastic samples characterization

Scanning electron microscopy (SEM) was applied to examine the plastics before and after the mechanical detachment of the biofilms: results are reported in Figure 1 for the green (Figure 1a-1c), yellow (Figure 1d-1f) and red samples (Figure 1g-1i) as selected plastics. Per each, the first picture shows the plastic with the biofilm at 5 or 10 μm scale, the second the plastic without biofilm at 10 or 20 μm scale while the third column shows the same plastic without biofilm at a lower resolution (200 μm). All plastics showed attachments of morphologically diverse rod-shaped prokaryotic cells, together with debris and filamentous structures that indicate a biofilm colonization (Figures 1a, 1d and 1g). SEM pictures taken at similar scales after stomacher extraction indicated that the procedure was effective in removing the cells and biofilm from the surfaces (Figures 1b, 1e and 1h). When the same samples were observed at a 200 μm scale a number of cavities were found on the plastic surfaces, especially for the red (Figure 1i) and the

green (Figure 1c) plastics, while in the yellow plastic (Figure 1f) and in the white and black (pictures not shown) the surface was less characterized by cavities possibly related to degradation events.

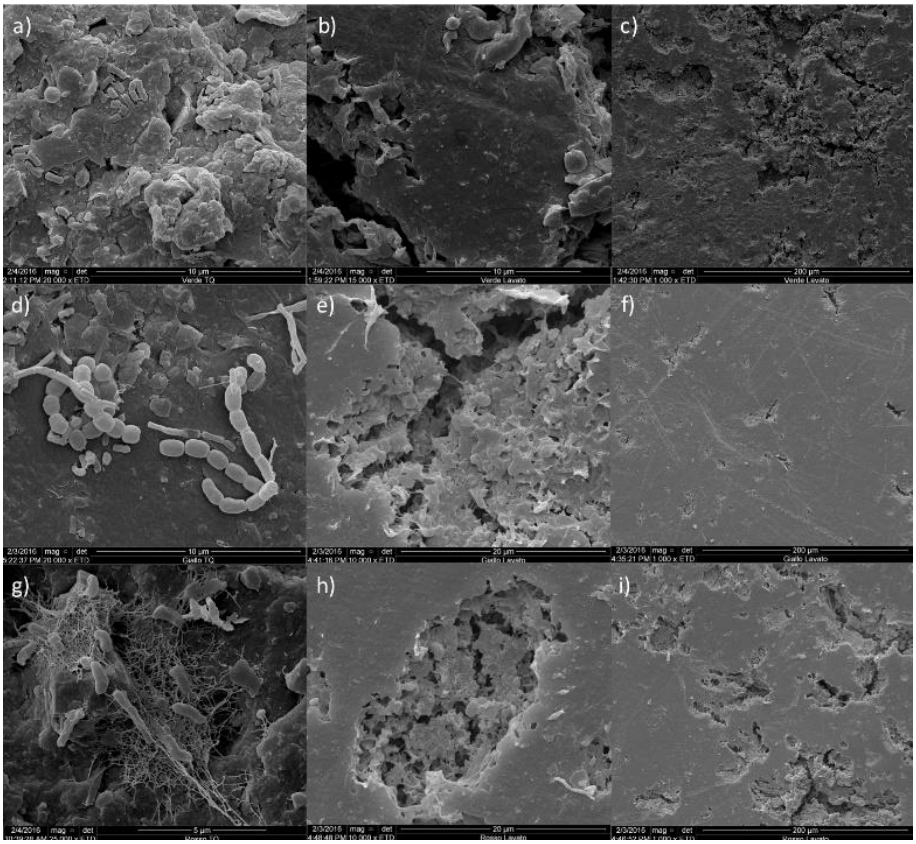

Figure 1. Scanning electron microscopy (SEM) images obtained with the gold coating technique; pictures show representative fields selected among all the observations performed.: a) green plastic with biofilm; b) and c) green plastic after biofilm detachment; d) yellow plastic with biofilm; e) and f) yellow plastic after biofilm detachment; g) red plastic with biofilm; h) and i) red plastic after biofilm detachment.

Plastic samples were further characterized by IR and TSC analyses. IR analyses (Figure 2) firstly confirmed that the analysed plastics were identifiable as polyethylene, since all the samples showed typical IR absorbance bands of polyethylene (Gulmine et al., 2002): 2916 cm^{-1} (CH_2 asymmetric stretching), 2848 cm^{-1} (CH_2 symmetric stretching), 1473 and 1463 cm^{-1} (bending deformation), 1370 cm^{-1} (wagging deformation) and 730-720 cm^{-1} (rocking deformation). Other bands indicating degradation occurred: a broad band around 3300 cm^{-1} present in all the samples which can be assigned to –OH, and a band around 1030 cm^{-1} appears which can be assigned to C-O. Except for the red polyethylene sample, all the other samples showed a band at 1650 cm^{-1}, which can be assigned to unsaturated C=C bonds. Furthermore, in yellow and black samples, carboxylic acids carbonyl absorbance band at 1714 cm^{-1} appeared; in red sample, esters carbonyl absorbance band at 1741 cm^{-1} occurred.

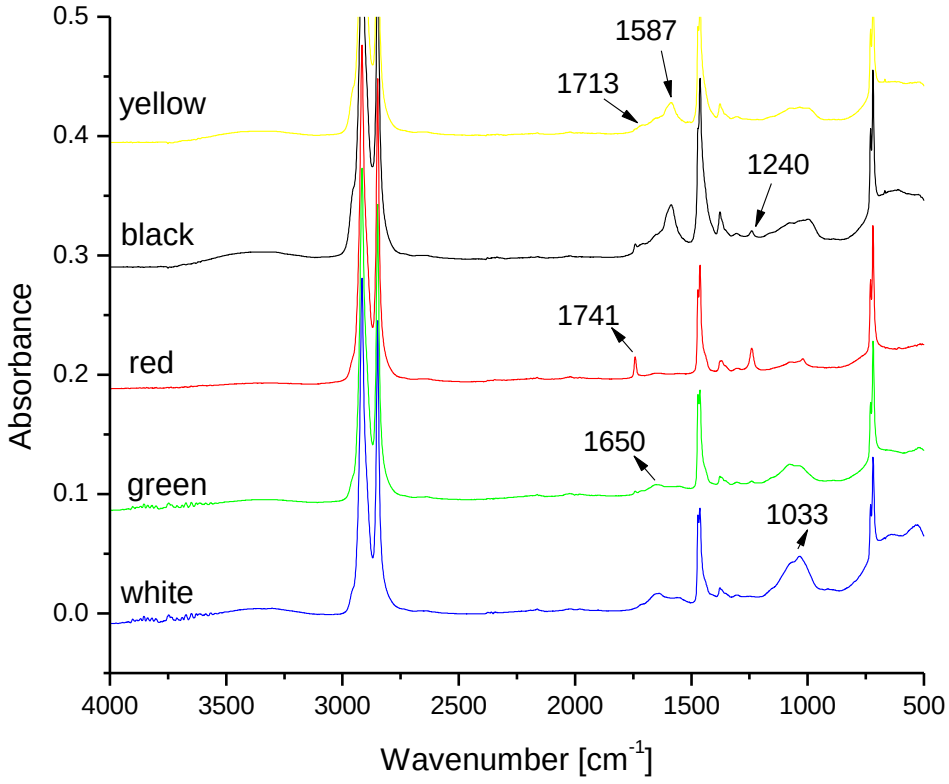

Figure 2. IR spectra of the analysed plastic samples

2.3.2 Molecular analyses of polyethylene-associated bacterial communities

Illumina MiSeq sequencing of the V3-V4 regions of 16S rRNA amplicons resulted in a total of 454,043 high-quality filtered reads, which were downscaled to 235,212 after a rarefaction to a common number of 11,202 reads per sample. No samples were eliminated due to the downscaling. The average coverage was 90.1% ± 2.4, indicating that the sequencing effort was sufficient to describe the vast majority of bacterial communities in the

samples. Of all the analyzed sequences, 94% were correctly classified at the order level, 76.1% at the family, 60.5% at the genus and 30% at the species level. The total number of OTUs per sample varied between a minimum of 1,379 ± 22 for the white plastic to a maximum of 2,097 ± 7 for the red plastic; higher number of OTUs were detected in the soil samples, respectively 2,653 ± 115 for the dump soil where the plastics were retrieved and 2391 ± 54 in the neighboring uncontaminated soil.

A multivariate principal component analysis (PCA) performed on the relative abundances of all analyzed OTUs revealed a strong differentiation between samples (Figure 3): each plastic hosted a different bacterial community, with the green and the yellow the black and white on the other being closer one to the other. The red plastic bacterial community was found in-between the plastic and the soil samples replicates; finally, the latter were very close one to the other but still separated in two different groups.

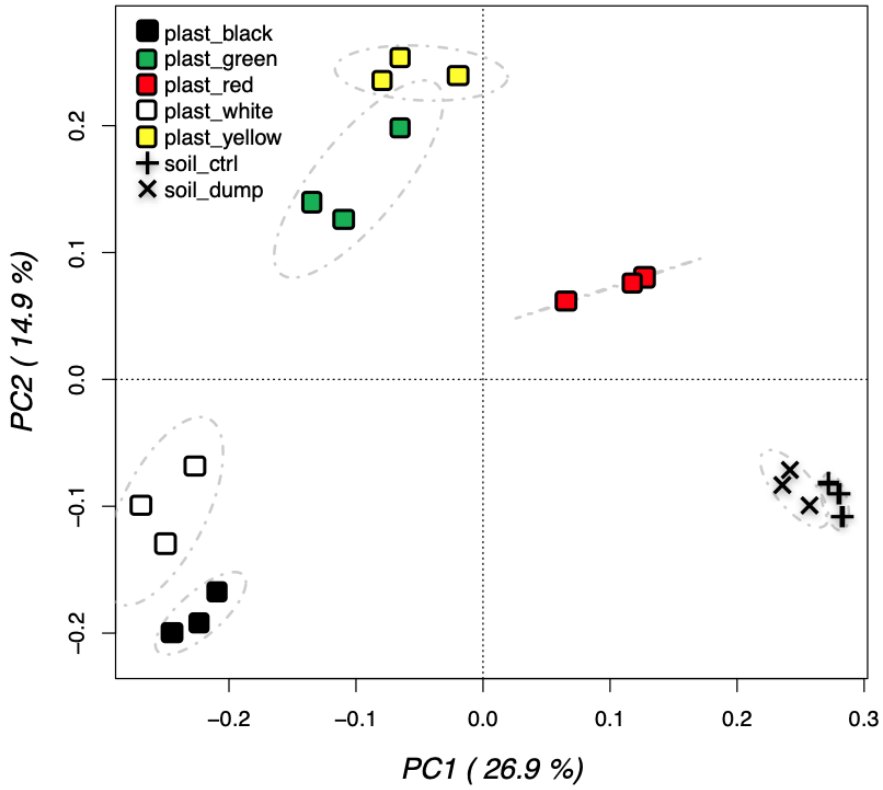

Figure 3. Principal component analysis performed on the total bacterial OTUs relative abundance data from plastic biofilms and soil. Replicates are labelled according to the samples.

This differentiation of bacterial communities was confirmed by hierarchical cluster analyses performed on sequences classified at the genus (Figure 4) and at the species level (Figure 5). In both cases the most abundant taxa, defined as those whose presence was >5% in one sample at least, are represented. In agreement with PCA results, the plastics communities clustered separately from the soil samples, and the 3 replicates from each sample (both plastic and soils) formed a separate cluster. A relevant

exception was represented by the red plastic, hosting a bacterial community that was closer to the soils than to the other plastics in terms of relative abundances of genera and species. Genus level analyses (Figure 4) revealed a significant enrichment of sequences classified as *Bacillus* on the plastic samples as compared to soil. In the control soil, an average of 1.9% of *Bacillus* sequences was detected: the relative presence of this genus increased to 3.2% in the dump soil and to 9.6% in the red plastic. Levels reached 22.4% in the yellow plastic, 24% in the green and then up to 47.2% and 57.5% in the black and white plastics respectively. Other genera significantly higher on plastic samples as compared to the soil were *Pontibacter* on the yellow and green plastics (15.6% and 2.2% respectively as compared to an average of 0.2% in the soils) and *Clostridium* in the black, white and red plastics (7.6%, 5.4% and 3.5% as compared to an average of 2.0% in the soils).

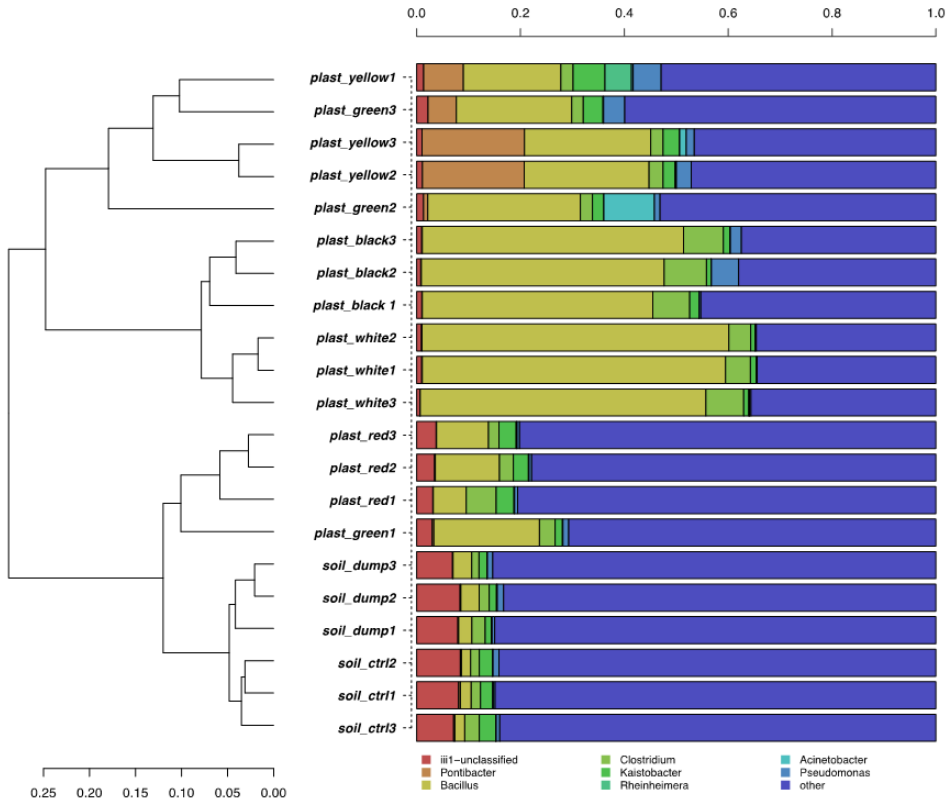

Figure 4. Genus level

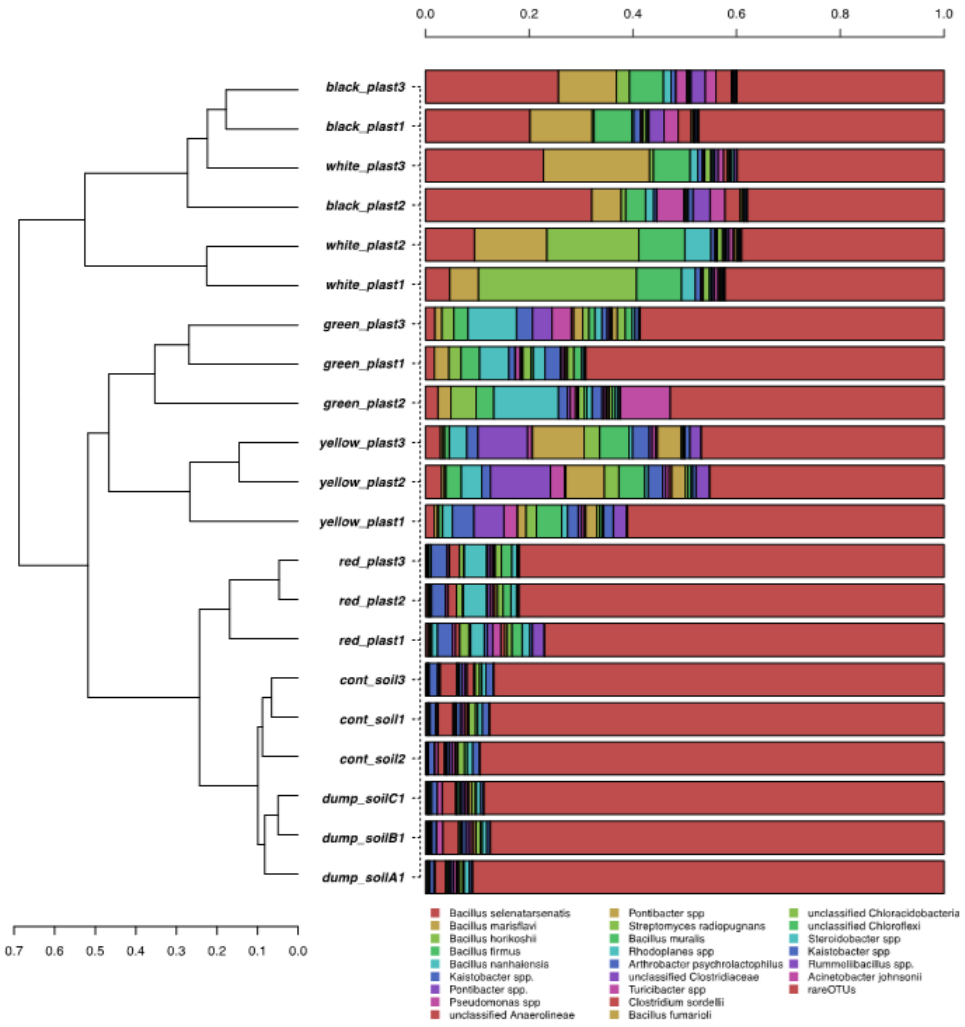

Figure 5. Species level

The clustering of the 24 most abundant OTUs is reported in Figure 5: 11 were classified at the species level, the rest were generally classified at the genus level. For the black and white plastics, these 24 OTUs represented up to the 60% of the total observed diversity; this was decreased to 50% in the yellow

and 40% in the green plastics. On the contrary, these 24 OTUs covered the 20% only of the total bacterial diversity for the red plastics, which is a value quite close to an 18% found in the soil samples. This outcome clearly indicated that the plastic in soils were colonized by a few species becoming dominant, and it was confirmed by α-diversity indexes analyses (Figure 6). When the comparisons were carried out between soil samples and plastics altogether, both Chao diversity (Figure 6a) and Simpson evenness (Figure 6c) were higher in the soil samples as compared to the plastics. Remarkably, the bacterial diversity in the dumpsite contaminated soil was significantly higher than in the neighboring uncontaminated soil (Figure 6a). When plastic samples were differentiated according to the color, further significant differences in bacterial indexes were detected. The Chao diversity index (Figure 6b) was higher in the green and red plastics, while the lowest index values were found for the black, the white and the yellow plastics. The situation was slightly different for the evenness as calculated with the Simpson index: the red plastic still had higher values than the other four, followed by the yellow and the green and afterwards by the black and white plastics. This result is in accordance with the PCA and clustering results presented above (Figures 3, 4 and 5).

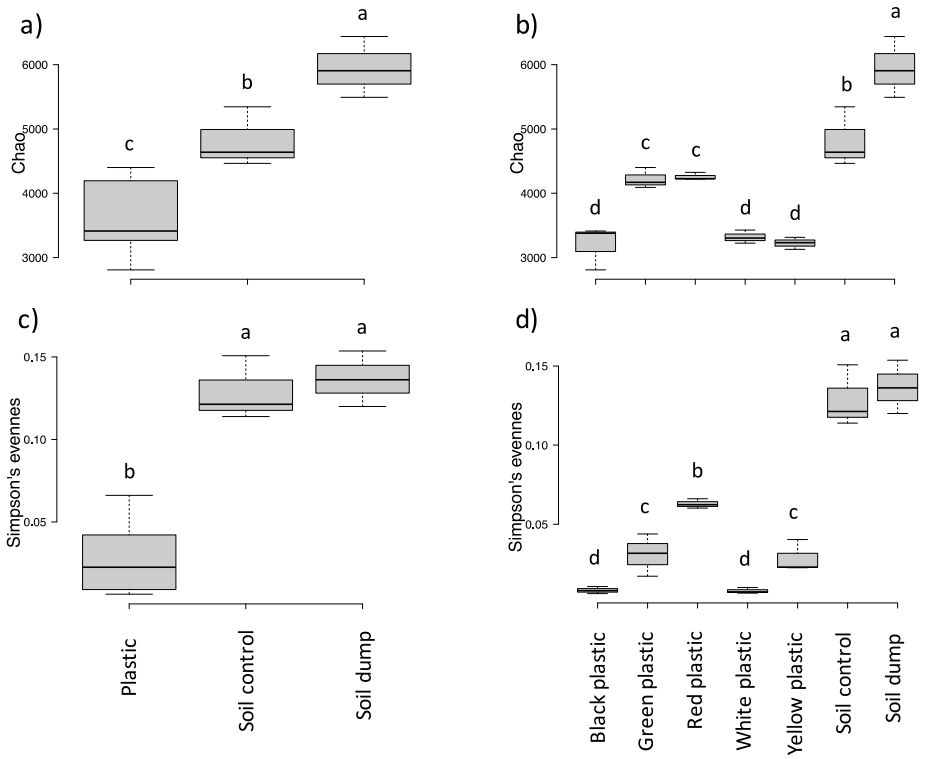

Figure 6: Chao diversity (a, b) and Simpson eveness indexes (c, d) of the bacterial communities of plastic and soil samples. Bar with the same letters are not statistically different according to Waller-Duncan test for comparison of means.

2.3.3 A focus on Bacilli as main colonizers of plastic wastes

Analyses of 16S PCR amplicons at the genus level (Figure 4) revealed that all plastic samples analyzed had high relative abundances of sequences classified as *Bacillus*, reaching a maximum of 57.5% of the total genera for the white plastics. For this reason, analyses were focused on the relative

abundances of bacilli that could be identified and classified at species level. Results are presented in Figure 7 for the 10 most abundant species detected: all except for one (the second mostly abundant) were correctly classified at the species level. The results presented in the upper graph (Figure 7a) confirmed that all the species of Bacillus were significantly higher on the plastics as compared to the control and dump soil. The most abundant was *Bacillus selenatarsenatis*, followed by *Bacillus firmus*, *Bacillus marisflavi*, *Bacillus horikoshii*, *Bacillus muralis*, *Bacillus fumarioli*, *Bacillus flexus* and *Bacillus cohnii*. When the data were disentangled by plastic type, it was revealed that even the relative distribution of these very abundant *Bacillus* species was controlled by the color of the polyethylene bags wastes (Figure 7b). In particular, *B. selenatarsenatis* and B. *marisflavi* were more abundant on the white and black plastics, while *B. firmus*, B. *horikoshii* and *B. cohnii* dominated on the white plastics only.

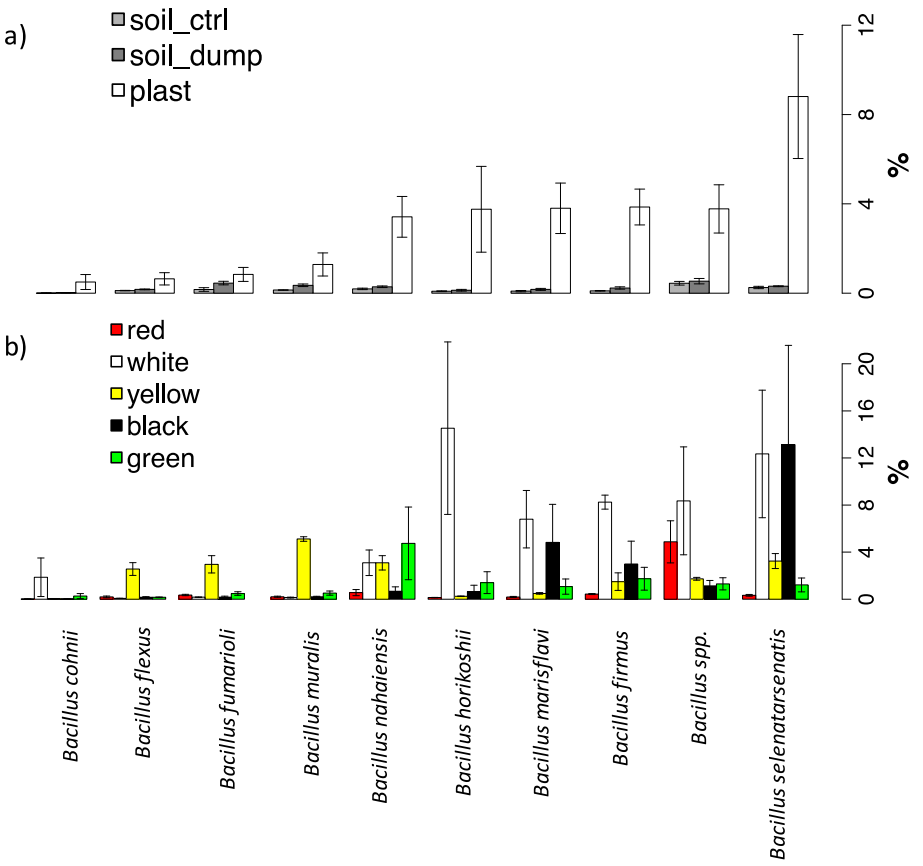

Figure 7: Relative percentages of the mostly abundant Bacillus species on the total bacterial community diversity. Data are presented by joining (a) or keeping separated (b) data from the 5 different plastic types.

The yellow plastics had quite a different structure of Bacillus communities, with the dominant species being *B. muralis*, *B. fumarioli* and *B. flexus*. It is worth noting that the red plastics, having higher levels of degradation (Figures 1 and 2) and a bacterial community much closer to the ones from

the soils (Figures 3, 4 and 5) had also a much lower relative abundance of these *Bacillus* species.

2.3.4 Cultivable bacteria isolated from the plastic surfaces

The same biofilm samples retrieved from the plastics and subjected to Illumina analyses of 16S rRNA PCR amplicons were used for the isolation of cultivable bacterial species. In order to focus not on the total bacterial community but on the species with affinities towards plastics, a selective minimal medium made up of salts and paraffin as sole carbon source was employed, with the latter being a model molecule used to select strains with potential PE-degrading abilities. Decimal dilutions were plated, and representative colonies were picked, purified by three consecutives subculturing, reduced by RAPD DNA analyses and finally identified by Sanger sequencing of PCR amplicons. The results reported in Table 1 show 27 different strains that were isolated from the 5 different plastic analyses: 6 from the yellow plastic, 3 from the black, 4 from the red, 5 from the green and 9 from the red. In agreement with the molecular analyses reported in Figures 2 and 4, *Bacillus* species dominated also the cultivable fraction, with 17 out of all 27 isolates belonging to this genus, with 12 different species identified (Table 1). The relative abundances of these species were also in agreement with the molecular data: *B. simplex* and *B. muralis* for the yellow plastics, *B. firmus* for the white and black. However, the most abundant *Bacillus* species according to the molecular data, *B. selenatarsenatis*, was not

detected among the isolates. Other isolates of interest were *Pseudomonas alcaligenes*, *Pseudodomonas gelcossicida*, *Pseudomoans thivervalensis*, *Arthobacter spp.* and *Lysinibacillus fusiformis*.

Table 1. Bacterial identification

CODE UC	Genus	Specie	plastic origin
UC 7509	*Bacillus*	*firmus*	White
UC 7531	*Lysinibacillus*	*fusiformis*	White
UC 7494	*Bacillus*	*luciferensis*	White
UC 7533	*Bacillus*	*firmus*	White
UC 7528	*Stenotrophomonas*	*maltophilia*	White
UC 7529	*Bacillus*	*firmus*	White
UC 7512	*Bacillus*	*sp.*	White
UC 7511	*Bacillus*	*subtilis*	White
UC 7521	*Bacillus*	*drentensis*	White
UC 7466	*Bacillus*	*pumilus*	Yellow
UC 7534	*Bacillus*	*simplex*	Yellow
UC 7530	*Arthrobacter*	*sp.*	Yellow
UC 7467	*Pseudomonas*	*alcaligenes*	Yellow
UC 7508	*Bacillus*	*simplex*	Yellow
UC 7517	*Bacillus*	*muralis*	Yellow
UC 7487	*Bacillus*	*aquimaris*	Black
UC 7476	*Bacillus*	*mycoides*	Black
UC 7520	*Bacillus*	*firmus*	Black
UC 7472	*Pseudomonas*	*plecoglossicida*	Red
UC 7473	*Acinetobacter*	*johnsonii*	Red
UC 7498	*Comamonas*	*testosteroni*	Red
UC 7513	*Arthrobacter*	*sp.*	Red
UC 7489	*Pseudomonas*	*thivervalensis*	Green
UC 7493	*Bacillus*	*boroniphilus*	Green
UC 7506	*Bacillus*	*megaterium*	Green
UC 7478	*Paenibacillus*	*woosongensis*	Green
UC 7503	*Bacillus*	*idriensis*	Green

2.4 Conclusion

The study represents one of the first fine scale assessment of microorganisms growing on plastics from a landfill site. The results revealed that after more 35 years of contamination, communities of plastic-loving bacteria established themselves on the surface of plastic of different colors. Each plastic type hosted a distinct community, thus indicating a strong relationship between the plastic properties (including the presence of colorants) and the colonizing populations. Plastics characterizations showed some significant changes associable the microbial activity. Most interestingly, a possible correlation between the level of plastic degradation and the bacterial community structure was found, with the most degraded plastic hosting a community that resembled more to the one of the uncontaminated soil.

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Chapter 3

3. An efficient method to isolate polyethylene degrading bacteria from landfill

3.1 Introduction

Polyethylene is a non-biodegradable synthetic plastic material. Over the last decades, its level of concentration in the environment has hugely increased (I. Gilan et al., 2004). In general terms, the biodegradation of polyethylene is usually performed by the action of microorganisms able to modify the structure and the properties of the polymer (Restrepo-Flórez et al., 2014).

However, the presence of the only CH_2 groups in the polyethylene, makes this polymer highly hydrophobic. Such a condition acts as a physical limit for microorganisms, becoming unable to attack the surface of the polymer (Arutchelvi et al., 2008). Abiotic treatment, such as UV irradiation (Cornell et al., 1984), chemical oxidation (Rajandas et al., 2012), prooxidant additives (Chiellini et al., 2006; Singh & Sharma, 2008) and thermal oxidation (Albertsson et al., 1998) increase the hydrophilicity of polyethylene. As a consequence, the potential of microorganism to degrade the polymer is increased. Moreover, the ability of microbial populations to form biofilm on solid surface, such as polyethylene, is usually correlated to a higher metabolic activity than that of suspended bacteria (I. Gilan et al., 2004), as a consequence, the carbon availability in biofilm is higher.

The aim of this study was to develop and apply an enrichment method for the isolation of a bacterial community able to metabolize PE as unique energy and carbon source. A thermal oxidized polyethylene was used in a part of the enrichment phase, to improve the selection of the best degradation bacteria.

3.2 Materials and Methods

3.2.1 Soil and plastic waste sampling

Sampling was carried out in Alseno (Province of Piacenza, Italy) in a landfill site abandoned since the 1980s. Soil samples of 100g were collected in 3 replicates in the landfill site and, as a control, in an uncontaminated and cultivated neighboring soil. Different plastic samples were retrieved from the site and identified by their color (Black B; White W; Yellow Y, Red R, Green G). Thermal oxidized (72 hours at 100°C) Low-density polyethylene film (LDPE) was carried out by Politecnico di Torino (PoliTO). Commercial polyethylene powder (PE) provided by Sigma-Aldrich (428043) was also employed in this study.

3.2.2 Plastic samples characterizations

Fourier Transform Infrared Spectroscopy (FT-IR) spectra were obtained using a diamond crystal on a Perkin Elmer Frontier instrument, with 32 scans per replicate in a scanning range of 4000-500 cm-1 and scan rate of 4 cm-1.

3.2.3 Bacterial culture media

Bacterial strains ability to use polyethylene sample as only energy and carbon source was tested in a minimal salt medium (SM) containing 4.0 g/L NH_4NO_3, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L K_2HPO_4, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.15 g/L KCl, and 1.0 mg/L of each of the following microelements: $FeSO_4 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$ and $MnSO_4$ (I. Gilan et al., 2004), where the only energy and

carbon source was constituted from polyethylene. In other experiments paraffin oil (*d*= 0.827-0.890 g/mL at 20 C, Sigma-Aldrich) at a concentration of 2% was added to the medium.

3.2.4 Two-step enrichment for isolation of plastic-degrading microorganisms

A two-step enrichment, first in solid phase with soil and then in minimal medium liquid culture, was carried out following with minor modifications the protocol of (I. Gilan et al., 2004). As a first step, 20 g of soil from the dumpsite were added to sterile plastic tubes with 8 mL of SM minimal medium. Two types of enrichment were performed for this first step: one (group A) using directly as inoculum a piece of about 3 cm^2 of waste plastic samples, the other (group B) using the biofilm retrieved from the waste plastic samples (extraction in stomacher with 100 mL of physiological solution) together with a piece of pure pre-oxidised polyethylene (PE). Microcosms were prepared in two different ways, with and without the addition of 2% paraffin oil, giving a total of 20 microcosms (8 from group A, 12 from group B) that were incubated for 5 months at 30°C without shaking, keeping the humidity level constant by periodically adjusting the weight with the addition of sterile water.

After 5 months of incubation, the second enrichment was carried out under sterile conditions in glass flasks containing 50 mL of SM each, a 4X4 cm piece of pure pre-oxidised polyethylene (LDPE) and as an inoculum 500 μL of the biofilm grown on the surface of the plastics samples used in the first

enrichment. From each 1st step microcosm, 3 replicated liquid microcosms were prepared, thus giving a total of 60 microcosms in this 2nd step. These 2nd step microcosms were incubated at 30°C for 30 days. At the end of the second enrichment step the 4X4 cm LDPE square was put in a small stomacher bag with 20 mL of physiological solution and biofilm was extracted.

3.2.5 Isolation and evaluation ability of cultivable bacteria

Biofilms retrieved from the plastic surfaces were plated by serial dilution on SM agar. Paraffin oil (2%) was added to the plates as carbon source, other than the complex sugars of agar, in order to isolate strains with potential degradation abilities. Plates were incubated at 30 °C in aerobic conditions.
Colonies grown on SM agar-paraffin oil (2%) were isolated in LB agar and tested in liquid cultures having as sole carbon source with PE as follows: an overnight culture of each bacteria was washed three times with sterile physiological solution and used as inoculum (1:100) in a Tube 25mL (Sarstedt) with 10 ml of sterile SM plus sterile PE 2% w/v. The tube was incubated in a shaker (30°C, 200 rpm) for 15 days. Absorbance was recorded at the end of incubation time. The best performing strains were tested in 100 mL flask with 50 mL of SM and 2% w/v of sterile PE. The flasks were incubated in shaker (30°C, 200 rpm) for 7 days and cells were counted by serial dilution and plating onto LB agar.

3.2.6 Characterization of isolate strains

DNA from isolates purified colonies was extracted with Microlysis kit (Microzone, Haywards Heath, UK) according to the manufacturer's protocol. Randomly Amplified Polimorphic DNA-Polymerase Chain Reaction (RAPD-PCR) was performed on each isolate using single stranded oligonucleotide primers RAPD2 (5'-AGC AGG GTC G-3') (Cocconcelli et al., 1995) and M13 (5'-GAG GGT GGC GGT TCT-3') (Huey & Hall, 1989). Amplification reactions with both primers were conducted in a programmable T100 thermocycler (BioRad) in volumes of 25 μL containing 1 μM of primer, 3 mM of MgCl2, 0.2 mM of DNTPs, 2.5 U of Taq DNA polymerase (Fermentas, Selangor, Malaysia). The PCR fragments electrophoretic profiles were captured and patter analysis was performed with the Fingerprinting II software (BioRad) in order to obtain similarity profiles of bands based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and select representative unique isolates.

The latter were identified by PCR amplification using the primers P0 5'-GAG AGT TTG ATC CTG GCT- 3') and P6 (5'-CTA CGG CTA CCT TGT TAC-3') as previously described (Di Cello & Fani, 1996). The PCR products were purified using the Wizard SV Gel and PCR Clean-Up system, according to the package insert (Promega Corpo- ration, Madison, WI) and sequenced at the BMR Genomics of Padova, Italy. The identification of sequences was performed by alignment against the Ribosomal Database Project (RDP) database using the Naïve Bayesian Classifier (Wang et al., 2007).

3.3 Results

3.3.1 Plastic samples characterizations from microcosms

To understand if the five-months microbial incubation in the microcosms led to a degradation of PE, the samples were analysed with FT-IR analyses. IR analyses (Figure 1) of all samples showed typical IR absorbance bands of polyethylene (Gulmine et al., 2002): 2916 cm^{-1} (CH2 asymmetric stretching), 2848 cm^{-1} (CH2 symmetric stretching), 1473 and 1463 cm^{-1} (bending deformation), 1370 cm-1 (wagging deformation) and 730-720 cm^{-1} (rocking deformation). Differences between polyethylene samples before and after the two-step enrichment were observed (Figure 1). The presence of a high peak at 1030 cm^{-1}, assignable to C-O group, indicated an oxidation that was carried out by microorganism. In detail, the C-O group was higher in the black sample (Figure 1 d). Also, it is possible to observe a higher 1033cm^{-1} peak in red and yellow polyethylene samples (Figure 1 b,c). The white polyethylene sample did not show significant differences before and after the enrichment step (Figure 1 a).

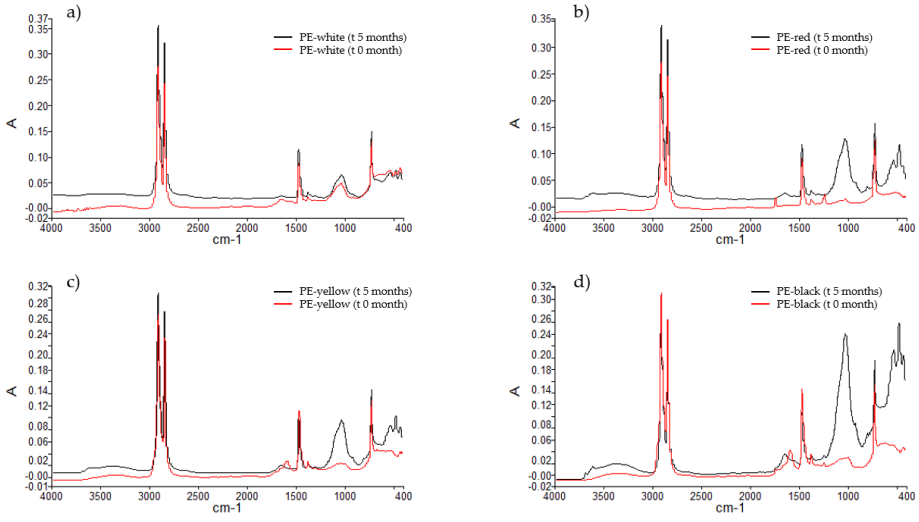

Figure 1. FT-IR analysis of plastic samples after two-step enrichments.

3.3.2 Bacteria isolation and identification

Since an effect of the bacterial population on PE was detected, a two-step screening experiment was designed, with the aim of isolating from the bacterial biofilm colonizing plastics a strain able to grow on PE as sole carbon source. The total amount of 165 colonies was collected and then tested on SM–PE. Finally, 23 bacterial isolates that showed an ability to grow in presence of polyethylene, reaching at least a value of 0.3 OD after one week of incubation, were selected for further studies. Twenty of these isolates derived from microcosms inoculated with a fragment of plastic from the landfill. The remaining three were isolated from microcosms inoculated with retrieval biofilm. The RAPD analysis was carried out on these bacteria cultures, with the purpose of avoiding strain replication among isolates.

Thus, as reported in Figure 2, 10 strains from a total initial number of 23 isolates were identified.

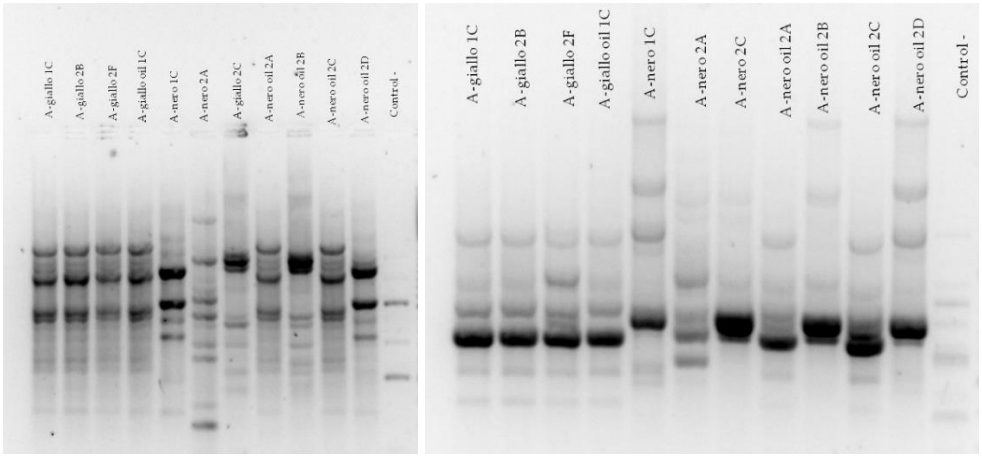

Figure 2. RAPD-PCR patterns generated by isolates from microcosm.

The Sanger sequencing of 16S rDNA amplicons allowed the taxonomical identification of the strains from the two-step enrichment procedure, as shown in Table 1.

Table 1. Bacterial identification

CODE UC	Genus	Specie	sample name
UC 4018	*Pseudomonas*	*manteilii*	A.black oil 2.A
UC 4019	*Achromobacter*	*xylosoxidans*	A. black oil 2.B
UC 4020	*Pseudomonas*	*manteilii*	A. black oil 2.C
UC 4021	*Ochrobactrum*	*anthropi*	A. black oil 2.D
UC 4022	*Ochrobactrum*	*anthropi*	A-white 3C G1
UC 4004	*Rhodococcus*	*ruber*	A- white 3C P1
UC 4023	*Ochrobactrum*	*anthropi*	A- white oil 2C G1
UC 4024	*Cellulosimicrobium*	*funkei*	A- white oil 2C P1
UC 4025	*Pseudomonas*	*putida*	A- white oil 3.C
UC 4026	*Pseudomonas*	*manteilii*	A-yellow 1.C
UC 4027	*Pseudomonas*	*manteilii*	A- yellow 2.B
UC 4028	*Achromobacter*	*xylosoxidans*	A- yellow 2.C
UC 4029	*Achromobacter*	*xylosoxidans*	A- yellow 2.C
UC 4030	*Pseudomonas*	*manteilii*	A- yellow 2.F
UC 4031	*Pseudomonas*	*manteilii*	A- yellow oil 1.C
UC 4032	*Ochrobactrum*	*anthropi*	A- black 1.C
UC 4033	*Achromobacter*	*xylosoxidans*	A- black 2.A
UC 4034	*Achromobacter*	*xylosoxidans*	A- black 2.C
UC 4035	*Pseudomonas*	*putida*	A-red 2.B G1
UC 4036	*Achromobacter*	*marplatensis*	A-red 2.B P1
UC 4037	*Pseudoxantomonas*	*daejeonensis*	B- black 2 C2b
UC 4038	*Pseudomonas*	*brenneri*	B- black 2 S2
UC 4003	*Pseudomonas*	*aeruginosa*	B- black 2 C2a

The genus *Pseudomonas* was the most abundant in the isolated strains, representing 43,48 % of the total. Other genera found in the isolates were *Achromobacter* with 26,08%, followed by *Ochrobactrum* with 17,39. Lastly, *Rhodococcus, Pseudoxantomonas* and *Cellulosimicrobium* each of the three representing 4,35% of the total.

Differently from what observed on plastics isolated from landfill, were members of *Bacillus* genus accounted for 57.5% of the total genera detected by 16SrRNA, none of the isolated strains belonged to the *Bacillus* genus.

The investigation in the genus *Pseudomonas* remarked that the found species need to be distinguished in *Pseudomonas manteilii* with 60% of total abundance, *P. putida* with 20%, and lastly *P. aeruginosa* and *P. brenneri* with 10% respectively.

3.3.3 Ability to grow on polyethylene

The 10 different selected strains were tested in minimal salt medium with the addition of polyethylene as only carbon and energy source. All of them confirmed the ability of growth on polyethylene, as shown in Figure 3. The cell density increased approximately of one log in all the tested strains, except for UC4003 and UC4004 that showed a higher growth rate (approx. 2 logs). The growth kinetics of the best performing strains, reported in Figure 4, revealed that the stationary phase was reached after four days of incubation.

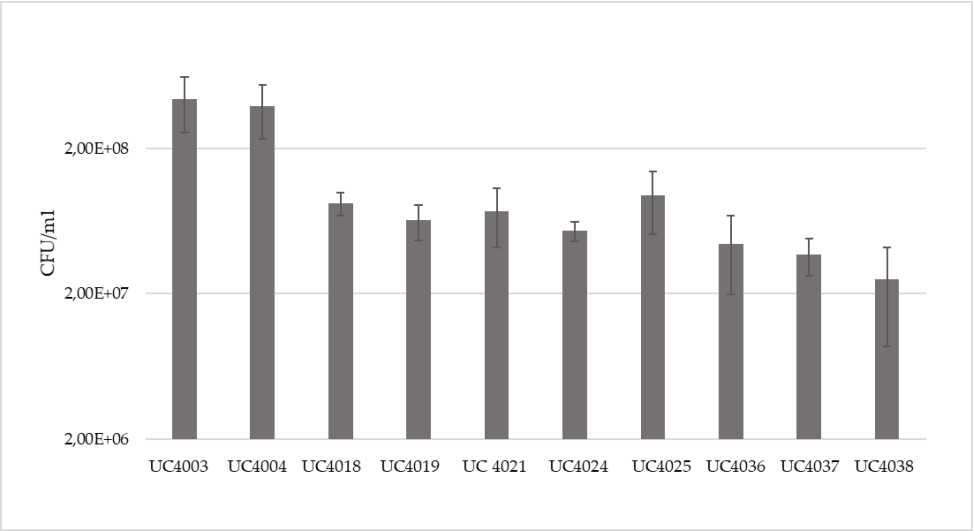

Figure 3. LOG increase of isolated strains on polyethylene as only carbon source.

A gram-negative and a gram-positive strain showed the highest and fastest growth. The gram-positive strain was identified as *Rhodococcus ruber* and called UC 4004; the gram-negative was identified as *Pseudomonas aeruginosa* and designated as UC 4003. The kinetics growth curves are reported in Figure 4.

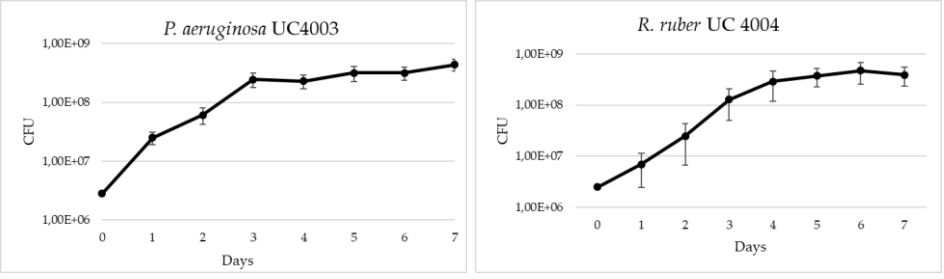

Figure 4. Kinetic growth curves of *P. aeruginosa* UC4003 and *R. ruber* on polyethylene as only carbon source.

The growth rate of *P. aeruginosa* UC4003, in presence of polyethylene, was 0.062 h^{-1} (doubling time, 11.1 h), whereas in the case of *R. ruber* UC4004, the growth rate was 0.050 h^{-1} (doubling time, 14 h).

3.4 Conclusion

The FT-IR analysis confirmed the capacity of the strains to generate changes in the functional groups of polyethylene. The presence of groups containing oxygen, revealed an activity of the microorganism suitable to degrade this hydrophobic substrate.

A two-step enrichment was found to be the right approach to obtain bacterial strains able to use polyethylene as only energy and carbon source. The use of paraffin oil (short-chain alkane) and pre-oxidized polyethylene gave more chances at microorganisms to uptake alkane. The data obtained from the use of a piece of plastic, or a biofilm, as inoculum showed a significant difference. However, the most performant strains, *P. aeruginosa* UC4003 and *R. ruber* UC4004, derived respectively from biofilm and plastic inoculum.

In conclusion, *P. aeruginosa* UC4003 and *R. ruber* UC4004 can be considered as two microorganisms to focus on in future works, with the purpose of promoting an efficient strategy for polyethylene degradation.

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Chapter 4

4. *Pseudomonas aeruginosa* UC4003: the role of *pra* in polyethylene metabolism.

This work was made in collaboration with Prof. Nikel's group, DTU Center for Biosustainability.

4.1 Introduction

Members of the *Pseudomonas* genus show relevant capacities in the bioremediation of short-chain alkane such as crude oil, naphthalene, simple hydrocarbons and other hydrophobic polymers (Wilkes & Aristilde, 2017). Long-chain alkane such as polyethylene, due to their highly hydrophobicity nature and their molecular weight, differently from short-chain alkane are unable to pass through the cell membrane. Thus, the first step of their degradation consists in the breakdown of the polymer into smaller oligomers (Kolvenbach et al., 2014; Lucas et al., 2008), thus enabling their entry into the cell.

Van Beilen and colleagues reported the ability of *P. putida* GPo1 to grow considerably on alkanes ranging from C_6 to C_{13}, (van Beilen et al., 2005). Furthermore Liu et al. isolated a *P aeruginosa* strain SJTD-1 able to grow on medium and long-chain alkanes (C_{12}–C_{30}) (Liu et al., 2014).

The addition of prooxidant additives or physical and chemical pretreatment increases the hydrophilicity of the long chain polymer of polyethylene, consequently improving polyethylene biodegradation. As a result, a chain scission of the polymer is obtained, together with the generation of carbonyl functional groups and low molecular weight components (Chiellini et al., 2006; Wilkes & Aristilde, 2017). Acid nitric pretreatment of low density polyethylene (LDPE), helps *P. aeruginosa* to degrade up to 50% of LDPE in two months (Rajandas et al., 2012). At the same time, some *Pseudomonas* sp., such as AKS2, express the capacity to degrade low density polyethylene

films up to 5% after 45 days of incubation without any prior oxidation (Tribedi & Sil, 2013).

The biodegradation of polyethylene involves several steps of oxidation, dehydrogenation and carbon-carbon bond breaking to produce acetic acid that can be integrated into the β-oxidation pathway (Wilkes & Aristilde, 2017). The key role of alkane hydroxylase in degradation of low-molecular weight polyethylene is widely recognised (Yoon et al., 2012).

However, despite the above-mentioned results, there is still a lack of information about the complete biological mechanism involved in polyethylene degradation.

The following part of the work focuses on *P. aeruginosa* UC4003 and its ability to degrade polyethylene.

4.2 Materials and methods

4.2.1 Bacterial culture media

P. aeruginosa UC4003 culture was maintained on Luria Bertani broth or nutrient agar. Minimal salt medium (SM) containing 4.0 g/L NH_4NO_3, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L K_2HPO_4, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.15 g/L KCl, and 1.0 mg/L of each of the following microelements: $FeSO_4 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$ and $MnSO_4$ was used as liquid culture.

Low-density (0.92 g/mL at 25 °C) polyethylene (LDPE) with an average molecular weight of 4,000 (PE 4,000) was used in this study. Commercial polyethylene powder provided by Sigma-Aldrich (427772). PE 4,000 was

sterilized by washing three times with 96% ethanol. Polyethylene powder was then dried under sterile condition before its inoculation.

The ability of growth of *P. aeruginosa* UC4003, on PE4,000 was assessed in 250 mL flasks containing 100 mL of minimal salt medium (SM) and 2g of PE4,000 sterilized; in case of overexpression strains, gentamycin (20ug/ml) and 3-Methylbenzoic acid (5mM) were added. As control SM with 2% glucose v/v was used.

Inoculation was made as follows: overnight cultures of *P. aeruginosa* UC4003 were centrifuged 5 min at 3200 rpm and then washed with sterile physiological solution (NaCl 9.0 g/L). This washing step was repeated three times, to limit the transfer of nutrients in SM. Then *P. aeruginosa* UC4003 was resuspended in physiological solution to an absorbance of 1 $OD_{600\ nm,}$ measured with the UV-Vis Spectrophotometer (Shanghai Metash Instruments CO., LTD) using the same solution as a blank. *P. aeruginosa* UC4003 was inoculated (1:100) in SM with PE4,000. The flasks were then incubated on a rotary shaker (180 rpm) for 72 hours at 30°C. The absorbance at of 600nm (OD 600nm), and the number of colony forming unit (CFU) by plating on LB medium was recorded at intervals. A 24 hours interval was considered for polyethylene, while for glucose, an interval of 8 hours was observed. Liquid medium was pelleted at 4500g for 10min at 4°C. Supernatant was recovered and filtered (0,22µm filter) to be used for protein and metabolomics analysis. Pellet was transferred in an Eppendorf of 1.5ml for RNA extraction.

4.2.2 Genome sequencing

The whole genome sequencing of *Pseudomonas aeruginosa* (UC4003) was carried out. DNA was extracted and purified using NucleoSpin Tissue, according to the package insert (MACHEREY-NAGEL GmbH & Co. KG). Illumina MiSeq V3 technology for 2 × 300-bp paired-end reads was used to sequence the whole genomes (Parco Tecnologico Padano, Lodi, Italy). SPAdes version 3.11 (Bankevich et al., 2012) was used to assemble the whole-genome sequences. Final draft genome was used for genome annotation employing RAST server (Aziz et al., 2008) and PATRIC server (Wattam et al., 2017).

4.2.3 Extracellular protein extraction

After a short-term glucose exposure (0, 16, 24, 48 hours), and a PE4,000 exposure (24, 48, 72 hours), a total amount of liquid culture from three replicates corresponding to each condition of *P. aeruginosa* UC4003 culture was transferred into a tube of 50 ml (Sarstedt) and after, pelleted at 10,000 rpm for 15 min at 4°C. Supernatant was recovered and filtered through a sterile polyethersulfone membrane filter (PES) (0.22 μm pore size, 33 mm diameter, Starlab). Acetone precipitation protocol (Wessel & Flügge, 1984) was carried out on supernatant. A volume of 10 ml of supernatant was transferred in a tube of 50 ml, then, four volumes of cold acetone 90% was added to the tube and the whole compound was vortexed well. The tube was incubated at -20°C for 15 hours at least. After that incubation, the tube was centrifuged at 14,000 x *g* for 30 min at 4°C, the supernatant was removed and

the pellet was air dried. The protein pellet was dissolved in 500 µl of ammonium bicarbonate 50mM (CARLO ERBA Reagents S.r.l.). Proteins were separated by one-dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins samples were loaded in the Mini-PROTEAN TGX 12% (Tris-Glycine eXtended) precast gels (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. For protein visualization, SDS-PAGE was stained with Oriole fluorescent gel stain solution (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. The gel results were visualized using a Gel Doc image analysis system (Bio-Rad Laboratories, Inc.).

4.2.4 Nano LC/QTOF proteomic mass-spectrometry (MS/MS)

Protein bands were excised from Oriole fluorescent gel stain gel, on a UV transilluminator table (UV transilluminator 2000, Bio-Rad Laboratories, Inc.).

Fifty micrograms of proteins were reduced with DTT, alkylated with iodoacetamide and overnight digested using trypsin (Promega, Madison, WI, USA). Tryptic peptides were analysed using nanoscale liquid chromatography coupled to a hybrid quadrupole-time-of-flight (Q-TOF) mass spectrometer, as reported in Lucini & Bernardo, 2015 work (Lucini & Bernardo, 2015). The search was conducted against the proteome of *P. aeruginosa* UC4003, and also *P. aeruginosa* sp. (UniProt, downloaded July 2017). The database was then concatenated with the reverse one and 1% false

discovery rate was selected for validation purposes. Finally, a label-free quantitation was carried out using summed peptide abundance (Zhang et al., 2018).

4.2.5 Recovery of degraded product and sample analysis

The PE4,000 powder was recovered after 72 hours incubation through centrifugation. The product was then washed firstly using 0.5% SDS and after with ethanol by centrifugation to remove the bacterial biomass. The obtained product was kept for overnight drying. Analysis of recovered PE samples were carried out by Politecnico di Torino. The analysis conducted were: X-ray diffraction (crystallinity) and Fourier Transform Infrared Spectroscopy (FT-IR), (Bhatia et al., 2014; Volke-Sepúlveda et al., 2002).

4.2.6 Gene expression analysis

RNA extraction was carried out on cell pellet, which was obtained after the same procedure explained in paragraph 2.4.

RNA was extracted and purified using NucleoSpin RNA (MACHEREY-NAGEL GmbH & Co. KG) according to the manufacturer's protocol and quantified with Qubit RNA HS Assay Kit (Thermo Fisher Scientific Inc). Complementary DNA (cDNA) was obtained via reverse transcription, 1 μg of total RNA was retro transcribed with Transcriptor First Strand cDNA Synthesis Kit (Roche Molecular Systems, Inc).

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was carried out on 0.25 ng of cDNA *pra*, *alkB*1 and *alkB*2 genes were amplified. Primers sequences are showed in Table 1. The PCR amplifications were performed in a T100™ Thermal Cyclerl Cycler (Bio-Rad Laboratories, Inc) thermocycler using the following temperature cycling parameters: initial denaturation at 94°C for 2 min followed by a total of 30 cycles of amplification in which each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min and primer extension at 72°C for 2 min. The final extension time was carried out at 72°C for 8 min. Agarose gel electrophoresis (1% w/v) was used to analyse PCR fragments.

Table 1. Oligonucleotide gene-specific primers used for PCR amplification

Primer name	Primer sequence
***pra*_fv**	5'- AAT CCC TAT CGG ATC TTT CC -3'
***pra*_rw**	5'- CAA ACA GGC AGG AAA TAT CG -3'
***alkB1*_fv**	5'- ATA ACC GGT GAT TCC TAT CG -3'
***alkB1*_rw**	5'- ATC GCT AAG GAT AGT CAA CC -3'
***alkB2*_fv**	5'- TAG AGG CTT TCC ACA TGT TG -3'
***alkB2*_rw**	5'- CCT GTA GGT CAG TCA GTT TG -3'

4.2.7 USER cloning for overexpression genes

Escherichia coli DH5α was used as host for plasmid propagation.

The plasmid pSEVA628 (Silva-Rocha et al., 2013) was employed as a backbone, PCR amplification was carried out using the primers pSEVA628_fw and pSEVA628_rv, that are listed in Table 2.

The three genes of interest, *pra* (489 bp), *alkB1* (1149 bp) and *alkb2* (1134 bp) were amplified by three PCR amplifications carried out in parallel using chromosomal DNA of *P. aeruginosa* UC4003 as template. The sequence of the oligonucleotide primers, *pra*_fw, *pra*_rv, *alkB1*_fw, *alkB1*_rv, *alkB2*_fw and *alkB2*_rv, all purchased from Integrated DNA Technologies, Inc., are listed in Table 2.

The reactions were performed using Phusion U Hot start DNA Polymerase (Thermo Scientific™) according to the manufacture's instruction. Additionally, 3% DMSO was included in all reactions.

Table 2. Oligonucleotide gene-specific primers used for USER cloning

Primer name	Primer sequence
***pra*_fv**	5'- AGG TAT TTT UAT GAA ATC CAT CAA GTC CCT
***pra*_rw**	5'- AGG GTT TUC GGA TTA CGG GTT GAC TAC G -3'
***alkB1*_fv**	5'- AGG TAT TTT UAT GTT TGA AAA TTT CTC TCC
***alkB1*_rw**	5'- AGG GTT TUA TTC TCA GGA AGC TGC CGG -3'
***alkB2*_fv**	5'- AGG TAT TTT UAT GTT TGC CTC GCT TTC CTC -3'
***alkB2*_rw**	5'- AGG GTT TUA AGG TGC CGT TTC CCA GG -3'
pSEVA628_fv	5'- AAA ACC CUG GCG ACT AGT CTT GGA CT -3'
pSEVA628_rv	5'- AAA AAT ACC UCC TTA CGT AAT ACG CTT TAC

The PCR products were column-purified using NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL) and quantified by NanoDrop 2000 (Thermo Scientific™).

The pSEVA628 amplicon was digested with DpnI for 1 hour at 37°C. Subsequently, a treatment with USER® Enzyme (New England Biolabs) was performed as described below for each gene, *pra, alkB1* and *alkB2*.

The final volume of ten microliter, consisting of a DpnI-digested vector and each gene PCR product mixed in a 1:10 molar ratio, was treated with 1 U of USER enzyme (Geu-Flores et al., 2007). Then, the reaction mixtures were incubated as reported in Table 3

Table 3. USER Reaction (PCR Protocol)

Temperature (°C)	Time (minute)
37	30.00
28	2.00
26	2.00
24	2.00
23	2.00
21.5	2.00
20	3.00
18	2.00
17	1.00
10	10.00

The whole USER-treated reaction mixtures were used to transform 100 μl of chemically competent *E. coli* DH5*α* cells by heat shock (Inoue et al., 1990),

which were plated on LB agar with 10 μg/ml gentamycin sulfate (Gm). The plates were incubated overnight at 37°C (Hansen et al., 2014; Nour-Eldin et al., 2010).

Colony PCRs, using OneTaq® 2X Master Mix with Standard Buffer (New England Biolabs) were performed on the colonies obtained after transformation; the primers used for each gene are reported in Table 2.

Three colonies that were obtained per each USER-reaction were transferred into 5 ml LB medium with 10 μg/ml Gm and grown at 37°C overnight; plasmids were column-purified using NucleoSpin® Plasmid (MACHEREY-NAGEL), quantified with a NanoDrop 2000 spectrophotometer (Thermo Scientific™) and ultimately sent for sequencing to verify the correct insertion and sequence integrity.

The plasmids obtained, pSEVA628::*pra*, pSEVA628::*alkB1* and pSEVA628::*alkB2*, were used to transform *P. aeruginosa* UC4003. *P. aeruginosa* UC4003 electrocompetent cell was prepared as described in a Martinez-Garcia & de Lorenzo work (Martinez-Garcia & de Lorenzo, 2012).

For electroporation, 500 ng of each plasmid DNA purified were mixed with 100 μl of electrocompetent cells. The mixture was transferred to a 2 mm gap width electroporation cuvette.

After having applied a pulse (settings: 25 μF; 200 Ω; 2.5 kV on a Bio-Rad GenePulserXcellk; Bio-Rad), 1 ml of room temperature LB medium was added at once, cells were transferred to a small (13 X 100 mm) plastic tube and shaken for 2 h at 30° C. Then, the cells were harvested in a microcentrifuge tube. A total of 900 Al of the supernatant was discarded and the cell pellet was resuspended in the residual medium. The entire mixture

was then plated on an LB + Gm20 plate. The plates were incubated at 30° C until colonies appeared (Choi et al., 2006).

To confirm the insertion of plasmids in *P. aeruginosa* UC4003, colony PCRs were conducted on growing colonies, the utilized forward primers are listed in Table 2. The reverse primer was **11pS2** 5′ CCC CTG GAT TCT CAC CAA TA 3′.

4.3 Results and discussion

4.3.1 Genome sequencing and annotation

An assembled genome for *Pseudomonas aeruginosa* UC4003 was submitted to the comprehensive genome analysis service at PATRIC (Wattam et al., 2017). Details of the analysis, including genes of interest (Specialty Genes), and a functional categorization (Subsystems), are provided below.

The assembled genome of *P. aeruginosa* UC4003 had 163 contigs, with the total length of 7,007,112 bp and an average G+C content of 65.99% (Table 4).

Table 4. Assembly Details

Contigs	163
GC Content	65.99
Plasmids	0
Contig L50	11
Genome Length	7,007,112 bp
Contig N50	200,305
Chromosomes	0

The *Pseudomonas aeruginosa* UC4003 genome was annotated using RAST tool kit (RASTtk) (Brettin et al., 2015) and assigned to a unique genome identifier of 287.6743. That genome was in the super kingdom and was annotated using genetic code 11. The taxonomy of the genome was reported as follows: *cellular organisms > Bacteria > Proteobacteria > Gammaproteobacteria > Pseudomonadales > Pseudomonadaceae > Pseudomonas > Pseudomonas aeruginosa group > Pseudomonas aeruginosa*

The genome showed to have 6,843 protein coding sequences (CDS), 61 transfer RNA (tRNA) genes, and 3 ribosomal RNA (rRNA) genes. The annotated features are summarized in Table 5.

Table 5. Annotated Genome Features

CDS	6,843
tRNA	61
rRNA	3
Partial CDS	0
Miscellaneous RNA	0
Repeat Regions	0

The annotation included 1,508 hypothetical proteins and 5,335 proteins with functional assignments (Table 6). The proteins with functional assignments included 1,349 proteins with Enzyme Commission (EC) numbers (Schomburg et al., 2004), 1,148 with Gene Ontology (GO) assignments (Ashburner et al., 2000), and 1,002 proteins that were mapped to KEGG

pathways (Kanehisa et al., 2016). PATRIC annotation included two types of protein families (Davis et al., 2016), and the investigated genome had 6,759 proteins belonging to the genus-specific protein families (PLFams) for, and 6,759 proteins -belonging the cross-genus protein families (PGFams).

Table 6. Protein Features

Hypothetical proteins	1,508
Proteins with functional assignments	5,335
Proteins with EC number assignments	1,349
Proteins with GO assignments	1,148
Proteins with Pathway assignments	1,002
Proteins with PATRIC genus-specific family (PLfam)	6,759
Proteins with PATRIC cross-genus family (PGfam)	6,759

A circular graphical display of the distribution of the genome annotations was provided (Figure 1). The figure includes, from outer to inner rings, the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to know virulence factors, GC content and GC skew. The colors of the CDS on the forward and reverse strand indicate the subsystem that these genes belong to (see Subsystems below).

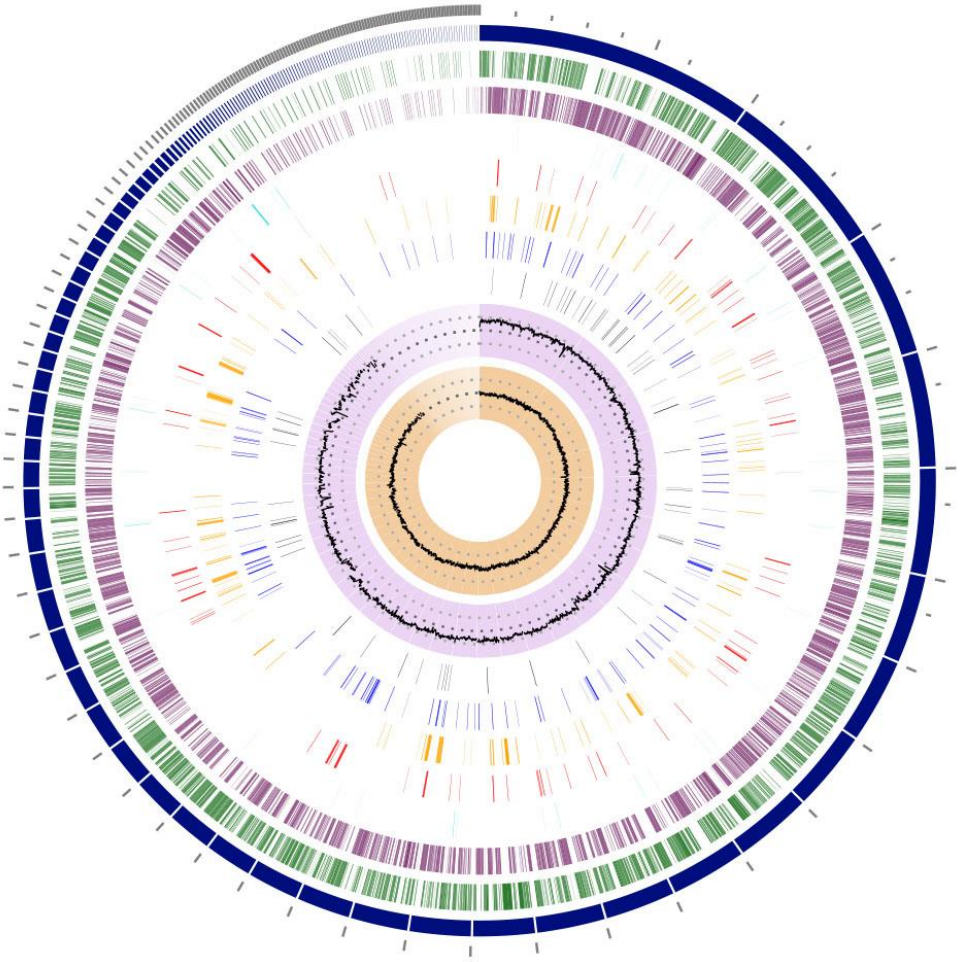

Figure 1. Graphical circular map of the *P. aeruginosa* UC4003 genome.

4.3.1.1 Subsystem Analysis

A subsystem is a set of proteins that together implement a specific biological process or structural complex (Overbeek et al., 2005) and PATRIC annotation included an analysis of the subsystems unique to each genome. An overview of the subsystems for this genome is expressed in Figure 2.

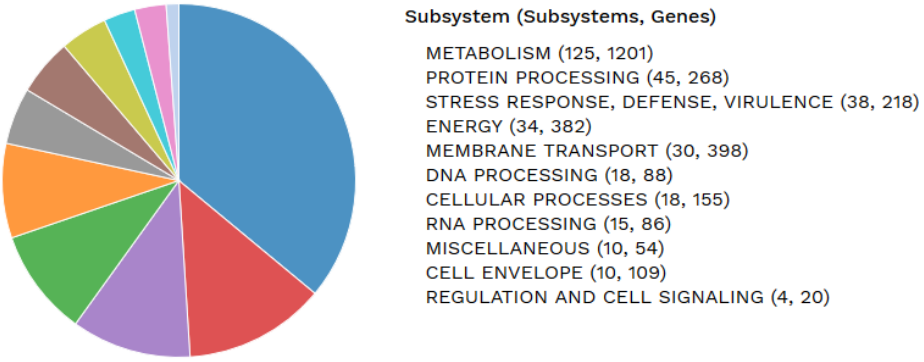

Figure 2. Subsystem overview of *P. aeruginosa* UC4003

4.3.1.2 Specialty Genes

Many of the genes annotated have homology to known transporters (Saier et al., 2016), virulence factors (Chen et al., 2016; Mao et al., 2015), drug targets (Law et al., 2014; Zhu et al., 2010), and antibiotic resistance genes (McArthur et al., 2013). The number of genes and the specific source database where homology was found, is reported in Table 7.

Table 7. Specialty Genes

	Source	Genes
	Victors	1
Antibiotic Resistance	CARD	54
Antibiotic Resistance	NDARO	5
Antibiotic Resistance	PATRIC	99
Drug Target	DrugBank	68
Drug Target	TTD	11
Transporter	TCDB	196
Virulence Factor	PATRIC_VF	1
Virulence Factor	VFDB	224
Virulence Factor	Victors	94

4.3.1.3 Oxygenase overview

Due to the ability of utilize alkanes, the pattern on oxygenases, monooxygenases and dioxygenases, was investigated. The 43 oxygenases identified during the annotation are reported in Table 8.

Table 8. Annotated oxygenase

Family ID	Description
PLF_286_00001167	Homogentisate 1,2-dioxygenase (EC 1.13.11.5)
PLF_286_00001284	Catechol 1,2-dioxygenase (EC 1.13.11.1)
PLF_286_00001330	Protocatechuate 3,4-dioxygenase beta chain (EC 1.13.11.3)
PLF_286_00001353	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase (EC 1.13.11.54)
PLF_286_00001540	Protocatechuate 3,4-dioxygenase alpha chain (EC 1.13.11.3)
PLF_286_00001700	Cysteine dioxygenase (EC 1.13.11.20)
PLF_286_00001906	Tryptophan 2,3-dioxygenase (EC 1.13.11.11)
PLF_286_00002184	4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27)
PLF_286_00002506	3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15)
PLF_286_00002775	Arachidonate 15-lipoxygenase precursor (EC 1.13.11.33)
PLF_286_00002808	Gentisate 1,2-dioxygenase (EC 1.13.11.4)
PLF_286_00003313	L-ornithine 5-monooxygenase (EC 1.13.12.-), PvdA of pyoverdin biosynthesis @ Siderophore biosynthesis protein, monooxygenase
PLF_286_00006109	4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27)
PLF_286_00098561	Nitronate monooxygenase (EC 1.13.12.16)
PLF_286_00238700	4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27)
PLF_286_00000072	Fatty acid desaturase (EC 1.14.19.1)
PLF_286_00001020	Phenylalanine-4-hydroxylase (EC 1.14.16.1)
PLF_286_00001041	P-hydroxybenzoate hydroxylase (EC 1.14.13.2)
PLF_286_00001322	Alpha-ketoglutarate-dependent taurine dioxygenase (EC 1.14.11.17)
PLF_286_00001360	FMNH2-dependent alkanesulfonate monooxygenase (EC 1.14.14.5)
PLF_286_00001485	FMNH2-dependent alkanesulfonate monooxygenase (EC 1.14.14.5)
PLF_286_00001511	Benzoate 1,2-dioxygenase alpha subunit (EC 1.14.12.10)
PLF_286_00001521	Benzoate 1,2-dioxygenase beta subunit (EC 1.14.12.10)
PLF_286_00001592	Benzoate 1,2-dioxygenase alpha subunit (EC 1.14.12.10)
PLF_286_00002278	DOPA 4,5-dioxygenase (EC 1.14.99.-)
PLF_286_00002361	Cyclohexanone monooxygenase (EC 1.14.13.22)
PLF_286_00002462	Alkane-1 monooxygenase (EC 1.14.15.3)
PLF_286_00002510	Cyclohexanone monooxygenase (EC 1.14.13.22)
PLF_286_00002529	4-hydroxyphenylacetate 3-monooxygenase (EC 1.14.14.9)
PLF_286_00002777	Flavodoxin reductases (ferredoxin-NADPH reductases) family 1
PLF_286_00002819	Linoleoyl-CoA desaturase (EC 1.14.19.3)
PLF_286_00003917	Vanillate O-demethylase oxygenase subunit (EC 1.14.13.82)
PLF_286_00004288	Alkane-1 monooxygenase (EC 1.14.15.3)

PLF_286_00004498	Aliphatic sulfate esters (C4-C12 chain lengths) dioxygenase (EC 1.14.11.-)
PLF_286_00005160	4-hydroxyphenylacetate 3-monooxygenase (EC 1.14.14.9)
PLF_286_00005161	Alpha-ketoglutarate-dependent dioxygenase AlkB (EC 1.14.11.33)
PLF_286_00005507	Gamma-butyrobetaine dioxygenase (EC 1.14.11.1)
PLF_286_00006169	Vanillate O-demethylase oxygenase subunit (EC 1.14.13.82)
PLF_286_00007581	Flavodoxin reductases (ferredoxin-NADPH reductases) family 1
PLF_286_00007664	Vanillate O-demethylase oxygenase subunit (EC 1.14.13.82)
PLF_286_00040648	Flavohemoglobin / Nitric oxide dioxygenase (EC 1.14.12.17)
PLF_286_00244601	Alpha-ketoglutarate-dependent taurine dioxygenase (EC 1.14.11.17)
PLF_286_00268667	Flavodoxin reductases (ferredoxin-NADPH reductases) family 1

Moreover, the analysis of the whole genome sequence revealed that *P. aeruginosa* UC4003 harbours the alkane hydroxylase complex, constituted from an integral membrane monooxygenase, 2 rubredoxins and a rubredoxin reductase (Van Beilen et al., 2003). Two alkane monooxygenases, AlkB1 and AlkB2 were found. The *alkB*1 gene was constituted by 1,149 bp, and the translation product was 382 peptides. *alkB*2, a gene with 1,134 bp, gave a translation product of 377 peptides. The monooxygenases AlkB drive the first reaction of *n*-alkane degradation. The *n*-alkane degradation starts by oxidation of a terminal methyl group, to carry out a primary alcohol. Primary alcohol is oxidized to an aldehyde and lastly converted into a fatty acid that was processed by β-oxidation, as summarized in Figure 3 (Tribelli et al., 2018).

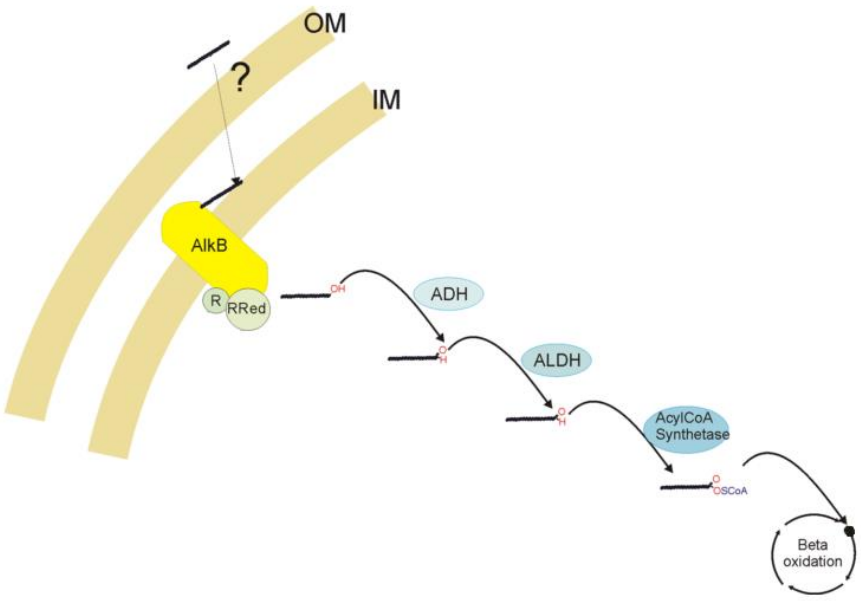

Figure 3. Alkane degradation pathway. (adapted from Tribelli et al., 2018).

A comparative analysis of *alkB*1 and *alkB*2 gene of *P. aeruginosa* UC4003 showed an identity of 72,5%. AlkB1 and AlkB2 peptides share 67,4% identity. Comparison gene analysis to *P. aeruginosa* PAO1 (taxid: 208964) revealed that the identities are 1146/1149 (99%) for *alkB*1, and for *alkB*2 identities are 1131/1134 (99%).

4.3.2 Growth kinetics

P. aeruginosa UC4003 and its ability to grow in SM and polyethylene as only carbon and energy source was investigated. Growth experiments showed that *P. aeruginosa* UC4003 can grow considerably on PE4,000, with a growth rate of 0,062 h^{-1} (doubling time, 11,1 h). Figure 4 reports a kinetic growth curve.

The research was conducted in the exponential phase, to examine the mechanisms involved in the first step of polyethylene assimilation.

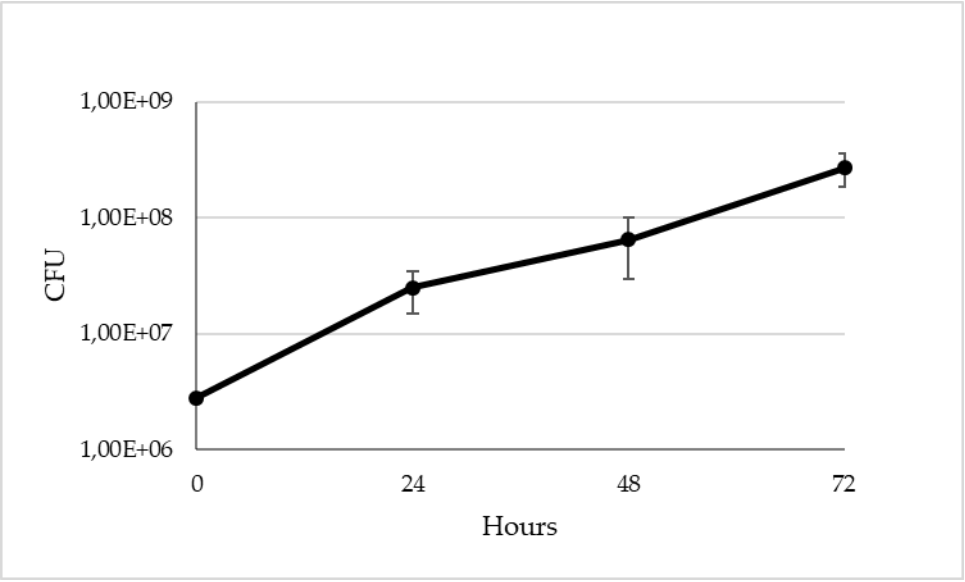

Figure 4. Grow kinetics of P. aeruginosa UC4003 in presence of PE4,000 as only carbon source.

4.3.3 Polyethylene analysis

The morphological changes of PE4,000 due to the biological treatment were evaluated as the change in percentage of crystallinity (Volke-Sepúlveda et al., 2002). Significant changes in PE 4,000 crystallinity by *P. aeruginosa* UC4003 exposure were observed after 72h, as showed in Figure 5. The decrease in PE crystallinity can be explained by the introduction of defects in the PE structure after exposure to bacteria. This can be ascribed to oxidation of PE chains or to the degradation of the shorter chains present in the PE matrix.

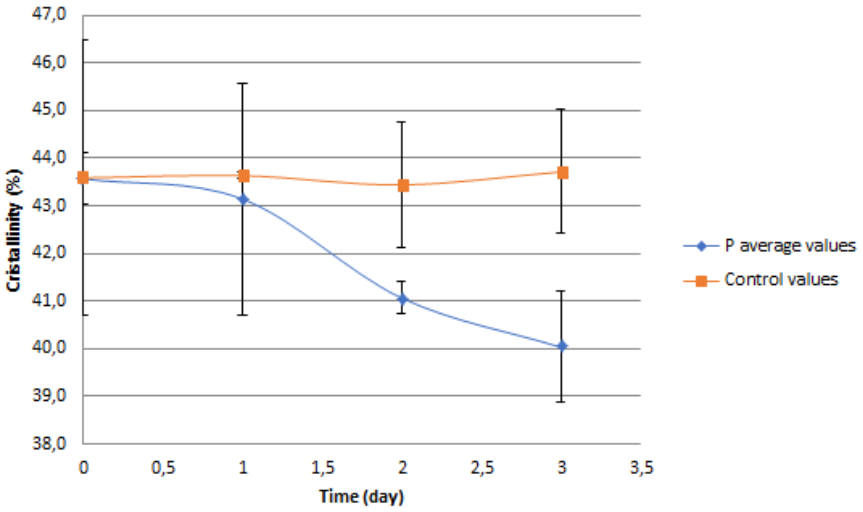

Figure 5. Crystallinity of PE4,000 samples incubated for 72 hours with *P. aeruginosa* UC 4003.

PE, after bacterial incubation (72 hours), was also analysed by Fourier transform infrared (FTIR) to quantify the content of carbonyl, double bond, and other functional groups during the PE4,000 degradation. Despite FTIR analysis, the biological treatment of PE 4,000 with *P. aeruginosa* UC4003 did not have a significant effect on the typical polyethylene spectrum (Figure 6). This is consistent with the hypothesis that the microbial metabolism utilized the shorter chains present in the PE matrix.

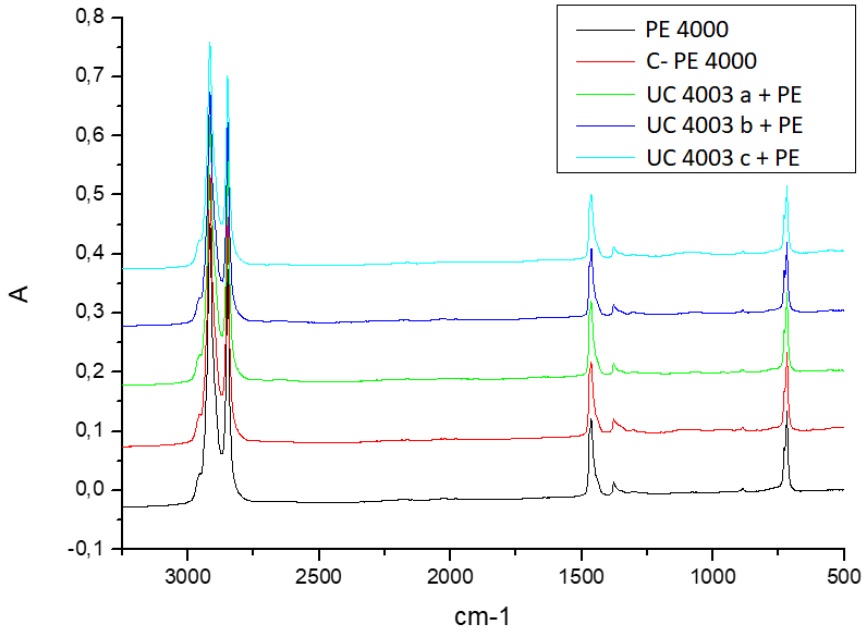

Figure 6. FTIR spectra of the PE 4000 exposed to *P. aeruginosa* UC 4003

4.3.4 Protein identification

To determine the possible proteins involved in polyethylene degradation, extracellular proteins were extracted. Oriole-stained one-dimensional gels of extracellular proteins identified distinctive band differences between the two conditions (Figure. 7).

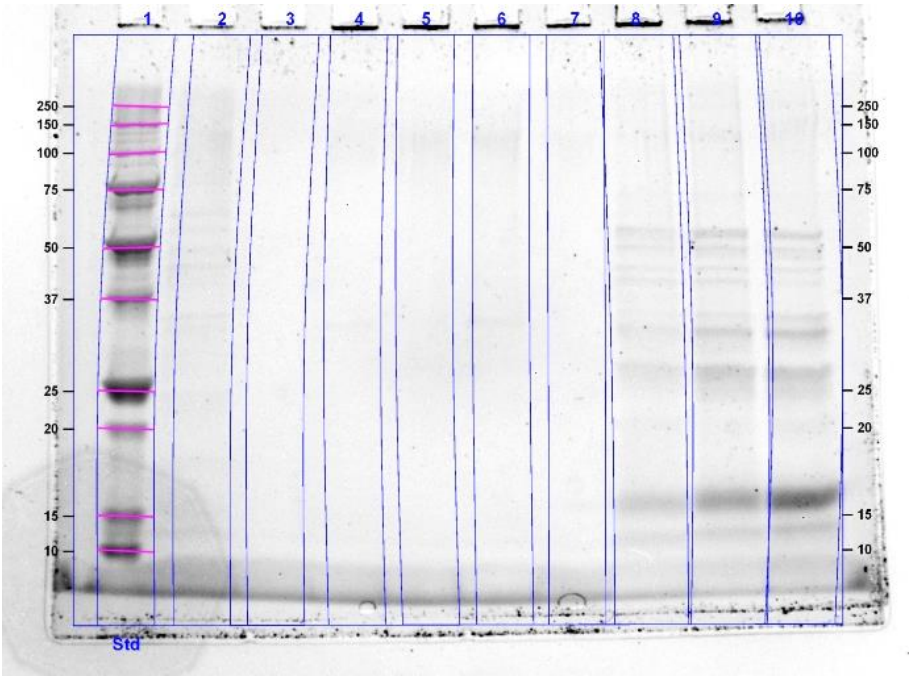

Figure 7. SDS-PAGE extracellular protein. Lane1: marker; lanes 2-6: UC4003 + 2% glucose (0, 16, 20, 24, 48h); lanes 7-10: UC4003 + PE4,000 (0, 24, 48, 72h)

Unique protein bands were found in the extracellular extract of polyethylene condition at approximately 54, 49, 44, 40, 34, 32, 27, 25, and 16 kDa (Figure 6). The major band that was displayed was a 16 kDa protein. Interestingly, the intensity of the 16 kDa was increased during the experimental time. After 24 hours, it was possible to observe a lower expression of this protein. The intensity of 16 kDa band was increased at 48 hours, and then reached its maximum expression at 72 hours. This molecular mass corresponds to a Protein activator or Protein-like activator for *n*-alkane oxidation (PA) (Hisatsuka et al., 1972).

4.3.5 Protein identification by MS/MS

With the aim of identifying the secret proteins involved in the PE metabolism, the secretoma of *P. aeruginosa* UC4003 was investigated using the Nano LC/QTOF proteomic mass-spectrometry. This approach led to the identification of 1691 single peptides. The most abundant 154 are reported in Table S1 (supplementary material).

Although the protein identification was carried out on extracellular proteins, the results showed a significant number of cytosolic proteins. This is probably due to the cell lysis in the limiting conditions of a media composed by salt and having PE as unique carbon compound. The most abundant protein found, was an extracellular protein defined as protein activator. This protein has a molecular mass of 16 kDa, in accordance with what was observed in the SDS-PAGE.

The protein, named Protein activator or Protein-like activator for *n*-alkane oxidation (PA), a was encoded by the *pra* gene, that was 489 bp (Hisatsuka et al., 1972) (Hardegger et al., 1994).

PA was described as a bioemulsifying protein. The role of PA as surface-active compound increased the local pseudosolubility of hydrocarbons and in conjunction with surfactants improved mass transfer to biodegrading bacteria (Holden et al., 2002). Several studies described PA as a protein that was secreted in presence of *n*-hexadecane (Holden et al., 2002). However, before this study, no evidence of expression of PA was certified in presence of polyethylene.

The *pra* gene showed a conserved sequence in *P. aeruginosa* strains, as reported in the phylogenetic tree (Figure 8). The PA of *P. aeruginosa* UC4003 presents a nucleotide sequence almost identical to the PA from a group of *Pseudomonas aeruginosa* strains that clusters separately. Four of the strains composing this cluster were isolated from clinical samples and one from rice.

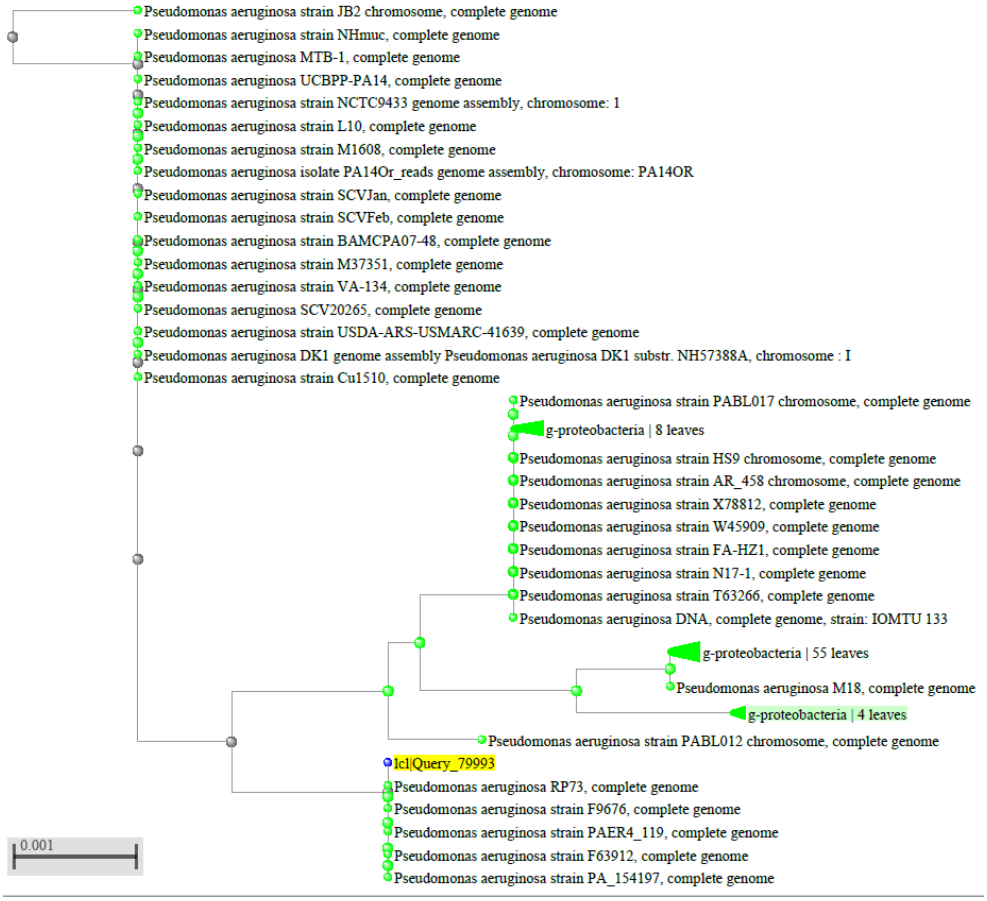

Figure 8. Phylogenetic tree of *pra* gene.

4.3.6 Expression studies

The expression of *pra* gene in presence of PE4,000 was studied using reverse transcriptase PCR. In presence of polyethylene, the gene was highly expressed if compared with cells grown in media with glucose, as shown in Figure 9. This evidence confirmed the results obtained by both SDS-PAGE and MS/MS.

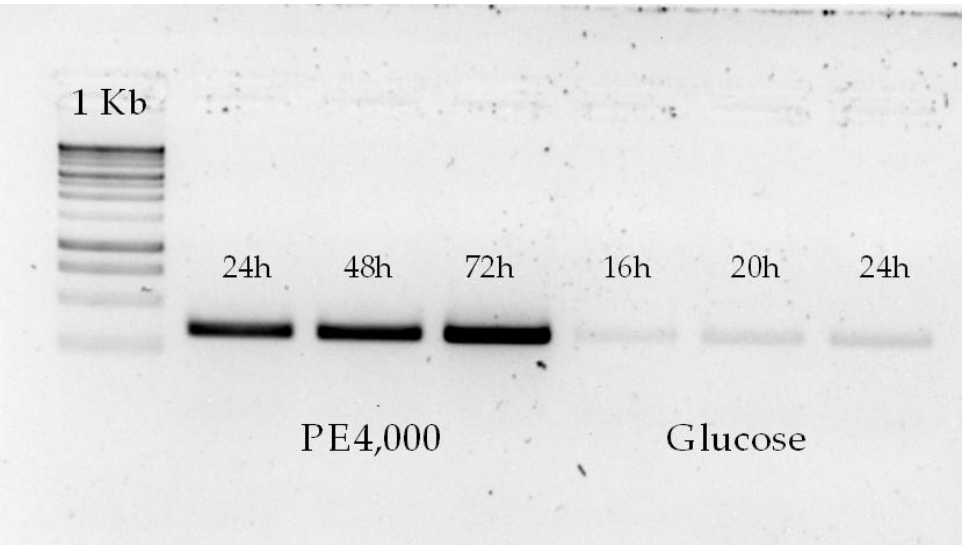

Figure 9. Comparison expression of *pra* gene in presence of PE4,000 and glucose 2%.

Subsequently, the expression of alkane monooxygenase, a key gene protein in alkane degradation, was investigated in presence of PE4,000. Reverse transcriptase PCR was carried out on a RNA from PE4,000 sample (72 hours) and glucose (24 hours), Figure 10.

AlkB1 showed a major activity in presence of glucose when compared to PE4,000. Differently, AlkB2 was found to be more expressed in presence of

polyethylene than in glucose. Such a difference of expression led to a new consideration of the activity and the structure of AlkB2. Rojo, assumed that the single mutation of amino acid in AlkB enzyme could modify the structure of the transmembrane enzyme allowed an oxidation of longer alkane (Rojo, 2005).

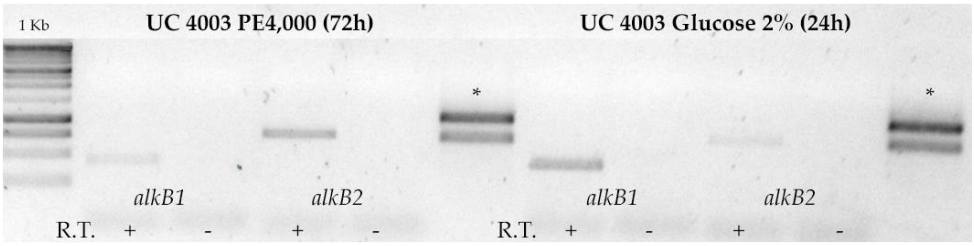

Figure 10. Comparison expression, of alkb1 and alkB2, in presence of PE4,000 and glucose 2%. * RNA was extracted.

4.3.7 Overexpression of *pra*, *alkB1* and *alkB2* in *P. aeruginosa* UC4003

The genes *pra*, *alkB1* and *alkB2* were cloned on a multicopy plasmid and reintroduced in *P. aeruginosa* UC4003 (wild type), to assess their role in PE metabolism. The coding regions of Gene Of Interesting (GOI), *pra*, *alkB1* and *alkB2*, were amplified using primers that contained a single deoxyuridine near the end 5'; the same procedure was carried out for the pSEVA628 plasmid.

The PCR products were mixed with a pre-digested USER-compatible plasmid and treated with the deoxyuridine-excising USER™ enzyme mix.

Ablation of the deoxyuridines from the PCR products resulted in the generation of 7-9 nt-long overhangs.

The 7-nt overhangs generated with the primers GOI_rv were designed to complement the 7-nt overhang generated with primer pSEVA628_fw. A similar design was made for the 9-nt overhangs generated with primers GOI_fw and pSEVA628_rw.

The incubation with USER enzyme carried out a stable circular hybridization product, pSEVA628::GOI, that was ready for transformation into *E. coli* DH5α.

The sequencing results of pSEVA628::GOI (not shown) reported a correct insertion. The pSEVA628::GOI was inserted into *P. aeruginosa* UC4003 by electroporation, and to confirm the insertion a colony PCR was conducted. The results reported in Figure 11 confirm the insertion of the plasmid into the cell.

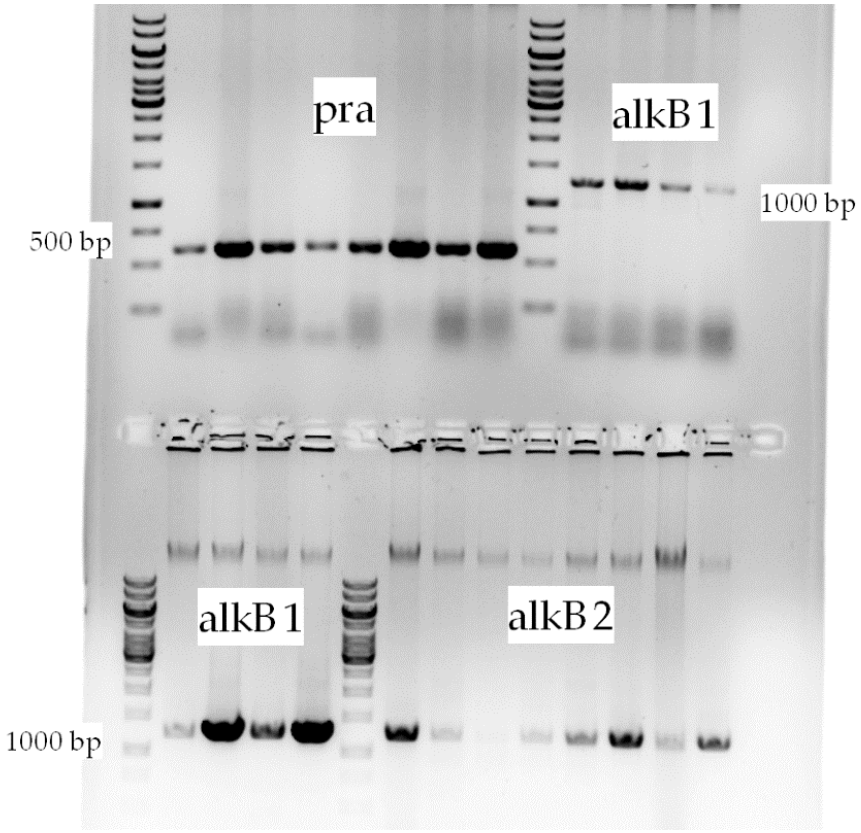

Figure 11. Validation of pSEVA628::GOI presence in *P.aeruginosa* UC4003

A schematic representation of plasmid construction is depicted in Figure 12

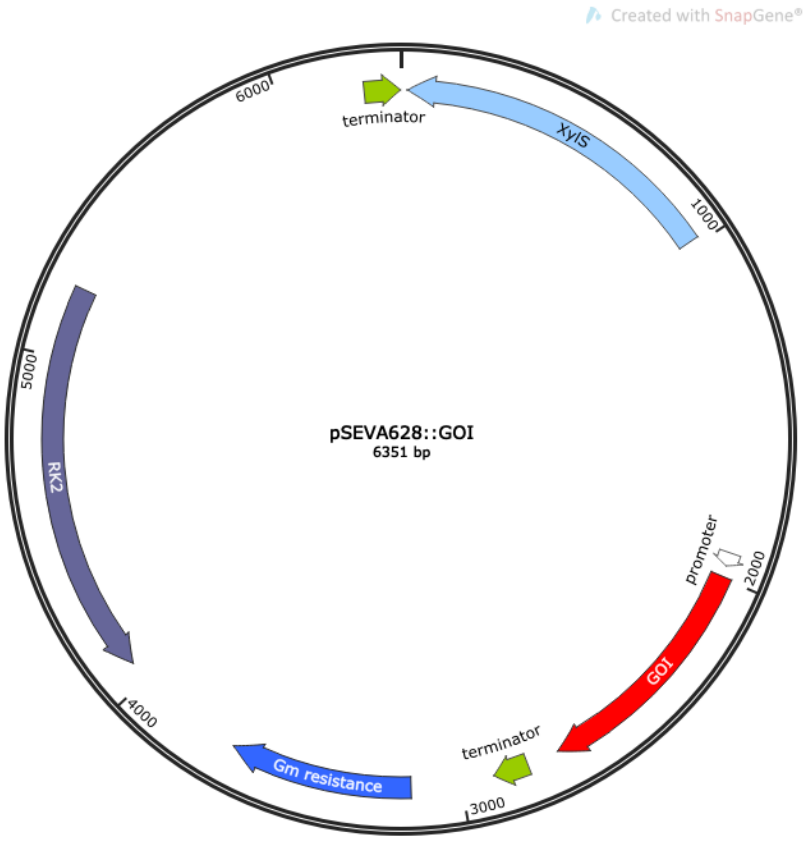

Figure 12. Schematic representation of PSEVA construct plasmid. GOI: Gene Of Interesting. T 0 and T 1: Terminator. Gm^R: Gentamycin resistance gene.RK2: origin of replication. XylS: Regulator. Pm: Promoter.

The growth kinetics of *P. aeruginosa* UC4003 strains harboring the plasmids overexpressing *pra* (*P. aeruginosa*::*pra*), *alkB1* (*P. aeruginosa*::*alkB1*) and *alkB2* (*P. aeruginosa*::*alkB2*), were compared against the wild type *P. aeruginosa*

UC4003 in presence of polyethylene as only carbon source over 72h of incubation in aerobic conditions, as shown in Figure 13.

The wild type showed a growth rate of 0.212 h^{-1} (doubling time, 3.27 h), while the derivatives displayed respectively, *P. aeruginosa*::*pra* growth rate 0.239 h^{-1} (doubling time, 2.9 h), *P. aeruginosa*::*alkB1* growth rate 0.198 h^{-1} (doubling time, 3.5 h) and *P. aeruginosa*::*alkB2* growth rate 0.194 h^{-1} (doubling time, 3.57 h).

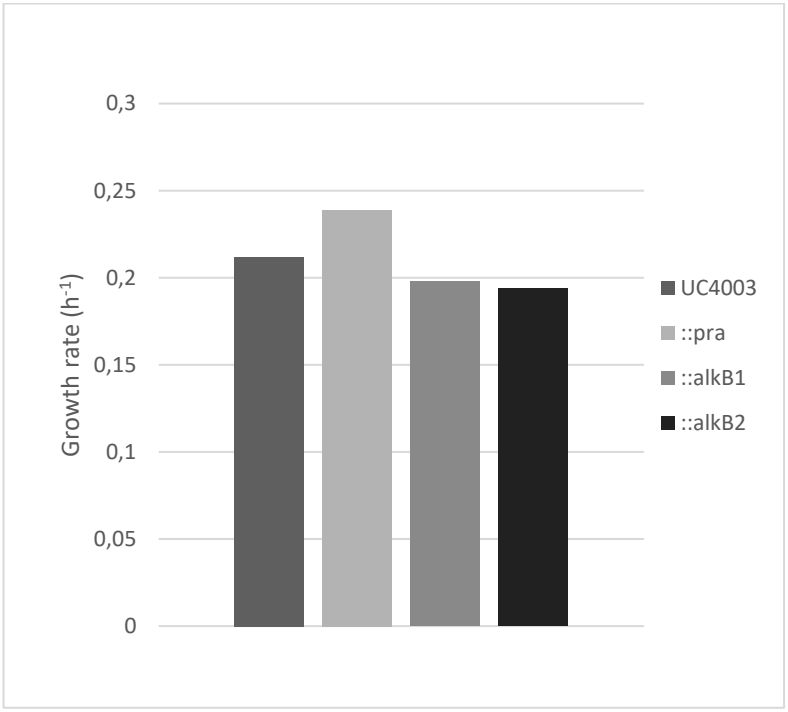

Figure 13. Growth rate comparison from wild type and overexpression strains.

The highest growth rate of *P. aeruginosa*::*pra*, supports the hypothesis that this small and significantly hydrophobic protein plays a key role in the release of alkane chains from the PE matrix.

4.4 Conclusion

The strain *P. aeruginosa* UC4003 studied in this thesis proved a relevant and rare capacity of using PE as nutrient, when grown in minimal salt medium were the only carbon source was constituted by this plastic. In a pretty short time period (72 h), this strain is able to reach the stationary phase, with an increase of 2 logarithmic orders. The bacterial growth was associated to a modification of PE structure with a clear decrease of crystallinity in the treated plastic. According to these experimental data a hypothesis may be drawn: the defects in the PE structure after exposure to bacteria can be the result of oxidation of the alkane chains or could be due to the degradation of the shorter chains present in the PE matrix. Since the FTIR analysis did not show a significant increase of functional groups (e.g: carbonyl, double bond) in the PE, the preferred hypothesis is that *P. aeruginosa* UC4003 is able to metabolise the shorter alkane chains embedded in the PE matrix.

The analysis of proteome supports this hypothesis, the most present polypeptide in the supernatant is PA, a 16 KDa highly hydrophobic protein that was demonstrated to be involved in the alkane utilisation, having a role as biosurfactant. This is the consequence of the high expression of the *pra* gene, coding for PA protein, in cells grown on PE. It can be hypothesised that PA protein with its surfactant activities swells the PE matrix and allows the

release of shorter alkane chains from the PE. The alkane can subsequently be metabolised by *P. aeruginosa* UC4003, using the already described pathway of *n*-alkane degradation in *Pseudomonas.* The observation that AlkB2 enzyme is overexpressed in presence of PE, supports this hypothesis.

The proteomic analysis revealed that a relevant number of other proteins were present in the supernatant and none of those showed homologies to known enzymes that seem to have a role in PE metabolism. However, almost one third of them are annotated as unknown function or hypothetical protein. Additional studies are necessary to gain detailed and in-depth insights of the role of other secreted proteins in the *P. aeruginosa* UC4003 polyethylene metabolism.

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Supplementary Material

Table. S1. Peptide sequence tag			
Spectrum intensity	Sequence	Protein MW (Da)	Protein Name (in *P. aeruginosa* UC4003)
1,97E+09	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
1,30E+09	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
9,26E+08	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
6,76E+08	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
4,50E+08	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
3,46E+08	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
2,55E+08	(K)VDGVGFK(I)	16481,5	No hits found
2,27E+08	(R)ITAVLSPR(I)	26565,3	No hits found
2,15E+08	(R)ITAVLSPR(I)	26565,3	No hits found
2,05E+08	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
1,95E+08	(K)LTVSSTTAGK(V)	16481,5	Protein activator
1,90E+08	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
1,83E+08	(K)SLSPAQTQTL(-)	5673,7	No hits found
1,00E+08	(R)AYDNATAAVAK(M)	48611,4	Lysyl endopeptidase
1,00E+08	(K)INSLSVKPTPAFVVNP(-)	16481,5	Lysyl endopeptidase
8,62E+07	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator

7,90E+07	(R)AYDNATAAVAK(M)	48611,4	Lysyl endopeptidase
7,86E+07	(K)LTVSSTTAGK(V)	16481,5	Protein activator
6,77E+07	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
6,40E+07	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
6,20E+07	(K)SLSPAQTQTL(-)	5673,7	No hits found
5,87E+07	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
5,77E+07	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
5,75E+07	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
5,58E+07	(R)ITAVLSPR(I)	26565,3	No hits found
5,31E+07	(R)YSEAEVARITDSAGALALAFR(L)	70462	No hits found
5,30E+07	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
4,73E+07	(R)AYDNATAAVAK(M)	48611,4	Lysyl endopeptidase
4,61E+07	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
4,46E+07	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
4,46E+07	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
4,40E+07	(R)ITAVLSPR(I)	26565,3	No hits found

4,29E+07	(K)SLSPAQTQTL(-)	5673,7	No hits found
3,91E+07	(R)DNLAKRPADAAPAPAVK(A)	43196,5	No hits found
3,42E+07	(R)YFTDSVR(N)	37867,1	No hits found
3,14E+07	(R)VNAVGYGESRPVADNATEAGR(A)	37587,6	Major porin and structural outer membrane porin OprF
3,14E+07	(R)VNAVGYGESRPVADNATEAGR(A)	37587,6	Major porin and structural outer membrane porin OprF
3,14E+07	(R)RATVLSPR(I)	79337,4	No hits found
3,00E+07	(K)LTVSSTTAGK(V)	16481,5	Protein activator
2,96E+07	(R)DNLAKRPADAAPAPAVK(A)	43196,5	No hits found
2,68E+07	(R)RATVLSPR(I)	79337,4	No hits found
2,64E+07	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
2,60E+07	(R)RATVLSPR(I)	79337,4	No hits found
2,51E+07	(K)SIGTVGIR(N)	13758,8	No hits found
2,46E+07	(R)DVLVNEYGVEGGR(V)	37867,1	Major porin and structural outer membrane porin OprF
2,16E+07	(R)RATVLSPR(I)	79337,4	No hits found
2,05E+07	(K)ASLDGQYGLEK(R)	37867,1	Major porin and structural outer

			membrane porin OprF
1,83E+07	(K)ASLDGQYGLEKR(D)	37867,1	Major porin and structural outer membrane porin OprF
1,80E+07	(R)DVLVNEYGVEGGR(V)	37867,1	Major porin and structural outer membrane porin OprF
1,80E+07	(R)RVEAEVEAEAK(-)	37867,1	Major porin and structural outer membrane porin OprF
1,78E+07	(K)ASLDGQYGLEKR(D)	37867,1	Major porin and structural outer membrane porin OprF
1,76E+07	(R)VTAVGPEVK(R)	43017,9	No hits found
1,50E+07	(R)NATLQWGK(F)	13758,8	hypothetical protein
1,48E+07	(K)LTVSSTTAGK(V)	16481,5	Protein activator
1,47E+07	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
1,40E+07	(K)SLSPAQTQTL(-)	5673,7	No hits found
1,36E+07	(R)RATVLSPR(I)	79337,4	No hits found
1,35E+07	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
1,35E+07	(K)LSHDFGSHHSGASSRGR(A)	108085,8	No hits found

1,35E+07	(R)VGPVLSPR(Y)	32335,4	No hits found
1,32E+07	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
1,31E+07	(R)DNLAKRPADAAPAPAVK(A)	43196,5	No hits found
1,29E+07	(K)LTVSSTTAGK(V)	16481,5	Protein activator
1,28E+07	(R)VDVQHAASQHRKPANETSTDGYTTLGASVGYR(F)	74471,4	Outer membrane receptor proteins, mostly Fe transport
1,18E+07	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
1,13E+07	(R)LIVEYPLQVL(-)	49818,2	Outer membrane porin, OprD family
1,13E+07	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
1,13E+07	(R)YSEAEVARITDSAGALALAFR(L)	70462	No hits found
1,12E+07	(R)YSEAEVARITDSAGALALAFR(L)	70462	No hits found
1,11E+07	(K)LTVSSTTAGK(V)	16481,5	Protein activator
1,09E+07	(K)SLSPAQTQTL(-)	5673,7	No hits found
1,09E+07	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
1,08E+07	(K)SGATNTWSFTPASADYAGITSGGNATGTDIGEVTLTLGR(F)	14692,8	hypothetical protein
1,05E+07	(K)LTVSSTTAGK(V)	16481,5	Protein activator

1,01E+07	(K)VVDDEIAAAGQK(A)	57852,5	Aminopeptidase Y (Arg, Lys, Leu preference)
1,01E+07	(K)LSHDFGSHHSGASSRGR(A)	108085,8	No hits found
9,97E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
9,87E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
9,54E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
9,23E+06	(K)SGATNTWSFTPASADYAGITSGGNATGTDIGEVTLTLGR(F)	14692,8	hypothetical protein
9,18E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
9,15E+06	(R)VNAVGYGESRPVADNATEAGR(A)	37587,6	Major porin and structural outer membrane porin OprF
8,99E+06	(K)SLSPAQTQTL(-)	5673,7	No hits found
8,93E+06	(R)VGDVFPETPVIQYGNSR(L)	49818,2	Outer membrane porin, OprD family
8,84E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
8,67E+06	(R)IPDGDRYFASLGAGYR(F)	49821,8	Long-chain fatty acid transport protein

8,64E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
8,56E+06	(R)GGFAYGPHQVLLSYQR(N)	49818,2	Outer membrane porin, OprD family
8,53E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
8,49E+06	(R)GGFAYGPHQVLLSYQR(N)	49818,2	Outer membrane porin, OprD family
8,28E+06	(R)VAVESLPR(Y)	57007	No hits found
8,05E+06	(K)SPLLVSTPLGLPR(C)	57852,5	Aminopeptidase Y (Arg, Lys, Leu preference)
8,00E+06	(K)DDAAGLQIANR(L)	40065,6	Flagellin protein FlaC
7,76E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
7,74E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	
7,65E+06	(R)GFTLVNDSLAK(G)	49818,2	Outer membrane porin, OprD family
7,62E+06	(K)SHGNTIVSIPYTYR(N)	49821,8	Long-chain fatty acid transport protein
7,61E+06	(K)VSVQPFPFTAYYPK(G)	57852,5	Aminopeptidase Y (Arg, Lys, Leu preference)
7,39E+06	(R)ISNTTTFGGR(K)	40065,6	Flagellin protein FlaC

7,34E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
7,34E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
7,28E+06	(K)LTVSSTTAGKVDGVGFK(I)	16481,5	Protein activator
7,24E+06	(R)YSEAEVARITDSAGALALAFR(L)	70462	No hits found
7,24E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
6,98E+06	(R)RATVLSPR(I)	79337,4	No hits found
6,94E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
6,56E+06	(K)SLSPAQTQTL(-)	5673,7	No hits found
6,42E+06	(K)LTVSSTTAGK(V)	16481,5	Protein activator
6,38E+06	(K)SGATNTWSFTPASADYAGITSGGNATGTDIGEVTLTLGR(F)	14692,8	hypothetical protein
6,19E+06	(K)VKENSYADIK(N)	37867,1	Major porin and structural outer membrane porin OprF
6,07E+06	(K)SGATNTWSFTPASADYAGITSGGNATGTDIGEVTLTLGR(F)	14692,8	hypothetical protein
6,02E+06	(R)TAVPAGYATLPGSPLEAAAAVAAAAHAPGSPPSASHLAR(A)	137481,9	Protein ImpG/VasA
5,92E+06	(R)NATLQWGK(F)	13758,8	hypothetical protein
5,80E+06	(K)YGVPGLTYR(V)	46848,9	Outer membrane porin, OprD family

5,72E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
5,69E+06	(K)LTVSSTTAGK(V)	16481,5	Protein activator
5,65E+06	(R)GGLYGGPSYCGAPTSQR(N)	48611,4	Lysyl endopeptidase
5,57E+06	(K)DDAAGLQIANR(L)	40065,6	Flagellin protein FlaC
5,51E+06	(R)GGLYGGPSYCGAPTSQR(N)	48611,4	Lysyl endopeptidase
5,46E+06	(K)ASLDGQYGLEKR(D)	37867,1	Major porin and structural outer membrane porin OprF
5,46E+06	(K)ASLDGQYGLEKR(D)	37867,1	Major porin and structural outer membrane porin OprF
5,25E+06	(R)SGAAGIDFFK(Q)	46848,9	Outer membrane porin, OprD family
5,05E+06	(R)VDVQHAASQHRKPANETSTDGYTTLGASVGYR(F)	74471,4	Outer membrane receptor proteins, mostly Fe transport
5,03E+06	(R)AGVAYDQTPTHNATR(D)	49821,8	Long-chain fatty acid transport protein
4,94E+06	(R)AAATPGYQASVDYVK(Q)	57852,5	Aminopeptidase Y (Arg, Lys, Leu preference)

4,79E+06	(R)SDSFESDLDEVR(L)	49818,2	Outer membrane porin, OprD family
4,79E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
4,79E+06	(R)VPQAIANAGLGSTVLMRYEVEPALGGRPLPSQER(A)	223492,9	DNA recombination and repair protein RecO
4,76E+06	(R)RVEAEVEAEAK(-)	37867,1	Major porin and structural outer membrane porin OprF
4,70E+06	(R)VDVQHAASQHRKPANETSTDGYTTLGASVGYR(F)	74471,4	Outer membrane receptor proteins, mostly Fe transport
4,69E+06	(R)VGDVFPETPVIQYGNSR(L)	49818,2	Outer membrane porin, OprD family
4,69E+06	(R)DVLVNEYGVEGGR(V)	37867,1	Major porin and structural outer membrane porin OprF
4,68E+06	(R)AYDNATAAVAK(M)	48611,4	Lysyl endopeptidase
4,64E+06	(K)DDAAGLQIANR(L)	40065,6	Flagellin protein FlaC
4,60E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
4,45E+06	(K)LTVSSTTAGK(V)	16481,5	Protein activator

4,39E+06	(R)ISNTTTFGGR(K)	40065,6	Flagellin protein FlaC
4,28E+06	(K)VDGVGFK(I)	16481,5	No hits found
4,16E+06	(R)SKEGFIEGSEVNLLLR(N)	49818,2	Outer membrane porin, OprD family
4,16E+06	(R)SKEGFIEGSEVNLLLR(N)	49818,2	Outer membrane porin, OprD family
3,88E+06	(R)YSEAEVARITDSAGALALAFR(L)	70462	No hits found
3,80E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
3,73E+06	(K)INSLSVKPTPAFVVNP(-)	16481,5	Protein activator
3,71E+06	(K)LDITLPESVDTSITHK(F)	45532,8	Long-chain fatty acid transport protein
3,67E+06	(K)LEDIASLNDGNR(A)	57852,5	Aminopeptidase Y (Arg, Lys, Leu preference)
3,58E+06	(K)VNGIAPALIQFNPDDSAEYR(A)	25636,6	FolM Alternative dihydrofolate reductase 1
3,53E+06	(R)LDSIPGQDVPGWLATTGSGRLLQVLADATPGSEVR(Q)	97960	Periplasmic nitrate reductase precursor
3,48E+06	(K)LTVSSTTAGKVDGVGFK(I)	16481,5	Protein activator
3,47E+06	(R)YFTQPYPAR(A)	13609,4	Endoribonuclease L-PSP
3,45E+06	(K)MGTFSWDDPWK(S)	13758,8	hypothetical protein
3,39E+06	(K)IAAAVNDANVGIGAFSDGDTISYVSK(A)	40065,6	Flagellin protein FlaC

3,39E+06	(K)QFTVNLSHPGNLPK(N)	16179,3	Azurin
3,38E+06	(R)SGAGGSVDLLPYDDQGRPQDDYSR(A)	47638,5	Outer membrane porin, OprD family
3,31E+06	(R)YFTQPYPAR(A)	13609,4	Endoribonuclease L-PSP

Chapter 5

5. General conclusions and future perspectives

This PhD thesis aimed to get a deeper insight in the microbial degradation of polyethylene.

The first part of the study identified a microbial community involved in polyethylene colonization. The data about plastic samples revealed a partial degradation due to microorganism's activity. Also, the rDNA approach gave us the possibility to study the community of non-culturable bacteria. Most interestingly, a possible correlation between the level of plastic degradation and the bacterial community structure was found. Also, the most degraded plastic hosting a community that resembled more the one of the soil.

In the second part of the study, the attention was focused on culturable microorganism involved in polyethylene degradation. The two-step enrichment was found to be the right approach to obtain several bacteria able to grow with polyethylene as only energy and carbon source. Furthermore, we assisted to a higher degradation of plastic samples; the number of functional groups was increased if compared to the beginning of the two step enrichments. At the end of this part of the work, 23 strains proved the ability to grow in minimal salt medium with polyethylene as only carbon source. Moreover, two strains presented the highest growth ratio, *P. aeruginosa* UC 4003 and *R. ruber* UC4004.

Finally, in the third part, we investigated the ability of *P. aeruginosa* UC4003 to grow on polyethylene. This strain proved to be a good model to explore the metabolic pathways involved in polyethylene degradation. During the research, *P. aeruginosa* UC4003, increased its capability to grow on polyethylene: At the very beginning of the project, *P.aeruginosa* needed 72 hours to reach the stationary phase, while in the last trials the strain revealed the ability to reach the stationary phase in only 24 hours. Thus, the time frame to achieve the stationary phase decreased during time research. The investigation of extracellular protein gave us the possibility to study the enzymes involved in the first step of biodegradation, highlighting the key role of *pra* gene, that was reinforced by overexpression approach.

Such outcomes are encouraging and could allow to more targeted researches to achieve a better understanding of long-chain alkane metabolic pathways. As a starting point for future researches, I would propose:

- To carry out a knockout of the gene *pra* in *P. aeruginosa* UC4003. This approach will hopefully confirm the leading role of *pra* in the first step of polyethylene colonization. Particularly, we have recently started a collaboration with the Novo Nordisk Foundation Center for Biosustainability, DTU, to shed light on this issue.

- To perform a knockout of the gene *alkB1* and *alkB2*, with a particular focus on *alkB2*. Albeit many of the latest studies have already confirmed their function in alkane degradation, it would be worthy

to get a deeper knowledge of their key role in PE degradation carried out by *P. aeruginosa* UC4003.

- To start additional studies for the purpose of gaining detailed insights on the role of the other secreted proteins identified in this thesis and resulting unknown or hypothetical.