Towards biorecycling of plastics: Isolation and characterization of *Pseudomonas capeferrum* TDA1, a bacterium capable to degrade polyurethane mono- and oligomers

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MSc. María José Cárdenas Espinosa

geboren am 24. Mai. 1986 in Quito, Ecuador

Dekan: Prof. Dr. Marc Schönwiesner

Gutachter: Prof. Dr. Lars M. Blank

Prof. Dr. Hauke Harms

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Maria José Cárdenas Espinosa

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Mismanagement of plastic waste has become a global threat, affecting all ecosystems. Polyurethane (PU), a synthetic polymer produced worldwide, reached 18.4 million metric tons (Mt) in 2019. Chemical and mechanical recycling has emerged as an alternative to process polyurethane waste; however, these methods require pre-treatments, high technology, pure feedstocks and high volumes of production to be cost-effective. With a view to the economic circularity, biological depolymerization is increasingly being considered as a sustainable option. This thesis focuses on the characterization a soil bacterium capable of degrading PU building blocks (2,4-diaminotoluene and polyurethane oligomer). The isolated strain utilizes the monomer and oligomer aforementioned as a carbon source. After 16S rRNA and whole genome sequencing, the strain was identified as *Pseudomonas capeferrum* TDA1. Using high-throughput technologies (RNA-sequencing), 712 differentially expressed genes (at least two-fold) were identified in bacterial cells grown on 2,4-TDA and 2,4-TDA + succinate compared to control (succinate). In the case of 2,4-TDA vs. succinate, 157 genes were upregulated over a four-fold. In addition, hydrolytic activity inside membranous structures called outer membrane vesicles (OMVs) was analyzed in bacterial cells grown on PU oligomer. OMVs yield on PU oligomer ($0.28 \pm 0.05\%$) revealed an increased activity compared to the control (succinate) ($0.09 \pm 0.01\%$), which can be considered as an adaptive mechanism of bacteria to stress conditions and propose a new PU degradation model based on OMVs as a supportive element. To sum up, this new approach shows a whole degradation scheme of PU building blocks by *Pseudomonas capeferrum* TDA1 using intra and extracellular enzymes.

Amor fati, hic et nunc

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Zusammenfassung

In den letzten 50 Jahren ist die Kunststoffindustrie exponentiell gewachsen und umfasst bis heute eine geschätzte Produktion von 8300 Millionen Tonnen. Trotz der großen Vielfalt der verfügbaren Polymere bestehen 99 % ausschließlich aus fossilen Brennstoffen, was ihre Abbaubarkeit nach dem Gebrauch beeinträchtigt. Die wichtigsten synthetischen Polymere, die heute verwendet werden, sind Polyethylen (PE), Polypropylen (PP), Polystyrol (PS), Polyvinylchlorid (PVC), Polyurethane (PU) und Polyethylenterephthalat (PET). Die derzeitigen Hauptentsorgungswege von Kunststoffabfällen sind hauptsächlich Deponierung, Verbrennung, mechanisches und chemisches Recycling. Obwohl diese Entsorgungsmethoden erheblich verbessert wurden, müssen noch einige Einschränkungen und Mängel überwunden werden.

Polyurethan (PU) ist ein synthetisches Polymer, welches als Rohstoff in verschiedenen Industrien verwendet wird. Im Jahr 2015 erreichte die weltweite Produktion von Polyurethan 27 Millionen Tonnen und ist damit der sechstmeistverwendete Kunststoff weltweit. Die Hauptbestandteile von Polyurethan sind Isocyanate, Polyole und Kettenverlängerer. Die zusätzliche Einbindung von aromatischen Ringstrukturen hat weitere Auswirkung auf die physikalischen und chemischen Eigenschaften des Polymers. PU gilt als sehr stabil, ist allerdings schwer zu recyceln, und bei unsachgemäßer Verbrennung entstehen giftige Gase, die für die menschliche Gesundheit schädlich sind. Darüber hinaus werden die meisten PU-Abfälle auf Mülldeponien entsorgt, was zu einem potenziellen Verlust für die Umwelt führt.

Bedauerlicherweise hat sich falsch entsorgtes Plastik in verschiedenen Lebensräumen ausgebreitet, darunter kalte Meeresgebiete und unbesiedelte Orte, wodurch Wildtiere und Ökosysteme bedroht werden. Um eine weitere Kontamination zu vermeiden, müssen Kunststoffabfälle recycelt werden, indem ihre funktionalen Eigenschaften wiederhergestellt, ein Mehrwert geschaffen und neue Anwendungsbereiche erkundet werden, die langfristig wirtschaftliche Vorteile bieten könnten.

Zusammenfassung

In den letzten 10 Jahren ist der Übergang von einer linearen Wirtschaft zu einer nachhaltigen, biobasierten Kreislaufwirtschaft von grundlegender Bedeutung geworden, um den durch fossile Brennstoffe verursachten Klimawandel und die globale Plastikverschmutzung zu bewältigen. Dieser Wandel beinhaltet industrielle und Grundlagenforschung, die sich stark auf Biotechnologie und Bioprozesse konzentriert. Bei diesem Übergang spielen Mikroorganismen aufgrund der großen Vielfalt an Enzymen und Stoffwechselwegen, die für die Entwicklung nachhaltiger Prozesse und Biomaterialien genutzt werden könnten, eine Schlüsselrolle.

In letzter Zeit wurden regelmäßig Mikroorganismen mit kunststoffabbauendem Potenzial in verschiedenen Umgebungen wie Abfallentsorgung, Deponien, Kunststoffraffinerien, offenen Mülldeponien etc. nachgewiesen. Selektiver Druck und die Entwicklung genetisch flexibler Mechanismen haben dazu beigetragen, anthropogene Verbindungen zu verstoffwechseln, was sich in mehreren enzymatischen Reaktionen niedergeschlagen hat, die für den effizienten Abbau einer Vielzahl widerspenstiger Substrate konzipiert sind und zu neuartigen Stoffwechselwegen führen.

Obwohl mehrere Bakteriengattungen für den Abbau von Umweltschadstoffen bekannt sind, gehören Pseudomonaden zu den meist zitierten Abbauern für aromatische Kohlenwasserstoffe und Kunststoffpolymere. Die Gattung Pseudomonas umfasst eine der komplexesten, vielfältigsten und ökologisch bedeutendsten Bakteriengruppen der Welt. Darüber hinaus wurden sie als wertvolle Plattform für biotechnologische Prozesse anerkannt.

Mitglieder dieser Gattung sind in großer Zahl in allen wichtigen natürlichen Lebensräumen (Land, Süßwasser und Ozean) zu finden und bilden enge Verbindungen mit Pflanzen und Tieren. Diese universelle Verbreitung deutet auf ein bemerkenswertes Maß an physiologischer und genetischer Anpassungsfähigkeit hin. Tatsächlich wurden Pseudomonas am häufigsten mit dem Abbau von PU in Verbindung gebracht.

Chemisch handelt es sich bei PU auf Polyesterbasis um halbkristalline Strukturen mit hydrolysierbaren Ester- und Urethanbindungen, die durch extrazelluläre Enzyme (Hydrolasen) aufgespalten werden, wodurch oligomere und monomere

Bausteine freigesetzt werden. Zum Beispiel werden beim PU-Abbau ständig Amine, Alkohole, Säuren, Aromaten und andere Rückstände wie EG (Ethylenglykol), 1,4-Butandiol (BDO), Adipinsäure (AA), 4′-Methylendianilin (MDA) und 2,4-Toluoldiamin (2,4-TDA) freigesetzt. MDA und 2,4-TDA gelten jedoch als Umweltschadstoffe, die eine große Gefahr für die Arten im aquatischen und terrestrischen Lebensraum arstellen. Darüber hinaus ist 2,4-TDA der Hauptrohstoff für die Herstellung von Toluoldiisocyanat (TDI), das bei der Herstellung von PU-Schäumen verwendet wird.

Diese Fragmentierung des Polymers wird als Depolymerisation bezeichnet und ist für die Stärkung von Recyclingprozessen, die Kunststoffabfälle als Ausgangsmaterial verwenden, von wesentlicher Bedeutung. Das breite Spektrum an Bausteinen könnte als Kohlenstoff- und Energiequelle für Mikroorganismen genutzt werden, die diese Verbindungen abbauen und/oder für die Herstellung höherwertiger Elemente verwenden. Letzteres gilt als vielversprechende Upcycling-Strategie zur Verringerung von Kunststoffabfällen aus fossilen Brennstoffen und zur Förderung neuer Abfallbewirtschaftungsstrategien.

Vorherige Studien haben gezeigt, dass extrazelluläre Enzyme für die Bildung von Biofilmen auf der Polymeroberfläche wesentlich sind und damit die Widerstandsfähigkeit und Haltbarkeit von Kunststoffen verringern. Dieser erste Schritt fördert die mikrobielle Anhaftung und die Nutzung der Polymere als Kohlenstoffquelle. Enzyme mit hydrolytischer und proteolytischer Aktivität wurden in kugelförmigen Strukturen, den so genannten äußeren Membranvesikeln (OMVs), bei mehreren Pseudomonas-Arten nachgewiesen.

Im Allgemeinen spielen OMVs eine Schlüsselrolle bei der inter- und intra-Kommunikation von Spezies, bei der Beschaffung von Nährstoffen, bei der Stressreaktion, bei der Abgabe von Toxinen, Adhäsions- und Virulenzfaktoren, bei der Biofilmbildung usw. Aufgrund dieser wichtigen Funktionen erhielten OMVs in den letzten Jahren große Aufmerksamkeit als Plattform für biotechnologische Anwendungen wie Impfstoffe, Systeme zur Medikamentenabgabe, Krebsimmuntherapien usw. Kürzlich durchgeführte Proteomics-Studien an aromatenabbauenden Bakterien (P. putida) zur Untersuchung der

Wechselwirkungen zwischen Mikroben und Lignin zeigten die Beteiligung von OMVs am Abbau von aromatischen Verbindungen.

Obwohl zahlreiche Bakterienstämme und Enzyme an den Abbauprozessen beteiligt sind, ist der vollständige Abbau-Mechanismus noch nicht vollständig bekannt. Im Mittelpunkt dieser Arbeit stehen die Charakterisierung von Außenmembranvesikeln für den extrazellulären Abbau eines Polyurethan-Oligomers und die Aufklärung des Abbauweges für das Polyurethan-Monomer 2,4-Diaminotoluol (2,4-TDA) durch Pseudomonas capeferrum TDA1. Im ersten Kapitel wurden die bakterielle Isolierung aus Bodenproben und die anschließenden Protokolle zur Quantifizierung des biologischen Abbaus der Polyurethan-Bausteine 2,4-Diaminotoluol (2,4-TDA) und eines Polyurethan-Oligomers ausführlich beschrieben.

Der isolierte Stamm war in der Lage, die bereits genannten Verbindungen als einzige Kohlenstoffquelle zu nutzen. 2,4-TDA dient dem Stamm ebenfalls als Stickstoffquelle. Diese Ergebnisse lieferten einen wichtigen Einblick in den katabolischen Mechanismus des Bodenbakteriums als potenzieller PU-Monomer- und Oligomer-Abbauer.

Das zweite Kapitel beschreibt die Identifizierung des isolierten Stammes als *Pseudomonas* sp. durch partielle Sequenzierung des 16S rRNA-Gens, des Membranfettsäureprofils und des Strukturgens der *cis/trans*-Isomerase (cti). Darüber hinaus wurde genomische DNA aus Bakterienzellen isoliert, die auf Succinat gewachsen waren, und für die Sequenzierung des Gesamtgenoms verwendet, um katabole Gene im Zusammenhang mit dem Abbau von aromatischen Verbindungen zu ermitteln. Vorläufig wurden Enzyme identifiziert, die am Stoffwechselweg beteiligt sind, ‚woraus der potentielle Abbauweg für *Pseudomonas sp.* entwickelt wurde, welches auf 2,4-TDA gewachsen ist.

Das Genom des isolierten Bakterienstamms wurde als *Pseudomonas capeferrum* (Typstamm WCS358) identifiziert. Der Stamm wurde als Pseudomonas capeferrum (Typstamm WCS358) anhand der vollständigen 16S rRNA-Gensequenz identifiziert.

Anschließend wurde dieser Stamm in einer Mischkultur getestet, die AA, EG, BDO und 2,4-TDA als Substrate enthielt. Obwohl 2,4-TDA aufgrund der Hemmung der Substratverwertung entfernt wurde, konnte das definierte mikrobielle Konsortium das PU-Hydrolysat in Rhamnolipide umwandeln.

Das dritte Kapitel berichtet über eine neue Methode zur RNA-Extraktion aus *Pseudomonas capeferrum* TDA1, welches auf 2,4-TDA wächst. Phenole und Brenzcatechine sind zentrale Zwischenprodukte des biologischen Abbaus von Aromaten, die leicht oxidiert werden können, um die entsprechenden Chinone zu erhalten, die mit Nukleinsäuren interferieren und dazu neigen, RNA auszufällen oder abzubauen. Der chemische Prozess wird durch die Aktivität von Polyphenoloxidasen-Enzymen gesteuert, die bereits in mehreren *Pseudomonas*-Arten identifiziert wurden.

Dieses optimierte Protokoll enthielt mehrere Modifikationen, einschließlich der Verwendung eines Trägers, gepoolter Proben und eines abschließenden Reinigungsschritts, der das Protokoll erheblich verbessern konnte. Es ergab eine qualitativ hochwertige RNA, gemessen an den A260/A280- und A260/230-Verhältnissen (2,02 ± 0,16 bzw. 1,95 ± 0,01) aus Zellen, die auf 2,4-TDA gewachsen waren, im Vergleich zu Standardassays. Außerdem wurden die RIN-Werte (RNA-Integritätszahl) analysiert, und Proben mit einem RIN-Wert von über 7,0 wurden für nachgeschaltete Anwendungen ausgewählt, was die RNA-Qualität bestätigte.

Anschließend wurden im vierten Kapitel die Transkriptionsveränderungen in *Pseudomonas capeferrum* TDA1, das auf 2,4-TDA gewachsen war, mithilfe von RNA-seq untersucht. Von allen exprimierten Genen wurde ein Drittel im Vergleich zur Kontrolle (Succinat) überexprimiert. Diese Veränderungen in der Genexpression zeigen, dass aromatische Verbindungen adaptive Reaktionen auslösen, die den Mechanismus der Transkriptionsregulierung modifizieren, einschließlich wichtiger Veränderungen nicht nur im katabolischen System, sondern auch in anderen Mustern, die mit der bakteriellen Zellphysiologie und Biofilmbildung zusammenhängen.

Zusammenfassung

Zur Bewertung des extrazellulären Abbaus wurden aus P. capeferrum TDA1, Zur Bewertung des extrazellulären Abbaus wurden die isolierten äußeren Membranvehikel (OMVs) aus *P. capeferrum*, gewachsen auf PU-Oligomeren, auf ihre hydrolytische Aktivität getestet. Gereinigte OMVs zeigten im Vergleich zu Zellpellets eine höhere Esteraseaktivität. Die relativen OMV-Ausbeuten in TDA1 stiegen in PU-Oligomer (0,28 ± 0,05 %) im Vergleich zu Succinat (0,09 ± 0,01 %) deutlich an. Diese dreifach erhöhte Aktivität könnte zeigen, dass die Freisetzung von OMV Teil der Anpassungsmechanismen der Bakterien an stressige Umweltbedingungen ist. Der makromolekulare Abbau kann durch die Wirkung sowohl periplasmatischer als auch membrangebundener Hydrolasen erfolgen, die sich in den OMVs befinden, und kann als unterstützender Mechanismus für den biologischen Abbau

Die gereinigten OMVs zeigten eine höhere Esteraseaktivität als die Zellpellets. Der makromolekulare Abbau kann sowohl durch periplasmatische als auch durch membrangebundene Hydrolasen erfolgen, die sich in den OMVs befinden, und kann als ein unterstützender Mechanismus für den biologischen Abbau angesehen werden.

Die Ergebnisse dieser Arbeit ermöglichen ein besseres Verständnis der Transkriptom-Reaktion in *P. capeferrum* TDA1, das einem PU-Monomer ausgesetzt ist und schlagen sowohl ein Modell den extrazellulären Abbau, an dem OMVs beteiligt sind, sowie einen vollständigen katabolischen Mechanismus für den biologischen Abbau von Pu auf Polyesterbasis vor, welcher intra- und extrazelluläre Enzyme enthält.

Darüber hinaus werden weitere Studien zum biologischen Abbau von PU dazu beitragen, Kunststoffe unter Berücksichtigung biologisch abbaubarer Bausteine neu zu gestalten und den biokatalytischen Abbau zu verbessern, was in den nächsten Jahren eine nachhaltige Nutzung von PU-Kunststoffabfällen ermöglichen könnte.

Summary

During the last 50 years, plastic industry has grown exponentially with an estimated 8300 million metric tonnes of plastic produced to date. Regardless of the large variety of polymers available, 99% are entirely fossil-fuel based which compromises its degradability after use. Major synthetic polymers in use today are polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyurethane (PU) and polyethylene terephthalate (PET). The current methods for disposing of plastic waste mainly include landfilling, incineration, mechanical and chemical recycling. Despite of the significant improvement of these technologies, it is still necessary to overcome several limitations and deficiencies.

Polyurethane (PU) is a synthetic polymer used as raw material in several industries. In 2015, PU global production reached 27 million metric tons, making the sixth most-used plastic worldwide. The main constituents of polyurethane are isocyanates, polyols and chain extenders. The additional incorporation of aromatic ring structures has further impact on the physical and chemical properties of the polymer. PU is considered highly stable, but difficult to recycle and improper incineration produces toxic gases that are harmful to human health. In addition, most of PU waste is constantly discarded to landfills, resulting in a potential loss into the environment.

Unfortunately, the mismanaged plastic has spread out in different habitats across our planet including cold marine areas and uninhabited places, threatening wildlife and ecosystems. In order to avoid further contamination, it is necessary to transform plastic waste by restoring functional properties, providing added value and exploring new application areas that could provide economic benefits in a long-term perspective.

In the last 10 years, a transition from a linear economy to a sustainable, bio-based circular economy has become fundamental to cope the fossil fuel-driven climate change and global plastic pollution. This transformation involves industrial and basic research strongly focused on biotechnology and bioprocesses. Within this transition, microorganisms are key players due to the wide diversity of enzymes

and metabolic pathways that could be used for the development of sustainable processes and biomaterials.

Recently, microorganisms with plastic-degrading potential have been regularly identified in different environments such as waste disposal, landfills, plastic refineries, open dumps, etc. Selective pressure and evolution of genetically flexible mechanisms have contribute to metabolize anthropogenic compounds, which it has been noted in several enzymatic reactions designed for the efficient degradation of a wide variety of recalcitrant substrates, leading to novel metabolic pathways.

Even though several bacterial genera have been reported in the degradation of environmental pollutants, *Pseudomonas* species are amongst the most cited degraders of aromatic hydrocarbons and plastic polymers. The genus *Pseudomonas* incorporates one of the most complex, diverse, and ecologically significant group of bacteria on the planet. Members of this genus are found in large numbers in all the major natural environments (terrestrial, freshwater, and marine) and form intimate associations with plants and animals. This universal distribution suggests a remarkable degree of physiological and genetic adaptability. In fact, *Pseudomonas* have been most frequently linked with PU degradation.

Chemically, polyester-based PUs are semi-crystalline structures containing hydrolysable ester and urethane bonds that are fragmented by extracellular enzymes (hydrolases), releasing oligomeric and monomeric building blocks. For instance, amines, alcohols, acids, aromatics, and other residues, such as EG (ethylene glycol), 1,4-butanediol (BDO), adipic acid (AA) ,4′-methylenedianiline (MDA) and 2,4-toluene diamine (2,4-TDA) are constantly present during PU degradation. However, MDA and 2,4-TDA are considered environmental pollutants, which represent a major risk for species in the aquatic and terrestrial areas.

This fragmentation of the polymer is known as depolymerization and it is essential for strengthening recycling processes that use plastic waste as feedstock. The broad spectrum of building blocks might be used as carbon and energy source for microorganisms that degrade these compounds and/or use them for the production of higher-value elements. This latter is considered a promising upcycling strategy to reduce fossil-fuel plastic waste and promote new waste management strategies.

Summary

Previous studies have revealed that extracellular enzymes are essential for biofilm formation on the polymer surface, reducing the resistance and durability of plastic materials. This first step promotes microbial attachment and further degradation. Enzymes with hydrolytic and proteolytic activity have been detected in spherical structures called outer membrane vesicles (OMVs) in several *Pseudomonas* species.

Generally, OMVs play a key role in establishing inter- and intra-species communication, acquisition of nutrients, stress response, delivery of toxins, adhesion and virulence factors, biofilm formation, etc. Due to these important functions, OMVs have gained attention during the last years as a platform for biotechnological applications such as vaccines, drug-delivery systems, cancer immunotherapy agents, etc. Lately, proteomics studies conducted in aromatic-catabolic bacteria (*P. putida*) to investigate microbe–lignin interactions revealed the participation of OMVs in the catabolism of aromatic compounds.

Even though numerous bacterial strains and enzymes are involved in degradation processes, the complete catabolic mechanism is not totally understood yet. This thesis also centers on the characterization of outer membrane vesicles for extracellular degradation of a polyurethane oligomer and elucidation of the degradation pathway for the polyurethane monomer 2,4-diaminotoluene (2,4-TDA) by *Pseudomonas capeferrum* TDA1.

In the first chapter, bacterial isolation from soil samples and the subsequent protocols to quantify biodegradation of polyurethane building blocks were fully described.

The isolated strain was able to use a PU oligomer and 2,4-TDA as sole source of carbon. The latter compound also served as nitrogen source. These results provided a key insight into the catabolic mechanism of the soil bacterium as a potential PU monomer and oligomer-degrader.

The second chapter described the identification of the isolated strain as *Pseudomonas* sp. by partial 16S rRNA gene sequencing, membrane fatty acid profile and structural gene for the cis/trans isomerase (*cti*). In addition, genomic DNA was isolated from bacterial cells grown on succinate and utilized for whole

genome sequencing in order to detect catabolic genes related to aromatic compounds degradation. Preliminary, enzymes involved in the metabolic pathway were identified, which eventually led to a suggested degradation pathway for *Pseudomonas* sp. grown on 2,4-TDA.

The strain was identified as *Pseudomonas capeferrum* (type strain WCS358) using the full 16S rRNA gene sequence.

The third chapter reported a new method of RNA extraction from *Pseudomonas capeferrum* TDA1 growing on 2,4-TDA. Phenols and catechols are central intermediates of the aromatics biodegradation that can be easily oxidized to yield the corresponding quinones, which interfere with nucleic acids and tend to co-precipitate or degrade RNA. The chemical process is regulated by the activity of polyphenol oxidases enzymes, which have been identified in several *Pseudomonas* species previously.

This optimized protocol incorporated several modifications including the use of a carrier, pooled samples and a final cleaning up step that could improve it significantly, yielded a high-quality RNA measured by A_{260}/A_{280}, $A_{260}/_{230}$ ratios (2.02 ± 0.16, 1.95 ± 0.01, respectively) from cells grown on 2,4-TDA compared to standard assays. Moreover, RIN (RNA integrity number) values were analyzed and samples with a RIN higher than 7.0 were selected for downstream applications, confirming the RNA quality.

Finally, the fourth chapter evaluated the transcriptional changes in *Pseudomonas capeferrum* TDA1 grown on 2,4-TDA using RNA-seq. From all the expressed genes, one third were overexpressed in comparison to the control (succinate). These alterations in the gene expression demonstrates that aromatic compounds trigger adaptive responses that modify the transcriptional regulation mechanism including important changes not only in the catabolic system, but also in other patterns related to bacterial cell physiology and biofilm formation.

In order to evaluate extracellular degradation, OMVs isolated from *P. capeferrum* TDA1 grown on a PU oligomer were tested for hydrolytic activity. Purified OMVs showed higher esterase activity compared to cell pellets. Relative OMV yields in TDA1 raised significantly in PU oligomer ($0.28 \pm 0.05\%$) compared to succinate

(0.09 ± 0.01%). This three-fold increased activity could demonstrate that the release of OMV is part of the adaptive mechanisms of bacteria to stressful environmental conditions. The macromolecular degradation may occur through the action of both periplasmic and membrane-bound hydrolases harbored inside of OMVs and can be considered as a supporting mechanism for biodegradation.

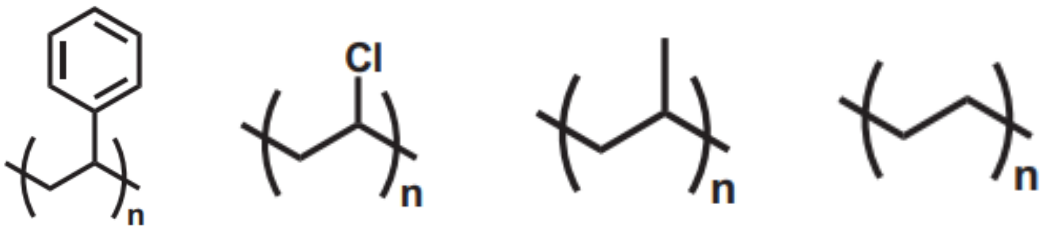
The results of this thesis present a further understanding of the transcriptome response in *P. capeferrum* TDA1 exposed to a PU monomer, suggest a model for extracellular degradation involving OMVs and propose a complete catabolic mechanism for the biodegradation of polyester-based PU containing intra and extracellular enzymes. Moreover, further studies on biological degradation of PU will contribute to redesign plastic polymers considering biodegradable building blocks and improving biocatalytic degradation, which could provide a sustainable use of PU plastic waste in the future.

1. Introduction

1.1 Plastic types, properties and applications

Synthetic polymers are macromolecule chains formulated from hundreds or thousands of organic subunits called "monomers" linked by chemical bonds. The assembly of the chemical structure can be performed by two reactions: polyaddition or polycondensation [1, 2]. The most common method involves addition polymerization of several small molecules together into a chain to form a large molecule, while as the condensation process combines different starting material to yield a single molecule. Nylons, some polyesters, and urethanes are examples of condensation polymers [2, 3, 4]. These polymers have been classified in two main groups: thermoplastic or thermosets.

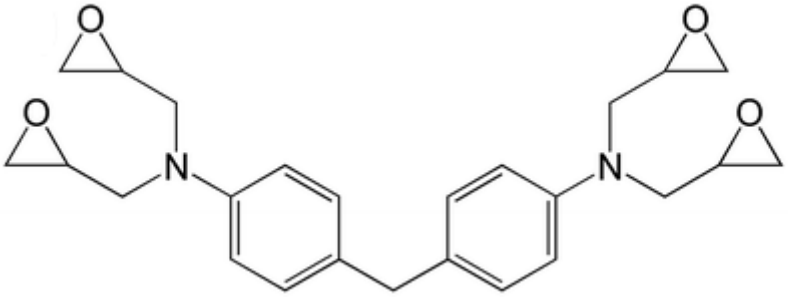
Thermoplastics are malleable polymers at high temperatures and can returned to this plastic state by reheating. This material offers excellent mechanical properties including improved strength values, high toughness, and hardness. Thermoplastic are flexible and recyclable several times, however, the quality will reduce ultimately [5, 6]. Common thermoplastics include polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyimide (PI), poly-methyl methacrylate (PMMA), polytetrafluoroethylene (PFE) and polyvinylidene chloride (PVDC) [1]. Due to the carbon-carbon backbone of thermoplastics, they are resistant to degradation or hydrolytic cleavage [1, 5].



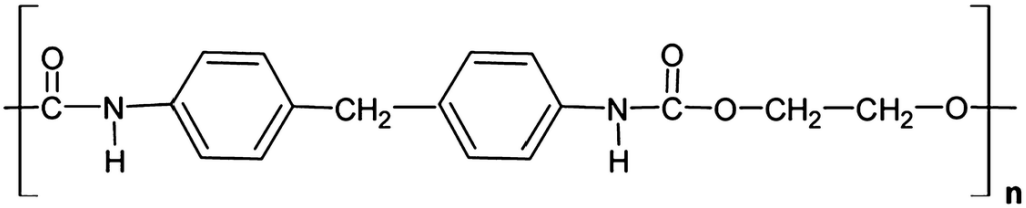
Polyester (PS) **Polyvinyl chloride (PVC)** **Polypropylene (PP)** **Polyethylene (PE)**

Figure 1.1. Structure of the most used thermoplastics.

In contrast, thermosets have an irreversible solidification process and cannot be molded due to their three-dimensional cross-linked structure. Further heating produces structural alteration and chemical decomposition [7, 8]. Due to their good adhesive strength and high temperature stability, thermosets have been classified amongst the most difficult materials to recycle [8]. There are several thermosets available such as, polyurethane (PU), polyester, acrylic, vinyl and epoxy resins. These polymers are potentially susceptible to hydrolytic cleavage due to amide or ester bonds in their structure [5].



4,4′-Diaminodiphenylmethane epoxy resin



Polyester polyurethane (Polyester PU)

Figure 1.2. Structure of common thermosets.

Thermoplastics and thermosets have been widely investigated for several high-performance applications including medical devices, insulation systems, electric applications, military aircrafts and satellites construction.

1.2 Current technologies for plastic waste treatment

Given the versatility and low cost of synthetics polymers, global manufacturing has grown exponentially, reaching 380 Mt year^{-1} in 2015 [2]. Nevertheless, the vast

majority of plastics are made from non-renewable fossil resources, which represents an emerging threat to the environment. Nowadays, only 18% of plastic waste is recycled, 24% is incinerated and 58% is discarded into landfills, open dumps, or natural environments [9, 10].

Mechanical recycling is the preferred method at present. It typically includes collection, sorting, washing and grinding of the material. Normally, plastic waste fractions are processed with no significant changes in their chemical composition [9, 11]. Common thermoplastics such as PET, PP, PE and PS are mechanically recycled and processed into secondary raw material. However, there are limitations to this technology including the low quality of the recycled plastic after many processing cycles and the limited commercial value [10, 12, 13].

As an alternative, chemical recycling can recuperate monomers or other chemicals that are used as secondary feedstock in the production of plastics and chemicals [14]. PET, PUR and nylon waste can be depolymerized by pyrolysis, glycolysis hydrolysis, ammonolysis, aminolysis, and hydrogenation [15]. During the last years, PU foams have been recycled using glycolysis and hydrolysis, but in spite of all the modifications and new technologies, the monomers recovered by these methods can only replace <50% of virgin polyol material for further application [16]. Other drawbacks such as the catalyst development, which defines the efficiency of the chemical process and use of polymer compatibilizers restrict its use for different polymers [10, 14].

Incineration is another method used routinely for disposal of plastic waste. It reduces the demand for landfills, but there are concerns due to the hazardous substances that are released into the atmosphere. Heavy metals, toxic carbon- and oxygen-based free radicals and greenhouse gases are some of the compounds produced during incineration [17]. Low-molecular compounds can be vaporized directly in the air, and depending on their structure, are able to form a combustible mixture, or oxidize in solid form. On the other hand, macro-molecular plastics have to decompose into small molecule compounds to initiate the combustion process [18, 19]. PE, PET, PP, PVC and PU are usually incinerated for heat recovery.

Finally, landfills represent the first option for plastic waste in some developing countries due to its low cost and operability. However, waste disposal by landfilling cause severe environmental issues such as uncontrolled release of methane in the atmosphere, leaching contaminants (benzene, toluene, xylenes, ethyl benzenes and trimethyl benzenes and BPA) that go directly into the soil and the ground water, unpleasant odors and spread of pathogenic microorganisms [17, 20]. The potential fate of plastic polymers in landfills and open dumps depends on their composition and the environmental conditions to which they are exposed [21]. For example, PE and PET have shown low degradation rates in the environment, but once plastic debris are accumulated and exposed to humidity and sunlight, the material tends to be susceptible to thermal-oxidative degradation or hydrolysis, which accelerates fragmentation and mass loss [2, 22]. In spite of this advantage, evidence has showed that complete physical degradation of PE requires than more than 20 years [22].

1.3 Bio-based plastics

The dramatic increase of synthetic polymers production and the lack of efficient strategies for plastic waste management have promoted new sustainable alternatives such as bio-based plastics and biodegradation. Nowadays, bioplastics are considered a potential replacement for petrochemical plastics, reducing fossil resource extraction and allow the transition towards a circular economy [23, 24]. Nevertheless, bioplastics represent only 1% of the total plastic production worldwide due to limiting factors such as price competitivity, decreased mechanical performance compared to fossil fuel and incompatibility with existing sorting infrastructures [24, 25].

The best known types of biodegradable polyesters are polyhydroxyalkanoates (PHAs), polylactic acid (PLA) and polybutylene succinate (PBS) [26, 27]. Although these materials are already used in several applications, the biodegradability of bioplastics can be highly affected by their physical and chemical structure, also environmental conditions such as temperature, pH, moisture and oxygen contents play a key role in the degradation rate [27]. Despite all the extensively research, biodegradation of bio-based plastics needs further studies focused on possible effects on animals and plants from different environments.

1.4 Microbial degradation of synthetic plastics

Plastic production and commercialization is one of the fastest growing fields of industry worldwide. Due to the accumulation and spread out of plastic debris in many environments including cold marine habitats [28] and uninhabited places [29], various microorganisms have evolved different biochemical pathways to degrade plastic waste. Some algae, bacteria as well as fungi have been classified as potential degraders of plastic polymers using enzymatic systems. Different bacterial species from *Pseudomonas*, *Escherichia* and *Bacillus* genera have important roles in plastic degradation due to their natural capacity of degrading long-chained fatty acids [2]. These species have been isolated from various sources such as open dumps, landfills and recycling sites [25]. Studies have shown that microbes continuously exposed to xenobiotic/recalcitrant compounds develop the capacity to either use this pollutant as energy sources or have genetic adaptation for degradation [30].

Initially, the superficial degradation of the plastic polymer initiates by bacterial cells attached to the surface that modify the physical properties of the polymer, especially molecular weight reduction and loss of mechanical strength[1]. Bacterial aggregates tend to proliferate using the polymer as sole carbon source. Once the biofilm is formed, it secretes exopolysaccharides (EPS) that promote stronger adhesion and cell surface attachment [31] (Figure 3). EPS are essential components that establish the functional and structural integrity of biofilms.

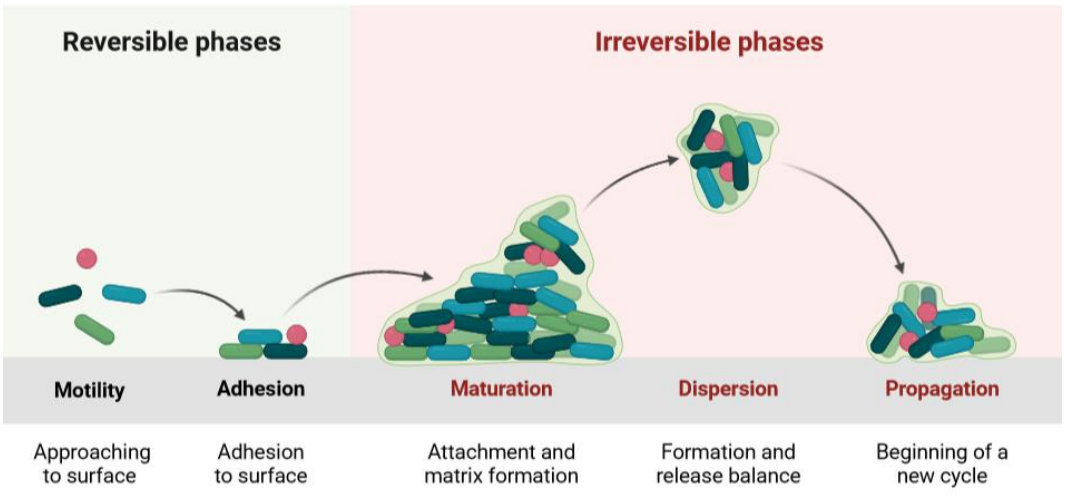


Figure 1.3 Biofilm formation. Adapted from Beitelshees, *et al* [32].

The presence of extracellular enzymes (hydrolases and depolymerases) catalyze the hydrolytic cleavage of the polymers into smaller compounds, which are susceptible to be deteriorated by microbial enzymes and further degradation. This reaction can occur either somewhere in the polymer chain (endo-attack), releasing small oligomers and monomers, or at the terminal (exo-attack), forming products that are not assimilated without further degradation [1, 31].

Once the plastic has been depolymerized into smaller molecules, some of these compounds can be transported and metabolized either as CO_2 or used for the biosynthesis of valuable products by different metabolic pathways [1, 31, 33]. Although the intracellular degradation process has not been explained completely for all the synthetic plastics, different membrane transportation systems facilitate the movement of these compounds for further processing. In *Rhodococcus* species, a group of transporters including an ATP binding cassette (ABC) family were upregulated during the assimilation of PE oligomeric intermediates [34]. A TPA (terephthalate) transporter was identified in *Comamonas* sp. and other genera as the responsible of the mobilization of terephthalic acid, a hydrolytic product of PET [35]. Inside the cell, hydrolytic compounds are transformed by peripheral pathways into key central intermediates such as catechol, protocatechuate and benzoyl-CoA. Then, central pathways cleave the organic compound through different routes, reaching the central metabolism (Figure 4).

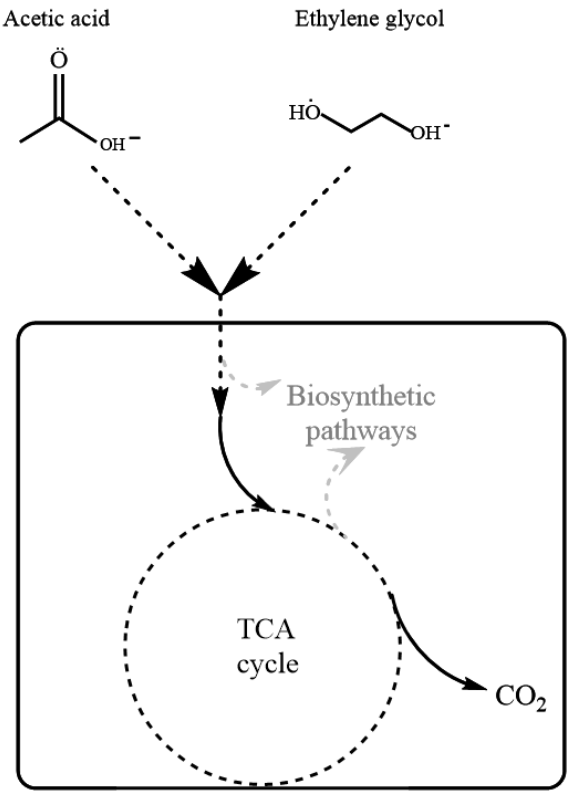


Figure 1.4 Acetic acid (PE degradation) and ethylene glycol (PU degradation) are suggested hydrolytic products that could be transported into the bacterial cell and processed though TCA cycle. Adapted from Wilkes, *et al* [31].

Over the years, different plastic-degrading enzymes (esterases, ureases, lipases proteases, etc.) have been identified in *Pseudomonas, Bacillus, Acinobacter*, *Rhodococcus* and other genera. For instance, an extracellular esterase and protease from *P. chlorophilis* was shown to degrade polyester PU [36]. Esterase activity was detected in *Bacillus sp*. increasing degradation rates of amorphous PET film [37]. Yoshida *et al*. reported a cutinase descendant enzyme (PETase) involved efficiently in the depolymerization process of PET at 30°C [38]. Alkane hydrolases (Alk family) in *Pseudomonas sp*. E4 degraded PE with high molecular weight [39]. Thermophilic cutinase (TfCut2) from *Thermobifida fusca*, catalyzed PET degradation [40]. A peroxidase of the lignin-decolorizing *Azotobacter beijerinckii* HM121 reduced the molecular weight of PS in a two-phase system [41]. *Anoxybacillus rupiensis* was able to use polyamide-6 (also known as nylon-6) as a sole carbon and nitrogen source [42].

Although most of the studies highlight the biochemical reactions of bacterial strains, there are other external factors such as UV irradiation, pH, temperature, moisture and polymer structure that play a significant role in polymer degradation [25, 43]. In the environment, prolonged exposure to specific weather conditions can modify plastic waste composition, which makes it more accessible for microbial and enzymatic degradation. Industrially, some of these factors are used as pre-treatments (physical, chemical and photo irradiation), that have a substantial impact on the polymeric structure and reactivity, preparing it for the consequent steps in the process to achieve plastic waste circularity [25]. However, pre-treatment processes must be economically safe and environmental friendly in order to be technically scalable.

1.5 Outer membrane vesicles

Several studies refer that microbial biofilm formation on the polymer surface is a prerequisite for biodegradation. Inside the complex nature of the EPS, small exosomes (20-240 nm) called outer membrane vesicles (OMVs) are released by gram-negative bacteria [44] (Figure 5). During bacterial growth, outer membrane vesicles are constantly discharged from the surface of the cell, increasing cell surface hydrophobicity.

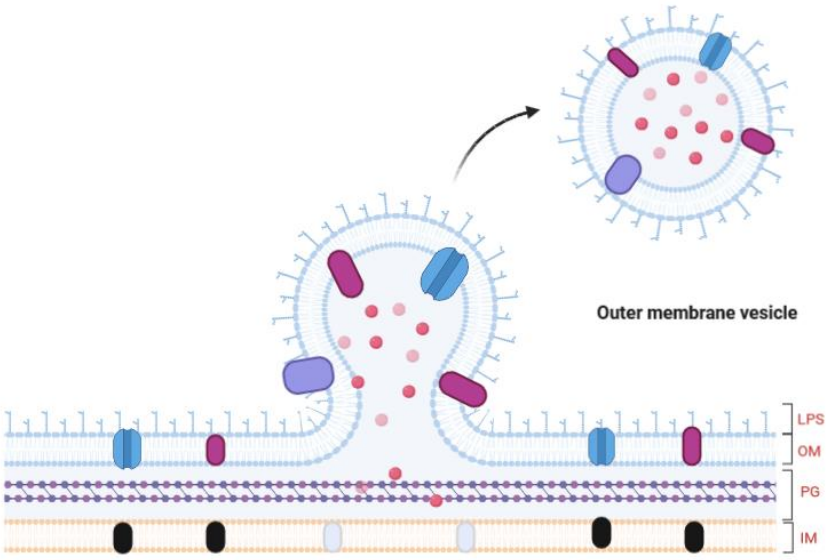


Figure 1.5 Schematic view of outer membrane vesicles (OMV) biogenesis. OMVs consist of outer membrane (OM) phospholipids, lipopolysaccharide (LPS) and periplasmic proteins. Proteins and lipids of the inner membrane (IM) and cytosolic content are excluded

from OMVs. Various cellular proteins associated with OM and peptidoglycan (PG) are represented as colored shapes. Adapted from Kuehn & Kesty [45].

Several enzymes and extracellular structures inside the OMVs have been identified in the degradation of aromatic substrates such as phenanthrene and lignin-rich media by different bacteria including *Pseudomonas*, *Rhodoccocus*, *Amycolatopsis* and *Delftia* [46, 47].

Furthermore, reports showed that OMVs may contribute to the biofilm formation [44, 48]. This property has led to recent interest into plastic degradation. Degradation rates of resilient plastic polymers such as PE and PS are improved by biofilm adhesion. For instance, *Pseudomonas sp*. AK2 showed an enhanced low-density PE degradation through an adapted biofilm compared to planktonic cells[49]. Other strains including *Bacillus sp*. grown on PS films presented a reduced polymer mass by 23% after 14 days [50]. PE film incubated with *Comamonas* sp. and *Delftia* sp. showed a decrease in crystallinity, while film incubated with *Stenotrophomonas* sp. showed an increased crystalline fraction [51].

1.6 Biodegradation of polyurethane

Polyurethane is a plastic polymer synthesized from the reaction between an diisocyanate and polyol molecule in the presence of either a catalyst or ultraviolet light activation [52] (Figure 6).

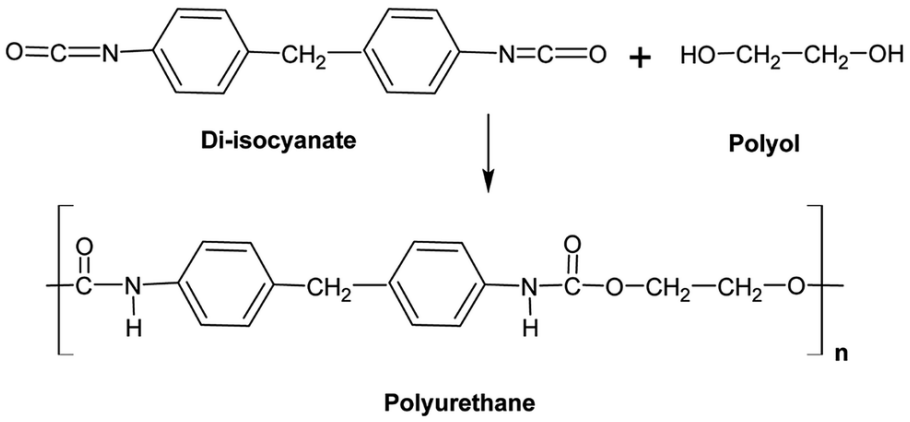


Figure 1.6 Common synthesis of polyurethanes. Adapted from Aikondo, *et al* [52].

Due to its excellent mechanical properties, stability and enhanced biocompatibility, PU ranks fifth among the most often produced synthetic polymers [33]. Depending on

the chemical structure of polyols (either polyether or polyester), PU degradation process could differ considerably. It is hypothesized that the ester bond in polyester-based PU (Figure 7) makes it more vulnerable to enzymatic enzymes compare to ether-linked PU [1].

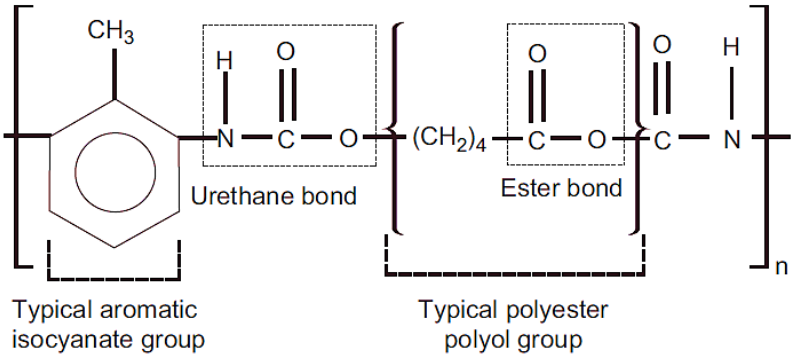


Figure 1.7 Typical polyester urethane polymer. Adapted from Gautam, *et al* [53].

There are numerous reports in the field of PU biodegradation using bacteria and fungi. Although fungi (namely *Aspergillus* sp. and *Penicillium* sp.) have been reported as the principal degraders of PU in nature, enzymatic activity has also been linked to different bacterial strains. *Pseudomonas*, *Corynebacterium*, *Rhodococcus*, *Bacillus* and other genera have shown substantial PU degrading capability [25]. Genes and enzymes contributing to microbial degradation of PU have been widely studied. For example, gene *pudA* encoded an esterase from *Comamonas acidovorans* TB-35 [54]. Two active genes *pueA* and *pueB* from *Pseudomonas fluoresecens* encoded three different esterases involved in Impranil DLN (polyester-base PU) [55]. A lipase from *Candida antartica* was used to degrade toluene diisocyante (TDI) based PU with weight loss of 25% [56]. Schmidt and colleagues used known polyester hydrolases (LC-cutinase, TfCut2, Tcur0390 and Tcur1278) and observed significant weight loss of the tested foils [57]. Extracellular esterases and lipases in *P. aeruginosa* were reported to facilitate aromatic-aliphatic polyester degradation [58].

As a result of enzymatic degradation, PU waste in landfills may continuously release environmental pollutants into soil or groundwater, such as 4,4′-methylenedianiline (MDA) and 2,4-toluene diamine (2,4-TDA) [59, 60, 61], which are considered as possible human carcinogen and pose an environmental risk for species in the aquatic and terrestrial areas [62]. Moreover, 2,4-TDA is the major raw

material for the production of toluene diisocyanate (TDI), which is used in the production of polyurethane (PU) foams [63]. To comprehend the fate of the diamines released from PU degradation and the role of OMVs can provide a new insight in biodegradation and could contribute with vital information that enhance catabolic efficiency.

1.7 Gene expression and RNA-sequencing

The central dogma of molecular biology states that genes are transcribed to RNA and then, RNA is translated into protein. Generally, the optimal expression level of a protein strongly depends on the external growth conditions [64, 65]. During temporal changes of nutrients, growth-inhibitory substances, stress or starvation; bacteria tends to orchestrate adaptation strategies that relies on sophisticated signaling pathways and gene regulatory networks [64]. The expression of a gene is a regulated process exerted by a countless of different mechanisms. RNA modifications placed in coding sequences of mRNAs have been identified as potential regulators of gene expression [66, 67]. It has been proven that transcript stability can be altered in response to stressors, while in other cases, genetic-specific modulations contribute to gene expression changes that bacteria need to adapt and survive in a new environment [68].

Over the past 10 years, regulation of gene expression in bacteria has been into the spotlight, with many discoveries in classic genetics and biochemical assays using high-throughput technologies [69].

Transcriptomic analyses, such as RNA-sequencing (RNA-seq), measure the expression of many genes and functions at once by detecting mRNA transcripts [70]. For instance, a carbon starvation model using *M. tuberculosis* showed changes in expression of regulatory genes with a reduction in mRNA species involved with energy metabolism and cell division [71]. RNA-sequencing studies in *Escherichia coli* O157:H7 undergoes to stress conditions revealed the differential expressions of 1217 genes were significant during ultrasonic disruption, including 621 up-regulated and 596 down-regulated genes [72]. Amrani *et al* [73] used RNA-seq to study the response of deep-sea bacteria under hydrostatic pressure. 65 differentially expressed genes were identified and mainly distributed in aromatic amino acid and

glutamate metabolisms, energy metabolism, signal transduction, and unknown functions.

RNA-seq data can identify and confirm active catabolic pathways, genetic responses and regulatory mechanisms. This promising technique reveals in-depth relationships between the gene expression levels and environment, which provides new insights in bacterial adaptation.

1.8 Aims of this study

Facing the persistent growth of global plastic production and considering the drawbacks of traditional methods to treat plastic waste, biological depolymerisation and conversion technologies have been increasingly discussed as a promising bio-upcycling strategy in both recalcitrant and bioplastics. However, the enzymatic mechanisms and microorganisms acting on synthetic polymers is still limited. The main bottleneck lies in the initial breakdown of high-molecular-weight polymers with robust chemical groups and crystallinity structure, which tend to resist hydrolysis even under conditions favoring microbial processes.

In recent years, considerable progress regarding plastic polymers with hydrolysable groups in their backbones such as polyurethane (PU) have been reported. Even though several bacterial species have been identified as a PU-degraders using different enzymes (esterases, lipases, proteases, cutinases, etc.), it is necessary to explore diverse environments to discover new plastic-degrading enzymes with high catalytic efficiency and stability. Moreover, the conversion of polyurethane degradation products into value-added compounds is a further step that still needs to be enhanced. In detail, it aimed:

1. To isolate and characterize a soil bacterium capable of degrading PU building blocks, identify catabolic genes and propose a degradation pathway for 2,4-TDA (Chapter 2.1 and 2.2).

2. To develop a RNA extraction protocol for *Pseudomonas capeferrum* TDA1 growing on aromatic substrates such as 2,4-TDA (Chapter 2.3).

3. To evaluate the transcriptional changes in *Pseudomonas capeferrum* TDA1 grown on 2,4-TDA and identify outer membrane vesicles (OMVs) with hydrolytic activity (Chapter 2.4).

2. Research chapters

2.1 Screening and cultivating microbial strains able to grow on building blocks of polyurethane

María Jose Cárdenas Espinosa, Andrea Colina Blanco, Hermann J. Heipieper, and Christian Eberlein.

Helmholtz Centre for Environmental Research—UFZ, Department of Environmental Biotechnology, Leipzig, Germany

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Screening and cultivating microbial strains able to grow on building blocks of polyurethane

María José Cárdenas Espinosa, Andrea Colina Blanco, Hermann J. Heipieper, and Christian Eberlein*

Helmholtz Centre for Environmental Research—UFZ, Department of Environmental Biotechnology, Leipzig, Germany

*Corresponding author: e-mail address: christian.eberlein@ufz.de

Contents

Abstract

The diverse benefits of synthetic polymers is overshadowed by the amount of plastic waste and its whereabouts. The problem can only be tackled by reducing and recycling of plastics. In this respect, investigating the (microbial) degradation of each type of polymer currently used may provide further understanding that fosters the development of new feasible recycling technologies. Here, we present a strategy to isolate bacteria from environmental samples that are able to degrade hydrolysis products and building blocks of polyurethane (PUR). Protocols are presented to enrich bacteria on the primary diamines 2,4-diaminotoluene (TDA) and 4,4′-diaminodiphenylmethane (MDA) as well as an oligomeric PUR (Sigma Aldrich, proprietary composition). For TDA and the oligomeric PUR, methods are suggested to monitor their concentration in bacterial enrichment cultures.

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1. Introduction

Synthetic polymers (plastics) are typically derived from petroleum oil and are an important pillar of our modern society. Among plastics, polyurethanes (PUR) are used as basic material (often as foams) in medical, textile, packaging and construction industries. In 2018 only, the demand for PUR in Europe increased to about 4 million tons (PlasticsEurope, 2019). As the building blocks for polyurethane synthesis are numerous and diverse, there is a huge range of different polyurethane polymers. The properties of PUR highly depend on the structure of the polymer backbone and they can be tailored to have a certain rigidity, strength, flexibility and toughness. However, the common characteristic that all PUR share is the presence of urethane bonds (also known as carbamate bonds). Precursors used to synthesize PUR are diisocyanates and polyols together with additives such as catalysts, cross linkers, and chain extenders, among others. Besides the mentioned urethane bonds, the polyols used for synthesis can additionally contain polyester (polyester PUR) or polyether bonds (polyether PUR). Regarding the diisocyanates employed for the PUR polymerization process, aliphatic and aromatic compounds are common. Fig. 1 shows the diisocyanates mainly used for PUR production: 4,4′-methylenediphenyl diisocyanate (MDI) and toluene-2,4-diisocyanate (TDI).

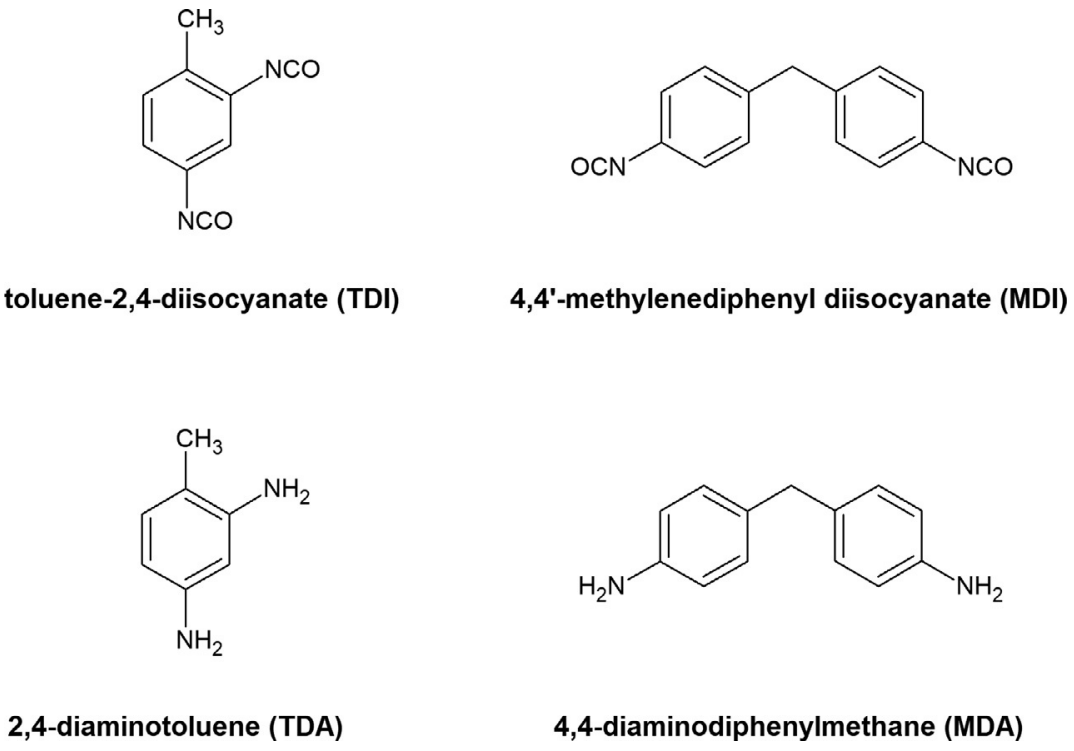

Fig. 1 Common aromatic diisocyanates used for PU synthesis and their corresponding diamines.

In order to investigate PUR biodegradation, two approaches are conceivable: to investigate the change in properties of bulk polymers or the release of degradation products including oligomers and monomeric building blocks. The first studies of PUR polymer degradation by microbial communities have been reported approximately 50 years ago with the exposure of polyester and polyether PUR to fungal activity (Kanavel, Koons, & Lauer, 1966; Ossefort & Testroet, 1966). The purpose of these initial studies was to improve the durability and quality of the materials rather than solving an environmental concern of plastic waste management. Since the 1990s, many studies focused their efforts on analyzing PUR degradation using Gram-positive and Gram-negative bacteria and are summarized in comprehensive review papers (Cregut, Bedas, Durand, & Thouand, 2013; Mahajan & Gupta, 2015; Wierckx et al., 2018). Some of them aimed to improve PUR properties. Other studies focused on the increasing accumulation of plastic waste in landfills or on the decreasing raw material supply (e.g., crude oil). Regarding the latter, a recycling strategy for polyethylene terephthalate (PET) to produce novel PUR polymers was recently accomplished. Enzymatic hydrolysis of PET, subsequent degradation of the building blocks and hydroxyalkanoyloxy-alkanoates (HAAs) production in a *Pseudomonas* strain led to the chemo-catalytic synthesis of a partially bio-based PUR (Tiso et al., 2020).

Generally, polymer degradation is caused by oxidation and hydrolysis reactions. However, many factors such as the chemical structure of the polymer (crystallinity, cross-linking, chemical groups in the molecular chains, etc.) and the abiotic conditions (pH, redox conditions, humidity, temperature, etc.) have an impact on the biodegradation reactions. The protocols presented in this chapter refer to standardized lab conditions in batch cultures of microbial (pure) cultures isolated from the environment. The following chapter describes the investigation of the biodegradation of building blocks of polyurethane polymers by isolating strains from the environment that are capable to use them as sole source of carbon and energy. However, diisocyanates are not stable in the environment due to their immediate reaction with water and the subsequent formation of the corresponding primary amine after decarboxylation (Kreye, Mutlu, & Meier, 2013; Ozaki, 1972). Therefore, diisocyanates such as TDI and MDI are not suitable as model substances to investigate PUR building block degradation in aqueous environments. Instead, their corresponding primary diamines 2,4-diaminotoluene (TDA) and 4,4′-diaminodiphenylmethane (MDA) were chosen. In addition, they are the environmentally relevant PUR hydrolysis products because these diamines were identified as

intermediates in the biodegradation of PUR material (Akutsu-Shigeno et al., 2006; Matsumiya, Murata, Tanabe, Kubota, & Kubo, 2010) and are common precursors in polyurethane production processes. In summary, due to the immediate reaction of PU monomers MDI and TDI with water, these monomers were replaced by MDA and TDA to investigate the PUR monomer metabolism (Utomo et al., 2020). Also, an oligomeric PUR was chosen as a model compound for PUR biodegradation experiments. The isolation of a bacterial strain on the oligomeric PUR was reported earlier (Mukherjee et al., 2011).

2. Methods

2.1 Isolation and characterization of bacteria from soil samples capable of degrading PU building blocks

2.1.1 Rationale

So far no pure culture was isolated that uses TDA or MDA as sole source of carbon and energy. Thus, nature's diversity was mined for a bacterial TDA/MDA degrader on a site rich in brittle plastic waste. This site was chosen to increase the chances to find a PUR building block consumer. To avoid the availability of an additional carbon source a mineral medium was chosen for isolation (Hartmans, Smits, van der Werf, Volkering, & de Bont, 1989).

2.1.2 Materials, equipment and reagents

UV/visible spectrophotometer
TDA (Sigma Aldrich, molar mass = 122.17 g/mol; 98% purity)
MDA (Sigma Aldrich, molar mass = 198.26 g/mol; purity ≥97.0%)
PU diol solution (Sigma Aldrich; 88% m/m; proprietary composition), aliphatic, dihydroxy-functional oligomeric PUR
Salts (Table 1) and trace elements (Table 2) to prepare mineral medium (Table 3)

2.1.3 Protocol

1. Prepare agar plates (Table 3) containing 10 mM of TDA or 10 mM MDA as well as agar plates not containing any additional carbon source
2. Take about 10 g of soil, preferably from a soil rich in brittle plastic waste and fill it into a 50 mL test tube
3. Proceed directly or store the sample at 4 °C

Table 1 Mineral salts solution, 10×.

Substance	Concentration (g/L distilled H_2O)
$Na_2HPO_4 \times 2H_2O$	70
KH_2PO_4	28
NaCl	5
NH_4Cl	10

Table 2 Trace elements solution, 500×.

Substance	Concentration (g/L distilled H_2O)	Volume (mL/L)
$MgSO_4 \times 7H_2O$	50.0	
$FeSO_4 \times 7H_2O$	5.0	
$MnSO_4 \times H_2O$	2.5	
$ZnCl_2$	3.2	
$CaCl_2 \times 6H_2O$	0.5	
$BaCl_2$	0.3	
$CoSO_4 \times 7H_2O$	0.18	
$CuSO_4 \times 5H_2O$	0.18	
H_3BO_3	3.25	
EDTA	5.0	
HCl (37%)	—	73

Table 3 Mineral medium used to isolate bacteria.

Substance	Quantity
Mineral salts, 10x	50 mL
Trace elements, 500x	1 mL
Distilled water	449 mL
Agar-agar (3.5% final concentration)	17.5 g

Carbon source was either TDA or MDA (10 mM in agar plates, 2 mM in liquid cultures) or PU diol solution (3 g/L).

4. Resuspend 1 g of each soil sample in 9 mL of 0.9% NaCl (m/v) and then dilute it 1:10 with 0.9% NaCl (m/v), this solution can be stored at 4 °C
5. Prepare a dilution series (10^{-1}, 10^{-2} and 10^{-3}) of the stock solution (prepared in number 4) and use 100 µL for inoculation of agar plates prepared in number 1
6. Store the plates at 30 °C
7. Check the plates after 5 days of incubation, discard the plates completely covered with fungi. From the plates with fungi and bacteria, transfer the latter to fresh plates containing the same carbon source
8. Check after a few days if the transferred bacteria grew and repeat the transfer two or three times more
9. Choose bacteria that grew after each transfer and check their growth on agar plates not containing a carbon source to avoid isolation of autotrophic strains
10. For growth in liquid culture prepare the same media as mentioned above without the addition of agar-agar (Table 3)
11. Prepare stock solutions of 200 mM for TDA and MDA in 10 mL methanol. These solutions must be prepared and used the same week that you inoculate the cultures. In addition, it should be made sure beforehand that methanol cannot be used as a carbon source for the strains isolated
12. Use 250 mL sterile flasks with 50 mL mineral medium containing 2 mM of TDA or MDA (5 mL from stock solution prepared in number 11 in 50 mL total culture volume) or 3 g/L PU diol solution
13. Pick a colony of a strain of interest and inoculate a liquid culture. Perform the experiment in triplicates
14. Incubate the cultures at 30 °C and 106 rpm
15. Measure optical density (OD) daily in a UV/VIS spectrophotometer at a wavelength of 560 nm during 14 days
16. To test if TDA or MDA are not only carbon and energy but also nitrogen source for the isolated bacteria, prepare mineral media as described above but without the addition of NH_4Cl
17. Compare the obtained optical densities between N-deficient media and N-containing media

2.1.4 Safety considerations and standards

TDA and MDA are toxic substances. Avoid contact with skin and eyes as well as formation of dust and aerosols.

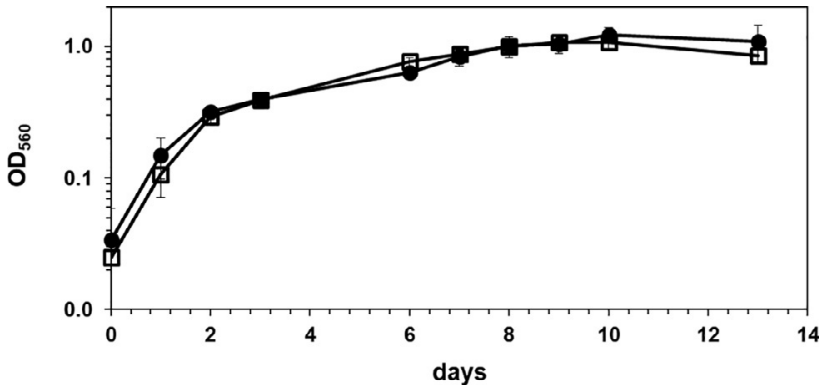

Fig. 2 Example for growth of isolated strain on 2 mM TDA in mineral medium containing an additional nitrogen source (filled circles) and in nitrogen-deficient mineral medium (empty squares). n = 3.

2.1.5 Analysis and statistics

In Fig. 2, optical density data from an isolated strain growing on 2 mM TDA in nitrogen-deficient and nitrogen-containing mineral media is shown (Cárdenas Espinosa et al., 2020). The growth yields are the same and from that, it can be derived that TDA not only serves as a carbon and energy but also as a nitrogen source for the isolated bacterial strain.

2.1.6 Alternative methods/procedures

In order to support the hypothesis that a bacterial strain was successfully enriched on a specific compound, a substrate concentration dependent growth yield test can be performed additionally to the protocol above. Here, the correlation between the concentration of TDA or MDA and the growth yields (OD values) of the isolated bacteria in the same phase of growth were compared. For example, when comparing 1 and 2 mM of TDA as substrate concentrations, the growth yield on 2 mM should roughly be twice the yield on 1 mM. Take into account that concentrations much higher than those used for isolating the strain could be toxic for the cells. For example lower OD values on high concentrations ($\geq$5 mM) of TDA or MDA in liquid cultures indicate that these concentrations are already toxic for the isolated bacteria.

2.2 Quantification of TDA degradation

2.2.1 Rationale

A further way to confirm the degradation of the PUR model compounds in the isolated strains is to measure their consumption via chromatographic methods. Here, a protocol is presented where TDA degradation was monitored by high performance liquid chromatography (HPLC) once the media were inoculated with the bacterial strains.

2.2.2 Materials, equipment and reagents

TDA (Sigma Aldrich, molar mass = 122.17 g/mol; 98% purity)
Methanol, Trimethylamine
Equipment for cultivation of bacterial isolates on TDA (see Section 2.1)
0.2 µm filters, for example, polyethersulfone membrane from Whatman™–GE Healthcare
Shimadzu HPLC with CBM-20A (communication bus module), SPD-M20A (photodiode array detector), CTO-20 AC (column oven), DGU-20A3 (degasser), LC- 20AB (liquid chromatograph) or equivalent
C18 column (LiChroCART® 125–4, RP-18e, 5 µm, Merck KGaA) or equivalent

2.2.3 Protocol

1. Inoculate 50 mL of 2 mM TDA media with a selected strain (see Section 2.1) and also prepare a sterile control
2. Collect 1 mL of the cultures daily for 14 days and perform $OD_{560\ nm}$ measurements as well to monitor the biomass growth
3. Prepare standards with TDA concentrations between 0.1 and 3 mM in mineral media to obtain a calibration curve
4. Centrifuge all samples (7 min at 15700 × *g*) and filter through a 0.2 µm polyethersulfone membrane
5. Quantify TDA concentrations by HPLC using a C18 column
6. HPLC program: Isocratic elution within 5 min with a mixture of 39.5% methanol, 59.5% distilled water, and 1% trimethylamine (flow rate: 0.65 mL/min; injection volume: 75 µL); temperature of the column constant at 25 °C. Detection was done with a photodiode array detector at 278 nm
7. Calculate TDA concentrations according to the calibration curve

2.2.4 Safety considerations and standards

TDA is a toxic and carcinogenic substance. Avoid contact with skin and eyes as well as formation of dust and aerosols.

2.2.5 Analysis and statistics

An example for TDA consumption in course of the cultivation is depicted in Fig. 3 and shows a depletion of 85% of the initial TDA concentration within 4 days. In contrast to that, the sterile control shows only a minor decrease in TDA concentration.

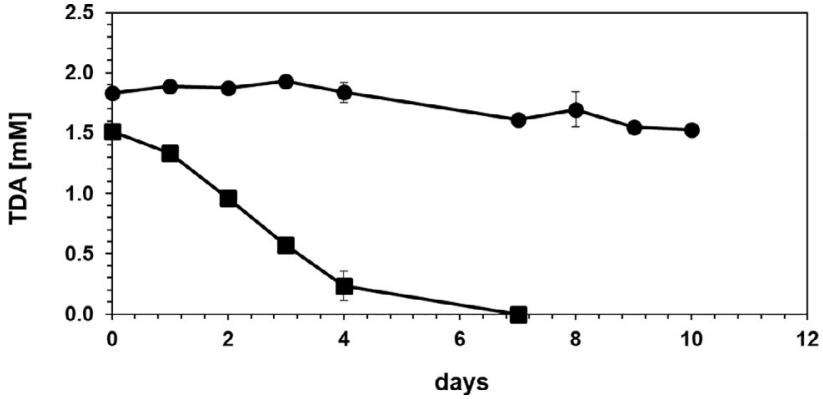

Fig. 3 Consumption of TDA measured via HPLC during the cultivation of an isolated strain on TDA as sole source of carbon and energy in mineral medium (filled squares) and in a sterile control containing TDA (filled circles). n = 3.

2.3 Quantification of PU diol degradation

2.3.1 Rationale

The proprietary composition of the commercially available PU diol solution makes it difficult to assess changes in its concentration during the course of cultivation with isolated bacteria. However, two methods were reported to solve this problem to a certain extent (Howard, Vicknair, & MacKie, 2001; Mukherjee et al., 2011), the determination of the concentration of the oligomeric PUR with high performance thin layer chromatography (HPTLC) and an agar plate assay.

2.3.2 Materials, equipment and reagents

PU diol solution (Sigma Aldrich; 88% m/m; proprietary composition), aliphatic, dihydroxy-functional oligomeric PUR

Ethyl acetate

Hexane

5% Ethanolic sulfuric acid

Equipment for cultivation of bacterial isolates on PU diol solution (see Section 2.1)

Aluminum-backed HPTLC sheets of 0.2 mm layers of silica gel 60 F_{254} (Merck) or equivalent

CAMAG® Linomat 5 (semi-automatic sample application system for bandwise spray-on application of the sample onto the chromatographic layer, including one dosing syringe 100 µL, 90–230 V) or equivalent

CAMAG® Twin Trough Chamber for 20 × 20 cm plates or equivalent

CAMAG® TLC Scanner 4 (densitometric evaluation of HPTLC plates, spectral range 190–900 nm, plate sizes up to 20 × 20 cm, absorbance and fluorescence mode, Software: CAMAG visionCATS) or equivalent

Separatory funnel

2.3.3 Protocol

1. Inoculate 50 mL of mineral media containing 3 g/L PU diol solution with a selected strain (see Section 2.1).
2. Collect 1 mL of the culture at different time points
3. Centrifuge the samples (7 min at 15700 × *g*).
4. Mix the supernatant equivoluminar with ethyl acetate in a separatory funnel, shake well, separate and continue with the ethyl acetate phase
5. Apply the extract of the supernatant (10 µL) on aluminum-backed HPTLC sheets of 0.2 mm layers of silica gel 60 F_{254} using the CAMAG® Linomat 5 sample applicator
6. Perform the chromatographic run in a CAMAG® Twin Trough Chamber, saturated with ethyl acetate and hexane (3:1).
7. Evaporate the solvent from the plates and incubated into freshly prepared solution of 5% ethanolic sulfuric acid and heat for 3 min at 150 °C
8. Wait for color development on the plates and then scan them in absorbance/transmittance mode at 450 nm with a scan speed of 40 mm/s using CAMAG® TLC Scanner 4

2.3.4 Analysis and statistics

In case of a successful PU diol degradation silica plates with samples of a later time point in the course of cultivation show lower values at 450 nm than those at the beginning of the cultivation. Examples can be seen in Mukherjee et al. (2011).

2.3.5 Alternative method

1. Prepare agar plates containing 50 mM phosphate buffer (pH 7), PU diol solution (3 g/L) and 0.001% (w/v) rhodamine B (results in an opaque, pink colored, solid medium).
2. Cut wells of 1 cm diameter in the agar plates and load either with cell suspensions or cell extracts
3. Incubate the agar plates at 37 °C for up to 72 h
4. Irradiate plates with UV light at 350 nm. PU cleavage activity can be detected as orange fluorescent halos surrounding the wells

3. Outlook

Further studies on the isolated strains like differential gene expression studies and transcriptomics as well as proteomics should be conducted to elucidate the degradation pathway of PUR monomers as well as the cleavage

of PUR oligomers and polymers. Information on the (extracellular) enzymes and metabolites involved could provide valuable information to foster biotechnical approaches to treat plastic waste.

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2.2 Toward Biorecycling: Isolation of a soil bacterium that grows on a polyurethane oligomer and monomer

María Jose Cárdenas Espinosa, Andrea Colina Blanco, Tabea Schmidgall, Anna Katharina Atanasoff-Kardjalieff, Uwe Kappelmeyer, Dirk Tischler, Dietmar H. Pieper, Hermann J. Heipieper and Christian Eberlein

Helmholtz Centre for Environmental Research—UFZ, Department of Environmental Biotechnology, Leipzig, Germany

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Toward Biorecycling: Isolation of a Soil Bacterium That Grows on a Polyurethane Oligomer and Monomer

María José Cárdenas Espinosa[1], Andrea Colina Blanco[1], Tabea Schmidgall[1], Anna Katharina Atanasoff-Kardjalieff[1], Uwe Kappelmeyer[1], Dirk Tischler[2], Dietmar H. Pieper[3], Hermann J. Heipieper[1] and Christian Eberlein[1]*

[1] Department of Environmental Biotechnology, Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany, [2] Interdisciplinary Ecological Center, TU Bergakademie Freiberg, Freiberg, Germany, [3] Microbial Interactions and Processes Research Group, Helmholtz Centre for Infection Research – HZI, Braunschweig, Germany

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Ren Wei,
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Weiliang Dong,
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***Correspondence:**
Hermann J. Heipieper
hermann.heipieper@ufz.de

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The fate of plastic waste and a sustainable use of synthetic polymers is one of the major challenges of the twenty first century. Waste valorization strategies can contribute to the solution of this problem. Besides chemical recycling, biological degradation could be a promising tool. Among the high diversity of synthetic polymers, polyurethanes are widely used as foams and insulation materials. In order to examine bacterial biodegradability of polyurethanes, a soil bacterium was isolated from a site rich in brittle plastic waste. The strain, identified as *Pseudomonas* sp. by 16S rRNA gene sequencing and membrane fatty acid profile, was able to grow on a PU-diol solution, a polyurethane oligomer, as the sole source of carbon and energy. In addition, the strain was able to use 2,4-diaminotoluene, a common precursor and putative degradation intermediate of polyurethanes, respectively, as sole source of energy, carbon, and nitrogen. Whole genome sequencing of the strain revealed the presence of numerus catabolic genes for aromatic compounds. Growth on potential intermediates of 2,4-diaminotoluene degradation, other aromatic growth substrates and a comparison with a protein data base of oxygenases present in the genome, led to the proposal of a degradation pathway.

Keywords: plastic, biorecycling, *Pseudomonas*, polyurethane, diaminotoluene, aromatics degradation, aromatic diamines

INTRODUCTION

Plastics are heavily used in our modern society and the global production rates increase since decades. With about 3.5 million tons polyurethanes were the fifth most demanded synthetic polymers in Europe in 2015 (Plasticseurope, 2016). The uses of polyurethanes are manifold with the major field of application being insulation materials. Common precursors used to synthesize polyurethanes are polyisocyanates and polyols together with additives such as catalysts, cross linkers and chain extenders, among others. Despite forming urethane bonds with the polyisocyanates, polyols additionally can contain ether or ester bonds, resulting

in polyether or polyester polyurethanes, respectively. On the other hand, the polyisocyanate compounds can have an aliphatic, polycyclic or aromatic nature. Two of the most widely used diisocyanates for PU synthesis are 4,4′-methylene diphenyl diisocyanate (MDI) and toluene-2,4-diisocyanate (TDI) and their precursors 4,4′-diaminodiphenylmethane (MDA) and 2,4-diaminotoluene (2,4-TDA), respectively. Next to an alcohol and carbon dioxide, primary amines are also formed after chemical hydrolysis of the urethane bond (Marchant et al., 1987).

Post-consumer plastics are already a major challenge for the environment and will be an even bigger one in the future. The biodegradation is often hampered by the durability, crystallinity and macroscopic structure of the polymers. For polyurethanes, the diverse chemical composition increases the obstacles for both, biological and chemical recycling. Reports on the degradation of polyurethanes mostly focus on polyester-based ones, fungal as well bacterial and enzymatic hydrolysis were reported (Wang et al., 1997; Russell et al., 2011; Krasowska et al., 2012; Shah et al., 2013; Magnin et al., 2019). The biodegradation of polyether-based PU is far less documented and was usually achieved by fungal activity (Matsumiya et al., 2010; Álvarez-Barragán et al., 2016).

The biodegradation of synthetic polymers in general is a two-step process. It involves the attack by extracellular enzymes overcoming the macromolecular structure of the polymers and providing monomers and oligomers for the second step, which is the mineralization of the latter inside the cell. The two steps can be carried out by a single species, or more likely by at least two. Regularly, aromatic monomers are released by the activity of extracellular enzymes. During microbial degradation of aromatic compounds typically mono- and dioxygenases are involved in ring hydroxylation and cleavage. The hydroxylation of the aromatic ring results in catecholic compounds (with at least two adjacent hydroxyl groups) reducing the aromatic character of the compound and facilitating the oxygenolytic cleavage of the ring. The latter can be intradiolic (*ortho*-cleavage) or extradiolic (*meta*-cleavage).

Studies that identified the products of PU hydrolysis found the diamines TDA and MDA (Matsumiya et al., 2010; Cregut et al., 2013; Magnin et al., 2019). Both amines have been proposed in the European Chemicals Agency to be identified as "Substances of Very High Concern," specifically in the category of "Carcinogenic, Mutagenic or toxic to Reproduction" (European Chemicals Agency, 2019). The carcinogenicity of TDA compounds was demonstrated with experimental studies in animals (Baua, 2008). To understand the fate of the diamines released from PU degradation and in order to investigate the monomer and oligomer metabolism in plastics degradation in general, we screened for bacteria capable to degrade both, 2,4-TDA and PU oligomer (Polyurethane diol solution, Sigma-Aldrich). From a site rich in brittle plastic waste, a *Pseudomonas* species was isolated on 2,4-TDA and positively tested for growth on the PU oligomer as the sole source of carbon and energy. Genome sequencing and the screening for potential carbon substrates led to a hypothetic degradation pathway of 2,4-TDA in the isolated *Pseudomonas* strain.

MATERIALS AND METHODS

Growth Conditions

The bacteria were grown in mineral media, as reported before (Hartmans et al., 1989), containing the following compounds (per liter demineralized water): 7 g $Na_2HPO_4 \times 2\ H_2O$; 2.8 g KH_2PO_4; 0.5 g NaCl; 0.1 g NH_4Cl; 0.1 g $MgSO_4 \times 7\ H_2O$; 10 mg $FeSO_4$; 5 mg $MnSO_4$; 6.4 mg $ZnCl_2$; 1 mg $CaCl_2 \times 6\ H_2O$; 0.6 mg $BaCl_2$; 0.36 mg $CoSO_4 \times 7\ H_2O$; 0.36 mg $CuSO_4 \times 5\ H_2O$; 6.5 mg H_3BO_3; 10 mg EDTA; 146 µl HCl (37%). The nitrogen-deficient mineral media did not contain NH_4Cl. As sole source of carbon and energy either 4 g/l disodium succinate (Sigma-Aldrich), 2 mM 2,4-TDA (Sigma-Aldrich) or 3 g/l PU oligomer (Sigma-Aldrich, dihydroxy-functional oligomer, aliphatic urethane of proprietary composition) was added. For growth on solid media 3.5% of agar was added. Cells were cultivated in 50 ml shaking cultures at 30°C at 150 rpm. All chemicals used were reagent grade and obtained from commercial sources. Optical density was measured at a wavelength of 560 nm (Perkin Elmer, Lambda 2S). Toluene, benzene, aniline, 2,4-dihydroxytoluene (4-methylresorcinol), methylsuccinate, sodium benzoate, 2-aminobenzoate (anthranilate), phenol, *o*-xylene, catechol, 4-methylcatechol and benzene-1,2,4-triol (hydroxyhydroquinone) were tested if they serve as sole source of carbon and energy for the isolated bacteria in 100, 200, and 300 mg/l concentrations and OD at 560 nm was measured to evaluate growth.

Bacterial Strain Isolation and Identification

For the isolation of bacteria from soil, three samples from a site rich in brittle plastic waste (Paunsdorf, Leipzig, Germany) were used. 1 g of each sample was dissolved in 9 mL of NaCl 0.9% m/V, diluted 1:10 and stored at 4°C. Afterward, dilution series of 10^{-1}, 10^{-2}, and 10^{-3} were prepared. 150 µL of the diluted soil solutions were added to agar plates containing mineral medium and different concentrations of 2,4-TDA (2, 5, and 10 mM) as sole carbon and energy source. The plates were stored at 30°C. After 5 days of incubation bacteria were transferred to fresh plates, agar plates without carbon source were used as control. The complete 16S rRNA gene sequence was obtained from the TDA1 genome and used for an alignment with other known Pseudomonas species by making use of the RDP data base (Wang et al., 1997).

Toxicity Test for 2,4-TDA

In order to test the toxic effect of 2,4-TDA on the isolated strain during growth with the readily metabolizable carbon source disodium succinate (4 g/L), 2,4-TDA was added at different concentrations to exponentially growing cultures as described earlier (Heipieper et al., 1995). The control was a culture growing with succinate as the carbon source without the addition of 2 4-TDA.

Membrane Lipid Fatty Acid Composition

The membrane fatty acid profile for selected strains was obtained. For the phospholipid fatty acids (PLFA) extraction, bacterial cells were harvested from an overnight culture and then centrifuged

for 7 min at 13000 rpm. The pellet was washed with 1.5 mL of 10 mM KNO_3, centrifuged and PLFA extraction was done as reported before (Bligh and Dyer, 1959), methylation was achieved by addition of 0.6 mL of 20% boron trifluoride in methanol (Morrison and Smith, 1964). The identification and quantification of the fatty acid methyl esters (FAME) was done using gas chromatography with flame ionization detector (GC-FID, Agilent Technologies, 6890N Network GC System, 7683B Series Injector). A CP-Sil 88 column (Varian CP7488) was used as stationary phase and helium as carrier gas. The temperature ramp programmed was: 2 min 40°C isotherm, a gradient increase to 220°C (8°C × min^{-1}) and 10 min 220°C isotherm.

Genome Sequencing of Selected Strain

Genomic DNA was extracted (DNeasy® Blood & Tissue Kit, QIAGEN) according to the manufacture's protocol for Gram-negative strains. The quantity of extracted DNA was checked by nanodrop followed by the library preparation with the Nextera XT DNA library kit (Illumina, San Diego, CA, United States). The library was checked with an Agilent technology Bioanalyzer 2100. Paired-end libraries were sequenced using Illumina v3 chemistry on a Illumina MiSeq sequencer with a 250-bp paired-end protocol according to the manufacturer's instructions. The sequencing reads were demultiplexed by MiSeq reporter software (Illumina). The draft genome sequences were assembled using the Velvet assembly program (Zerbino and Birney, 2008). The RAST queue (Aziz et al., 2008) was used to annotate by using *P. putida* KT2440 as reference strain. For the annotation of dioxygenases the AROMADEG data base was used in addition (Duarte et al., 2014). To reveal similarities to known enzymes (mono- and dioxygenases, enzymes involved in aromatics degradation) amino acid sequences of genes present in the genome of TDA1 were compared to UniprotKB database or by using the basic local alignment search tool (BLAST) data base in NCBI as reported before (Altschul et al., 1997). The suggestion of genes possibly involved in the degradation was based on significant amino acid sequence similarities, i.e., a high coverage (at least 80%) and similarity (at least 30%) as well as a low *E* value (1×10^{-8} or lower) given by BLAST when compared to the sequences to known and described enzymes. Dioxygenases or enzymes with an aromatic substrate were analyzed mainly by deploying the AROMADEG data base.

HPLC Measurements

2,4-TDA degradation was monitored by measuring the decrease in concentration. The experiment was performed in triplicates. 50 mL of 2 mM 2,4-TDA media were inoculated with the isolated bacterial strain. 1 mL of the culture was collected and mixed with an equal amount of methanol. A calibration curve for the concentrations between 0.1 mM and 3 mM of 2,4-TDA was prepared. All the samples were centrifuged (7 min, 13000 rpm) at room temperature and filtered through a 0.45 µm polyethersulfone membrane syringe filter (Whatman™-GE Healthcare). 75 µl of the sample was analyzed by high performance liquid chromatography (HPLC; LC- 20AB, Shimadzu). All the samples and standards were measured using a C18 column (LiChroCART® 125-4, RP-18e, 5 µm, Merck KGaA). Isocratic elution of 2,4-TDA was conducted with 39.5% methanol, 59.5% distilled water and 1.0% triethylamine at a flow rate of 0.65 ml min^{-1} (Freedman et al., 1996). The temperature of the column was kept constant at 25°C. Detection was done with a photodiode array detector, using a deuterium lamp as light source, at 278 nm (SPD-M20A, Shimadzu).

RESULTS

The screening performed with soil samples taken from a site rich in brittle plastic waste led to the isolation of two bacterial strains that grew on agar plates containing mineral medium with 2,4-TDA as sole carbon and energy source and showed growth in liquid media containing 2 mM 2,4-TDA. Any isolated bacteria that did grow on agar plates without any carbon source were discarded to exclude autotrophic growth on 2,4-TDA agar plates. One strain, named TDA1, was chosen for further investigations. **Figure 1** shows the growth of the TDA1 isolate on 2 mM 2,4-TDA as sole carbon and energy source. The growth rate was 0.04 h^{-1} corresponding to a generation time of 14 h^{-1} during exponential growth phase. The degradation of 2,4-TDA was quantified using HPLC. The 2,4-TDA was consumed by the bacterial strain whereas the sterile control only shows a minor decrease in 2,4-TDA concentrations (**Figure 1**). 2,4-TDA at a concentration of 2 mM was shown to be the optimal concentration, because lower and higher concentrations yielded lower optical densities (data not shown). This was also verified in toxicity tests where 2,4-TDA was added to cells growing exponentially with succinate as carbon and energy source (**Figure 2**). The growth rate with succinate in the presence of 2 mM 2,4-TDA was reduced by 55% compared to the untreated control whereas higher concentrations caused significantly higher growth inhibition.

Remarkably, strain TDA1 was also able to grow in a nitrogen-deficient mineral media containing only 2,4-TDA

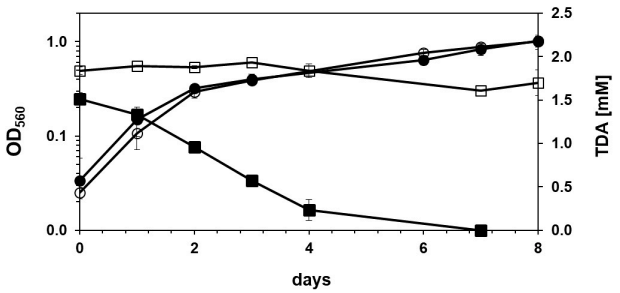

FIGURE 1 | Growth of *Pseudomonas* sp. TDA1 on 2,4-TDA. Circles: Growth of *Pseudomonas* sp. TDA1 on 2 mM 2,4-TDA as sole source of carbon and energy in mineral medium containing an additional nitrogen source (filled circles) and in nitrogen deficient mineral media (empty circles). Squares: Consumption of 2,4-TDA measured via HPLC during the course of cultivation of *Pseudomonas* sp. TDA1 on 1.5 mM 2,4-TDA as sole source of carbon and energy in mineral medium (filled squares) or in a sterile control containing 1.8 mM 2,4-TDA (empty squares) *n* = 3.

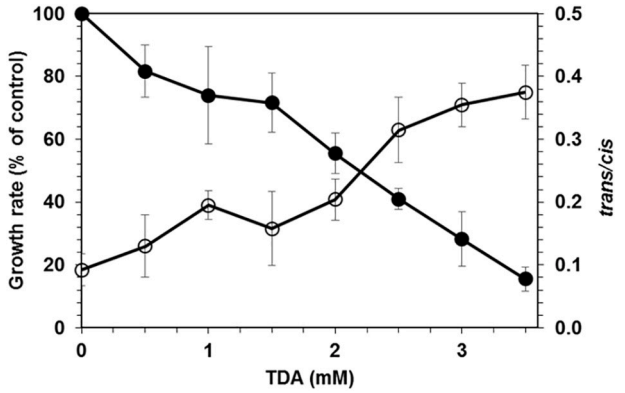

FIGURE 2 | Effect of 2,4-TDA on *P. putida* KT2440. Filled circles: Effect of 2,4-TDA on growth of *P. putida* KT2440. The growth rate after the addition of 2,4-TDA to exponentially growing cells is given relative to a control without 2,4-TDA. Empty circles: Effect of different 2,4-TDA concentrations on the *trans/cis* ratio of unsaturated fatty acids of *P. putida* KT2440.

as sole carbon and nitrogen source. Optical densities were similar to those obtained with ammonium chloride as nitrogen source (**Figure 1**). Next to 2,4-TDA also other (aromatic) compounds were tested if they serve as sole source of carbon and energy for the isolated strain. Toluene, benzene, aniline, 2,4-dihydroxytoluene and methylsuccinate did not support growth of the TDA1 strain, whereas benzoate, 2-aminobenzoate (anthranilate), phenol, *o*-xylene, catechol, 4-methylcatechol and benzene-1,2,4-triol served as a growth substrate (**Table 1**). In addition, the strain grew on an aliphatic oligomeric PU substrate of proprietary composition (PU diol solution, Sigma-Aldrich). Optical densities of about 0.8 were obtained with a concentration of 3 g/l (about 9 mM) of the oligomeric PU as sole carbon and energy source (data not shown).

The whole genome sequence has been deposited at DDBJ/ENA/GenBank under the accession WOVH00000000. The version described in this paper is version WOVH01000000. The gene locus tag is GNP06_XXXXX, the corresponding five-digit number is given in the text for each gene mentioned. Using the complete 16S rRNA gene sequence (gene 02555), the strain was identified as *Pseudomonas* sp. strain that shows high similarity with *P. oryzihabitans* and various *P. putida* strains. The strain TDA1 will be referred to as *Pseudomonas* sp. TDA1 in this paper. In addition, the phospholipid fatty acid profile of the strain TDA1 showed the presence of the following fatty acids: C14:0, C16:0, C16:1*trans*, C16:1*cis*, 17*cyclo*, C18:0, C18:1*trans*, C18:1*cis*, and 19*cyclo* (data not shown) comprising more than 95% of the total fatty acids of the strain. The fatty acid composition and pattern of TDA1 was the same as the one of strain *P. putida* KT2440 which was used as a control and benchmark. In addition, the gene for the *cis-trans* isomerase of unsaturated fatty acids (CTI), an important marker gene for the genus *Pseudomonas* (Palleroni, 2015; Eberlein et al., 2018), is present in the TDA1 genome (gene 13840) revealing more than 90% amino acid sequence identity with several *Pseudomonas* CTIs already present in the protein BLAST database (for example: Accession numbers Q8RJN7, A0A059V043, and F8FYU0). Also, the CTI phenotype, regularly given as solvent stress-depending increase in the *trans/cis* ratio of unsaturated fatty acids, was detected in the presence of 2,4-TDA in *P. putida* KT2440 (**Figure 2**).

Among pathways for degradation of central catecholic intermediates, genes encoding enzymes of the catechol branch of the 3-oxoadipate pathway (catechol 1,2-dioxygenase, muconate cycloisomerase and muconolactone isomerase, genes 25335, 25340, 25345) as well as those encoding the protocatechuate branch (α- and β-subunit of protocatechuate 3,4-dioxygenase, 3-carboxymuconate cycloisomerase and 4-carboxymuconolactone decarboxylase; genes 17435, 17430, 07520, and 07510) and the respective 3-oxoadipate enol-lactone hydrolases (genes 20490 and 07515, respectively) were identified. In addition, genes encoding enzymes for the formation of homogentisate (4-hydroxyphenylpyruvate dioxygenases, genes 05520 and 05730) and a homogentisate 1,2-dioxygenase pathway (genes 17645, 17650, and 17655) as well as a homoprotocatechuate pathway including a LigB type 3,4-dihydroxyphenylacetate 2,3-dioxygenase (gene 05110) were observed. Genes encoding enzymes of the corresponding *meta*-cleavage pathway for homoprotocatechuate were found: 5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase (gene 05115), 5-carboxymethyl-2-hydroxymuconate isomerase (gene 05105), 5-carboxymethyl-2-oxo-hex-3-ene-1,7-dioate decarboxylase (genes 05120 or 05125), and 2-oxo-hepta-3-ene-1,7-dioic acid hydratase (gene 05095). An additional dioxygenase was identified (gene 06545) which according to AROMADEG (Duarte et al., 2014) belongs to a family of extradiol dioxygenases of the vicinal oxygen chelate superfamily of extradiol dioxygenases comprising, among others, enzymes using miscellaneous substrates such as 2,3-dihydroxybenzoate and clustered with proteobacterial extradiol dioxygenases of unknown function (comprising among others YP_046462, an extradiol dioxygenase of *Acinetobacter baylyi* ADP1).

Genes coding for archetype catechol 2,3-dioxygenases such as *xylE, catE,* or *nahA*, extradiol dioxygenases belonging to family I.2 of the vicinal oxygen chelate superfamily, showing a

TABLE 1 | Growth spectrum for *Pseudomonas* sp. TDA1.

Carbon source	Growth
Toluene	–
Benzene	–
Aniline	–
2,4-Dihydroxytoluene (4-Methylresorcinol)	–
Methylsuccinate	–
Sodium benzoate	+
2-Aminobenzoate (Anthranilate)	+
Phenol	+
o-Xylene	+
Catechol	+
4-Methylcatechol	+
Benzene-1,2,4-triol (Hydroxyhydroquinone)	+

Aromatic substrates that were tested as sole source of carbon and energy for strain TDA1. Plus, growth. Minus, no growth.

preference for monocyclic substrates and specifically cluster in XXII according to the revised phylogeny of AROMADEG (Eltis and Bolin, 1996; Vaillancourt et al., 2004; Pérez-Pantoja et al., 2009), are not present in the genome of TDA1. Neither ring cleaving dioxygenases involved in aminoaromatic degradation like 5-aminosalicylate 1,2 dioxygenase (Stolz and Knackmuss, 1993), 2-aminophenol 1,6-dioxygenase (Takenaka et al., 1997; Wu et al., 2005) nor hydroxybenzoquinol 1,2-dioxygenase (Travkin et al., 1997; Kitagawa et al., 2004; Wang et al., 2007; Pérez-Pantoja et al., 2009) are encoded in the TDA1 genome.

At least seven genes encoding putative α-subunits of Rieske non-heme iron dioxygenases are present in the genome of TDA1. They were analyzed using AROMADEG (Duarte et al., 2014): it was shown, that genes 26235, 17905, and 06615 are distantly related to enzymes of the phthalate family of Rieske dioxygenases. Gene 26235 probably encodes a vanillate O-demethylase with 76.2% amino acid sequence similarity to P12609 from *Pseudomonas* strain ATCC 19151. The product of gene 06615 shows significant amino acid sequence similarity of 47% with toluene 4-sulfonate monooxygenase TsaM1 (accession P94679) from *Comamonas testosteroni* T-2 (Locher et al., 1991a,b). Among enzymes of documented function, also the product of gene 17905 shows similarity to toluene 4-sulfonate monooxygenase TsaM1, however, only to a low extent of 33%. The gene product of 06600 clearly is a member of the phthalate family of Rieske oxygenases. According to AROMADEG, it belongs to a cluster comprising putative phthalate 4,5-dioxygenase from *Ralstonia eutropha* JMP134 (accession YP298987). Gene 19420 encodes a protein with 73.8% similarity with CntA carnitine monooxygenase (accession D0C9N6) of *Acinetobacter baumannii* ATCC 19606 and thus may be responsible for carnitine transformation to form trimethylamine and malic semialdehyde. The protein encoded by gene 25270 belongs to cluster I of the benzoate family of Rieske dioxygenases (enzymes involved in indole acetic acid degradation and related enzymes). Gene 08315 encodes a benzoate 1,2-dioxygenase (cluster XI, benzoate and 2-chlorobenzoate dioxygenases of the benzoate family of Rieske dioxygenases) with 97.1% identity with BenA of *P. putida* GJ31 (accession AAX47023).

Neither gene clusters encoding proteins involved in the side-chain oxidation of methyl-substituted aromatics, namely the two-component xylene/*p*-cymene monooxygenase, which consist of a hydroxylase related to AlkB alkane hydroxylase and a reductase (Worsey and Williams, 1975; Eaton, 1996) were observed in the genome, nor are multicomponent soluble diiron benzene/toluene or phenol/methylphenol monooxygenases encoded. However, five genes coding for flavin depending monooxygenases were detected (genes 05080, 17225, 06905, 06505, 06585). Gene products of 05080 and 06585 show high amino acid sequence similarity to 4-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii* (accession Q6Q272) of 72.1% and 72.6%, respectively (Thotsaporn et al., 2004). The product of gene 17225 exhibits high sequence identity to documented 4-hydroxybenzoate 3-monooxygenases such as the enzymes P00438 from *P. fluorescens* (74.9%) or P20586 from *P. aeruginosa* (74.6%). In contrast to that, the function of flavin monooxygenases 06505 and 06905 remains unknown.

The release of nitrogen from aromatic amines can occur before ring cleavage in form of ammonia (Aoki et al., 1983; Chang et al., 2003; Takenaka et al., 2003), but also after ring opening (Takenaka et al., 2000). The latter is done by 2-aminomuconate deaminase during 2-aminophenol degradation by *Pseudomonas* sp. AP-3. This enzyme belongs to the YjgF/YER057c/UK114 family (also known as the Rid family). Five members of this family were observed to be encoded in the genome of the strain TDA1 (genes 01225, 03255, 14860, 17920, 05035). For two of these gene products significant similarities to 2-aminomuconate deaminase of *Pseudomonas* sp. AP–3 (accession Q9KWS2) could be documented: 36% for the gene product of 14860 and 32% for the gene product of 05035.

DISCUSSION

A bacterial strain capable of degrading both, an oligomeric PU and a PU building block was obtained from soil samples. According to our knowledge, this is the first report on the isolation of a bacterial pure culture for the polyurethane precursor 2,4-TDA. A powerful metabolic potential of the strain is given because of the ability to use both as sole source of carbon and energy, a monomer and an oligomer of PU. 2,4-TDA was used not only as the carbon and but also as a nitrogen source. That concentrations higher than 2 mM 2,4-TDA did not increase the optical densities further, might be due to toxic effect. Also for *P. putida* KT2440 it was shown, that concentrations above 2 mM 2,4-TDA diminished growth. The isolate was identified as *Pseudomonas* sp. strain by 16S rRNA gene sequence analysis and by comparing the fatty acid profile to the one of *P. putida* KT2440. The isolation of a *Pseudomonas* strain from the same oligomeric PU material was reported before (Mukherjee et al., 2011). Moreover, microbial attack on polyurethanes by species of the genus *Pseudomonas* was documented earlier (Howard and Blake, 1998; Howard, 2002; Gautam et al., 2007; Peng et al., 2014; Hung et al., 2016). The fact that PU polymers or components do not only meet the carbon but also the nitrogen demand was confirmed in this study. Earlier reports also had shown that polyisocyanates may serve as nitrogen source (Darby and Kaplan, 1968; Crabbe et al., 1994; Nakajima-Kambe et al., 1995; Kloss et al., 2009).

Considering the genomic potential and the substrate spectrum a degradation pathway for 2,4-TDA with candidate genes encoding the enzymes involved can be suggested (**Figure 3**). Although also a monooxygenation of an aromatic ring lacking hydroxyl groups has been reported in the case of styrene (Beltrametti et al., 1997), an initiation of the degradation of not yet activated aromatics by flavin monooxygenases is rather unlikely (Van Berkel et al., 2006). In contrast to that, hydroxylation of substituents at the aromatic ring, like the methyl group of toluene, is common (Assinder and Williams,

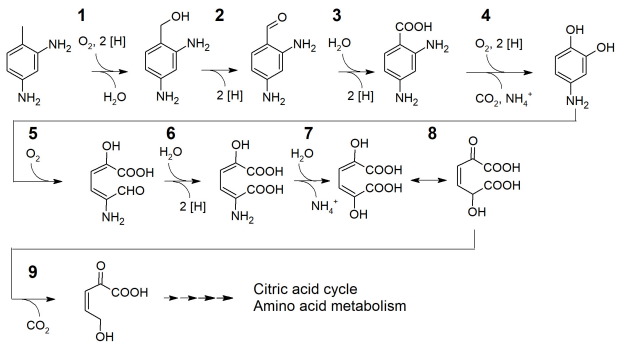

FIGURE 3 | Proposed degradation pathway including extradiol cleavage of 4-aminocatechol for 2,4-TDA in the putative *Pseudomonas* sp. TDA1. **1**: Toluene 4-sulfonate monooxygenase (candidate gene GNP06_06615) with toluene-4-sulfonate monooxygenase reductase (candidate gene GNP06_06620) and subsequent alcohol dehydrogenase **(2)** and aldehyde dehydrogenase **(3)** activity (encoded elsewhere in the genome). **4**: Benzoate 1,2-dioxygenase with anthranilate dioxygenase activity (candidate genes for α- and β-subunit as well as a ferredoxin reductase component GNP06_08305, GNP06_08310 and GNP06_08315). **5**: Extradiol 2,3-dioxygenase (candidate gene GNP06_06545 or GNP06_05110). **6**, **8** and **9**: Enzymes for the homoprotocatechuate *meta*-cleavage pathway (candidate genes GNP06_05115, GNP06_05105 and GNP06_05120/25). **7**: 4-amino-2-hydroxymuconate deaminase (candidate genes GNP06_14860 or GNP06_05035).

1990). However, strain TDA1 does not grow on toluene (**Table 1**) and the only putative methyl group oxidizing enzymes encoded are those with similarity to toluene 4-sulfonate monooxygenase TsaM1 (accession P94679) from *Comamonas testosteroni* T-2 (Locher et al., 1991a,b). Therefore, it can be assumed that the methyl group is hydroxylated to a primary alcohol (candidate gene 06615) with the help of an electron transferring unit. For the latter, a gene encoding for a protein sharing 48.1% sequence similarity with toluene-4-sulfonate monooxygenase reductase subunit TsaB1 (accession P94680) in *Comamonas testosteroni* is located adjacent to 06615. Obviously, the methyl oxidizing enzyme present needs a substituent in *para* position on the aromatic ring to function as the strain cannot grow on toluene. The following steps yielding 2,4-diaminobenzoate (4-aminoanthranilate) would be catalyzed by an alcohol dehydrogenase and subsequently by an aldehyde dehydrogenase encoded elsewhere in the genome. Strain TDA1 uses anthranilate as the sole source of carbon and energy which typically is catalyzed by an anthranilate 1,2-dioxygenase (Cain, 1968; Eby et al., 2001; Schühle et al., 2001; Chang et al., 2003; Liu et al., 2010; Costaglioli et al., 2012; Kim et al., 2015). No such enzyme is encoded in the genome of TDA1. However, some benzoate dioxygenases are reported to transform anthranilate to catechol (Yamaguchi and Fujisawa, 1980; Haddad et al., 2001) and a gene cluster encoding a benzoate 1,2-dioxygenase α- and β-subunit as well as a ferredoxin reductase component (genes 08305, 08310, 08315) is conserved in the genome. It is therefore conceivable that 2,4-diaminobenzoate is transformed by benzoate 1,2-dioxygenase yielding 4-aminocatechol as central intermediate.

Studies showed that aromatic compounds with electron-donating substituents, such as amino groups, are preferably degraded via the *meta*-cleavage pathway (Ribbons, 1965; Seidman et al., 1969; Bugg and Ramaswamy, 2008; Shukla et al., 2016). It can therefore be speculated that the putative intermediate 4-aminocatechol is transformed by an extradiol dioxygenase; and a respective extradiol dioxygenase of the vicinal chelate superfamily is actually encoded in the genome (candidate gene 06545). A second extradiol dioxygenase, a homoprotocatechuate 2,3-dioxygenase of the LigB superfamily (Roper and Cooper, 1990), is encoded by gene 05110 located within a gene cluster encoding enzymes for the further metabolism of the homoprotocatechuate ring-cleavage product via the *meta*-cleavage pathway. Several publications state that homoprotocatechuate 2,3-dioxygenase is promiscuous and may accept 4-nitrocatechol as a substrate (Groce et al., 2004; Henderson et al., 2012; Kovaleva and Lipscomb, 2012; Mbughuni et al., 2012). If the 06545 extradiol dioxygenase or a promiscuous homoprotocatechuate dioxygenase is involved in 2,4-TDA degradation by strain TDA1 remains to be elucidated. Further degradation of the putative ring-cleavage product 4-amino-2-hydroxymuconate semialdehyde may then be performed by homoprotocatechuate *meta*-cleavage pathway enzymes with 5-carboxymethyl-2-hydroxymuconic semialdehyde dehydrogenase encoded by gene 05115 forming 4-amino-2-hydroxymuconate. As the next step, the formed 4-amino-2-hydroxymuconate could be deaminated by an aminomuconate deaminase (candidate genes 14860 or 05035) similar to the deamination after ring cleavage in the degradation pathway of aminophenol in *Pseudomonas* sp. AP-3 (Takenaka et al., 2000) or in nitrobenzene degradation in *Pseudomonas pseudoalcaligenes* JS4 (He and Spain, 1997). For the latter, the enzyme 2-aminomuconate deaminase does not depend on cofactors and deamination of its substrate even happens spontaneously in acidic environments (Ichiyama et al., 1965). In the metabolization of 4-amino-3-hydroxybenzoic acid in *Bordetella* sp. 10d the amino group is cleaved off already from the muconic semialdehyde intermediate by a 2-amino-5-carboxymuconicsemialdehyde deaminase (Orii et al., 2006). The resulting intermediate 2,5-dihydroxy-muconate probably undergoes tautomerization (gene 05105) and could be further subjected to a decarboxylation step (gene 05120 or 05125). Following the *meta*-cleavage pathway, a hydroxylation would take place after the decarboxylation and the corresponding hydratase is also present in the genome of TDA1 (gene 05095). However, how exactly the degradation pathway is continued to lead to central metabolites of the citric acid cycle or amino acid metabolism needs to be elucidated in further studies.

To sum up, a preliminary degradation pathway of 2,4-TDA is proposed. In the peripheral pathway 4-aminocatechol is formed after oxidation of the methyl group of diaminotoluene and subsequent dioxygenation with concomitant decarboxylation and deamination. Ring cleavage of 4-aminocatechol in TDA1 would be possible in an extradiol manner (candidate gene 06545) and further employment of the homoprotocatechuate

meta-pathway (genes 05115, 05105, 05120/25) with the second deamination potentially taking place after the formation of 5-amino 2-hydroxymuconate (candidate genes 14860 or 05035).

The majority of the enzymes involved in the proposed pathway must be promiscuous regarding their substrate specificity, i.e., they need to accept especially amino substituted analogs. Due to the low steric hindrance of an additional amino group substrate promiscuity might be favored. Enzymes involved in aromatics degradation exhibiting significant activity with substituted substrate analogs were reported before (Pascal and Huang, 1986; Smith et al., 1990; He and Spain, 1997; Eby et al., 2001; Chang et al., 2003; Guzik et al., 2011). However, the proposed degradation pathway of 2,4-TDA in the putative *Pseudomonas* strain TDA1 needs further confirmation via proteomic, transcriptomic analysis or *in vitro* assays with potential intermediates of the proposed pathway. Identifying the key enzymes for the degradation of both, 2,4-TDA as putative degradation product as well as precursor of PUs (Matsumiya et al., 2010; Magnin et al., 2019) and for the oligomeric PU could help to equip well known and biotechnological used lab strains like *P. putida* KT2440 for monomer degradation in two-step biorecycling processes.

DATA AVAILABILITY STATEMENT

The whole genome sequence has been deposited at DDBJ/ENA/GenBank under the accession WOVH00000000. The version described in this article is version WOVH01000000.

AUTHOR CONTRIBUTIONS

MC, CE, UK, and HH conceived and designed the experiments. MC, AC, TS, and AA-K performed the experiments. DT performed the genome sequencing and annotation. DP, UK, CE, and HH analyzed the data. HH and CE contributed reagents, materials, and analysis tools. MC, CE, DP, and HH wrote the manuscript.

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2.3 An optimized method for RNA extraction from the polyurethane oligomer degrading strain *Pseudomonas capeferrum* TDA1 growing on aromatic substrates such as phenol and 2,4-diaminotoluene

María José Cárdenas Espinosa, Tabea Schmidgall, Georg Wagner, Uwe Kappelmeyer, Stephan Schreiber, Hermann J. Heipieper, Christian Eberlein

Helmholtz Centre for Environmental Research—UFZ, Department of Environmental Biotechnology, Leipzig, Germany

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RESEARCH ARTICLE

An optimized method for RNA extraction from the polyurethane oligomer degrading strain *Pseudomonas capeferrum* TDA1 growing on aromatic substrates such as phenol and 2,4-diaminotoluene

María José Cárdenas Espinosa[1], Tabea Schmidgall[1], Georg Wagner[1], Uwe Kappelmeyer[1], Stephan Schreiber[2], Hermann J. Heipieper[1], Christian Eberlein[1]*

1 Department of Environmental Biotechnology, Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany, **2** Department Molecular Systems Biology, Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany

* christian.eberlein@ufz.de

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Abstract

Bacterial degradation of xenobiotic compounds is an intense field of research already for decades. Lately, this research is complemented by downstream applications including Next Generation Sequencing (NGS), RT-PCR, qPCR, and RNA-seq. For most of these molecular applications, high-quality RNA is a fundamental necessity. However, during the degradation of aromatic substrates, phenolic or polyphenolic compounds such as polycatechols are formed and interact irreversibly with nucleic acids, making RNA extraction from these sources a major challenge. Therefore, we established a method for total RNA extraction from the aromatic degrading *Pseudomonas capeferrum* TDA1 based on RNAzol® RT, glycogen and a final cleaning step. It yields a high-quality RNA from cells grown on TDA1 and on phenol compared to standard assays conducted in the study. To our knowledge, this is the first report tackling the problem of polyphenolic compound interference with total RNA isolation in bacteria. It might be considered as a guideline to improve total RNA extraction from other bacterial species.

Introduction

During several decades, various microorganisms have evolved metabolic pathways to degrade environmental pollutants derived from anthropogenic activities (e.g. agriculture, solid waste, untreated industrial effluents, oil and solvent industry, etc.) which are present in different habitats [1]. In nature, aromatic compounds belong to the most persistent and hazardous pollutants, causing deleterious effects on human and animal health [2,3]. Their biodegradation has been intensely studied, however the removal of organic compounds has been focused on the role of bacteria due to their quick adaptation, metabolic versatility and genetic plasticity

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allowing them to use aromatic substrates as their sole carbon and energy source [4,5]. Hundreds of bacterial species of several phylogenetic origins have been identified as being capable to aerobically degrade all different kinds of aromatic compounds. Among all these, *Pseudomonas* species are by far the best studied ones because of their unique properties to degrade and tolerate a wide variety of xenobiotic compounds [6,7].

The mayor challenge in bacterial degradation is to overcome the resonance energy that stabilizes the aromatic ring [8,9]. To do so, aerobic bacteria rely on the addition of either one or two atoms of molecular oxygen by mono or dioxygenases. They transform aromatic compounds into central intermediates of aromatics degradation such as catechol, protocatechuate and gentisate [10]. Then, ring-cleaving dioxygenases catalyze ring fission via the *ortho*- or *meta*-cleavage pathway. During *ortho*-cleavage pathway, the aromatic ring fission occurs between two hydroxyl groups while during *meta*-cleavage this is done between one hydroxylated carbon and other adjacent non-hydroxylated carbon. Both ways are catalyzed by intradiol and extradiol dioxygenases, respectively, using Fe^{+3} and Fe^{+2} at the active site [2,11,12]. Finally, ring cleavage products are transformed into aliphatic molecules that can be channeled to the central metabolism. Some central intermediates of the biodegradation of aromatics such as phenols and catechols can be easily oxidized to yield the corresponding quinones. This reaction is regulated by the activity of enzymes known as polyphenol oxidases (PPOs) which are the principal basis of the browning reactions in plant tissues and extracts [13–16]. Such oxidases were also observed in *Pseudomonas* and other bacterial species [17]. Several authors have suggested that enzymatic oxidation of phenolic compounds and the presence of polysaccharides and other secondary metabolites represent a major problem in molecular studies [18–21]. A modified and improved RNA isolated protocol have been described in a bacterial culture containing pyrene as a carbon source [22].

Nucleic acid isolation is regularly the starting point for all downstream applications. However, isolation of intact RNA can be a challenge due to several factors including hydrolysis susceptibility, enzymatic and heat degradation [23]. To overcome these problems, reliable extraction methods such as commercial RNA extraction kits and organic solvents yield high quality RNA from different types of samples including cell lines, plant and mammalian tissues, bacteria, virus, etc. Nevertheless, phenolic compounds, polysaccharides, proteins and other secondary metabolites interfere with nucleic acids tend to co-precipitate or degrade RNA, restricting its yield and quality [13,23–27].

In many follow up applications including cDNA library construction, gene expression studies and next generation sequencing, the reproducibility and validity of the data depend on the quality of the RNA extracted [26]. In addition, the accurate assessment of RNA integrity and the correct quantification are key elements for further molecular analysis.

In this study, total RNA extraction was conducted for the *Pseudomonas capeferrum* TDA1 growing on three different carbon sources including phenol, succinate and 2,4-diaminotoluene (2,4-TDA); an aromatic diamine and precursor for the production of polyurethane. In previous reports, this compound was degraded by *Pseudomonas capeferrum* TDA1 and a preliminary degradation pathway was suggested [28,29]. Regardless of the proposed pathway, mono- and dioxygenases are involved for sure, leading to polycatecholic/phenolic intermediates. Those are likely to be subjected to the activity of oxidases present in the strain's genome and responsible for the formation of polyphenolic compounds observed as dark precipitation during growth on 2,4-TDA. In order to obtain high quality RNA, commercial kits and conventional methods were tested but all of them failed for the cells grown in 2,4-TDA media, most likely due to the presence of polyphenolic compounds that could interfere with the RNA [30–34]. In order to solve this problem, a simple and effective RNA extraction method from bacterial cultures grown on 2,4-TDA was developed. This procedure uses a mixture of guanidine

thiocyanate and phenol in a monophasic solution, which is a frequent protocol for some varieties of biological samples considered as a "challenge" due to several factors. It provides purified total RNA suitable for RT-PCR, qPCR and cDNA libraries.

Materials and methods

Bacterial strain and growth conditions

Prior to the experiment, *Pseudomonas capeferrum* TDA1 was cultivated in Hartman's mineral salts medium [28] and succinate (4 g/L) as carbon source at 30˚C and 150 rpm overnight. Afterwards, two milliliters of each culture were centrifuged (7 minutes at 18,000 g) and the resulting cell pellets were washed with KNO_3 (10 mM) while the supernatant was discarded.

The pellets were added to mineral media containing only one carbon source (4 g/L succinate, 2 mM 2,4-TDA or 2 mM phenol) and incubated for 8 hours (succinate) and 7 days (2,4-TDA) until they reached the exponential phase (OD_{560} = ~0.8). The cells grown on phenol were harvested after 5 days and added to a fresh medium, reaching the exponential phase 4 days later. After that, two milliliters of the culture of every single media were centrifuged (3 minutes at 18,000 g), re-suspended in RNA Later (Sigma-Aldrich, St. Louis, MO, USA) and stored at -80˚C.

RNA isolation

The following standard laboratory kits and methods were tested for RNA extraction from *Pseudomonas capeferrum* growing on succinate, phenol and 2,4-TDA following the manufacturer's instructions: RNeasy, RNeasy PowerPlant (Qiagen, Düsseldorf, Germany), peqGOLD TriFast (VWR, Leuven, Belgium) and phenol chloroform protocol [35]. Additionally, the improved protocol was applied for all three carbon sources mentioned. This was done by using RNAzol® RT (Sigma-Aldrich, St. Louis, USA) with the following modifications: Initially, the cell solution was centrifuged (5 minutes at 20,000 g) to collect cells and discard the supernatant. 0.5 milliliters of RNAzol® RT were added to the pellets and re-suspended in the reagent. Each solution was transferred to the lysing matrix B tubes and homogenized using the FastPrep-24 (MP Biomedicals, Inc) during 35 seconds at 6.5 m/s. After homogenization, the samples were transferred to 1.5 mL micro-centrifuge tubes and 0.2 mL of RNase-free water were added for DNA, protein, and polysaccharide precipitation according to the protocol [36].

Afterwards, the supernatant was transferred to a new 1.5 mL low binding micro-centrifuge tube with an equal volume of isopropanol and 1 μL of glycogen (molecular biology grade, Thermo Fisher, Waltham, United States). The samples were incubated at −80˚C for 40 minutes and centrifuged at 12,000 g for 10 minutes at room temperature. The RNA pellets were washed twice with 0.4 mL of 70% ethanol (v/v) and centrifuged at 8,000 *g* during 2.5 minutes at room temperature. The supernatant was removed carefully and the pellets were solubilized by adding RNase free water (45 μL) (Fig 1). Finally, the samples were cleaned up using the RNA Clean & Concentrator™-5 kit (Zymo Research, California, USA) following the protocol suggested by the manufacturer for total RNA extraction. Due to the low RNA concentration (2.5–3.0 ng/μL) yielded from 2,4-TDA samples, a pooling step (2 or 3 samples per pool) was added to the protocol before the cleaning up process.

RNA quantification and RIN determination

Total RNA was quantified using a fluorescent RNA-binding dye Qubit Fluorometer (Thermo Fisher, Waltham, United States) according to the manufacturer's instructions. $A_{260}/_{280}$ and $A_{260}/_{230}$ values for RNA samples were measured using Nanodrop ND-1000

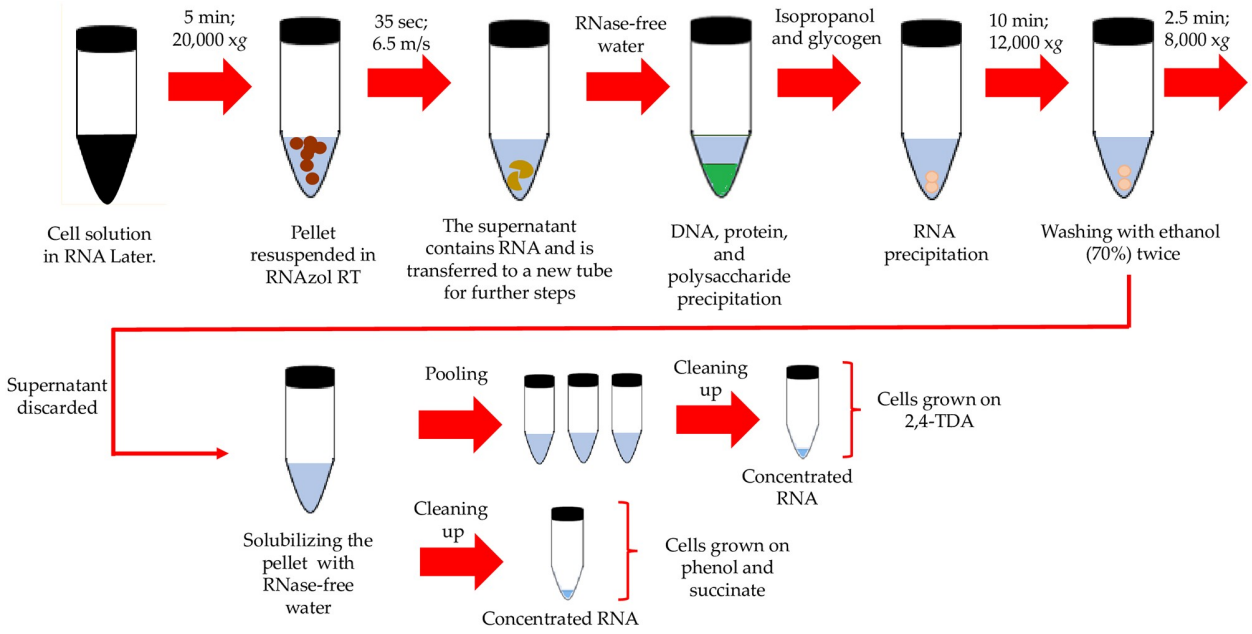

Fig 1. Optimized RNA extraction method from *Pseudomonas capeferrum* TDA1.

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Spectrophotometer (Peqlab, Erlangen, Germany). The quantification of RNA was done in triplicates. After RNA concentrations in the samples were analyzed, RNA integrity was determined in 1 μl of total RNA using the RNA Nano (succinate and phenol) and Pico chips (2,4-TDA) assays and Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, United States) in accordance with the manufacturer's protocols. Samples with an integrity number (RIN) above 7.0 were selected for further downstream applications.

Results and discussion

Yield and quality of total RNA isolated from *P. capeferrum* TDA1

Routine molecular applications such as RT-PCR, NGS, RNA-seq require RNA with high purity and integrity [23,26]. At present, several methods and kits are available for extracting RNA from samples rich in polysaccharides, phenols and other secondary metabolites but they are mostly applied to plants tissues, leaves and woody species [20,21,23,26,33,37,38]. For this particular reason, five RNA isolation methods were compared in order to obtain high-quality RNA from *Pseudomonas capeferrum* TDA1 growing on aromatic compounds (phenol and 2,4-TDA) and succinate.

First, two commercial kits (RNeasy and RNeasy power plant kit, Qiagen) based on spin columns following the manufacturer's recommendations yielded low RNA quantity for phenol (ranged from 5.3 to 18.9 ng/μL) and 2,4-TDA samples (ranged from 2.0 to 2.1 ng/μL), compared to RNA isolated from cells grown on succinate that showed higher concentrations for both protocols.

In addition, one method based on phenol/chloroform [35] and another containing guanidium-thiocyanate-phenol and chloroform (TriFast, VWR) were tested and the RNA yields were the lowest for cell samples grown on the aromatic compounds among all assays conducted. Finally, the modified RNAzol RT method achieved high RNA concentrations for *P. capeferrum* TDA1 grown on succinate, phenol and 2,4-TDA (Table 1).

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Table 1. Total RNA quantity (ng/μL) and purity (A_{260}/A_{280} and A_{260}/A_{230}) for different RNA isolation methods applied on cells from *Pseudomonas capeferrum* TDA1 growing on different carbon sources (succinate, phenol and 2,4-TDA). Values represent mean ± SD.

Method	Concentration (ng/μL)			A_{260}/A_{280}			A_{260}/A_{230}		
	Succinate	Phenol	TDA	Succinate	Phenol	TDA	Succinate	Phenol	TDA
RNeasy[1]	67.5±14.17	5.3±1.77	2.0±1.74	1.98±0.19	1.60±0.12	2.71±1.44	2.02±0.13	0.33±0.18	0.2±0.13
RNeasy P.Plant[1]	48.7±12.35	18.9±3.02	2.1±0.25	2.25±0.06	2.06±0.09	1.30±0.46	2.12±0.05	2.02±0.18	2.48±1.58
Phenol/Chloroform	54.0±25.07	37.3±25.66	0.3±0.14	1.67±0.15	1.20±0.57	LOD	1.63±0.52	0.81±0.62	LOD
Trifast[2]	102.7±9.38	88.2±1.17	1.1±0.71	1.66±0.37	1.85±0.07	LOD	2.17±0.08	1.65±0.28	LOD
Modified RNAzol RT[3]	130.0±32.07	73.6±8.80	5.3±0.16	2.11±0.02	2.10±0.11	2.02±0.16	2.32±0.07	2.10±0.27	1.95±0.11

[1]Column purification;

[2] Guanidium thiocyanate, phenol and chloroform;

[3] Guanidine thiocyanate and phenol. LOD: below limit of detection.

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A_{260}/A_{280} and A_{260}/A_{230} ratios are often used for providing a rough indication of purity. A ratio of 2.0–2.2 is generally accepted as pure RNA [26,39,40]. Table 1 shows the A_{260}/A_{280} ratio for the five protocols demonstrating that RNeasy kit (1.98 ± 0.19; succinate), RNeasy power plant kit (2.25 ± 0.06; succinate, 2.06 ± 0.09; phenol) and modified RNAzol RT (2.11 ± 0.02; succinate, 2.10 ± 0.11; phenol, 2.02 ± 0.16; 2,4-TDA) method were effective inhibiting protein and phenol contamination [39,40].

The A_{260}/A_{230} ratio is a sensitive indicator of contaminants such as: guanidine thiocyanate (GTC), guanidine hydrochloride (GuHCl), EDTA, polysaccharides and other secondary metabolites. The RNeasy kit (2.02 ± 0.13; succinate), RNeasy power plant kit (2.12 ± 0.05; succinate, 2.02 ± 0.18; phenol) and modified RNAzol RT (2.32 ± 0.07; succinate, 2.10 ± 0.27; phenol, 1.95 ± 0.11; 2,4-TDA) showed values that correspond to high RNA purity (Table 1). On the other hand, phenol/chloroform and TriFast methods revealed lower A_{260}/A_{280} ($\leq$ 1.85) and A_{260}/A_{230} ($\leq$ 1.65) ratios for succinate and phenol samples, which indicates organic contamination that compromises the RNA quality [23,26,38,41,42]. For these two methods, the ratios could not be measured for 2,4-TDA because the RNA concentration was below the detection limit [43].

RNA isolation of cells grown on 2,4-TDA using spin columns presented A_{260}/A_{280} and A_{260}/A_{230} ratios out of the acceptable range, suggesting possible problems in the extraction due to the presence of polyphenolics, polysaccharides and secondary metabolites that precipitated with the nucleic acids [26,42].

These results are consistent with previous reports which demonstrated that commercial kits using spin columns are not suitable for RNA extraction from plants rich in polysaccharides and polyphenols. Phenolic substances reduce the efficiency of the column and can bind irreversibly to proteins and nucleic acids, leading to degradation and subsequent low-quality RNA [23,26,44]. Therefore, the low RNA concentration yielded by commercial kits has been proven previously in several plant tissues, seeds, roots and woody perennials with high content of polysaccharides and polyphenols [23,25–27,42,45].

However, not all RNA yields from cells grown on aromatic compounds had the same result. In the case of 2,4-TDA, the poor RNA yield and quality demonstrated protein and organic contamination. During incubation, a browning effect in the media was observed, which suggests the presence of PPO enzymes catalysing the oxidation of diphenols to quinones [28,46] that can irreversibly bind to the RNA and interfere with the extraction process and downstream applications [18,23,31]. Studying the annotated genome of *Pseudomonas capeferrum* TDA1 reveals the presence of the gene *yfiH* encoding for a polyphenol oxidoreductase laccase

(EC 1.10.3.2) (data not showed). This enzyme oxidizes a broad range of phenolic and non-phenolic compounds and has been isolated from several *Pseudomonas* species [47]. In this study, the browning effect was not visible in phenol samples probably due to the hydroxylation of monophenols to diphenols that produces colourless intermediates [46,48]. Previous work also demonstrated that high-quality total RNA could be isolated from bacterial strains grown on phenol and benzoate using commercial kits [49–51], however not all the metabolic pathways related to bio-degradation of aromatic compounds have been identified and many intermediates as well as secondary metabolites are still unknown.

Methods (phenol/chloroform and TriFast) based on guanidinium thiocyanate–phenol–chloroform were less efficient for the isolation of RNA from *P. capeferrum* TDA1. RNA extracted from cells grown on phenol presented low A_{260}/A_{280} and $A_{260}/_{230}$ ratios (Table 1) for both protocols. These results are consistent with previous reports that suggested that chloroform can affect the isolation and quantification of the RNA [23,35]. Thus, polysaccharides can co-precipitate with RNA during the phenol/chloroform extraction steps [25]. Despite of these drawbacks, some modified protocols have been tested in plant leaves [52], seeds [37] and seedlings [53] obtaining high-quality RNA.

In the case of 2,4-TDA samples, the phenolic compounds and secondary metabolites in the oxidized form could interfere with the RNA yield. Negligible quantities of RNA extracted from plant tissues was reported earlier using a reagent based on guanidinium thiocyanate [54]. Also, it has been demonstrated that this substance participates in the precipitation of considerable amount proteins with the nucleic acids, reducing the RNA isolation efficiency [23,55].

Further analysis of RNA integrity using an Agilent 2100 Bioanalyzer showed RIN values $\geq$ 8.50 (Fig 2A–2C) for RNA from cells grown on phenol (TriFast and modified RNAzol RT) and for 2,4-TDA (modified RNAzol RT) indicating no degradation of RNA. Generally, RNA with a RIN value above 7.0 is suitable to ensure sequencing quality [42]. The three remaining methods revealed RIN values below 5.50.

On the other hand, RNA isolation from succinate samples (RNeasy and RNeasy power plant kit, TriFast and modified RNAzol RT) revealed RIN values $\geq$ 7.90, which confirms the complexity of high-quality RNA extraction from cells grown on aromatic compounds. In contrast, the phenol/chloroform protocol obtained low RIN values ($\leq$ 5.20) for all the samples.

RNA samples (succinate and phenol) extracted with the TriFast method exhibited $A_{260}/_{280}$ and $A_{260}/_{230}$ ratios out of the acceptable range for high-quality RNA (lower than 1.8 or over 2.0) and a RIN over 8. This finding is compatible with other studies which propose that RNA purity and RNA integrity are unrelated and there is no significant correlation between them [56,57]. However, RNA quality control (integrity and purity) is critical and must be assessed independently in order to assure reliable and reproducible results. Different reports suggest that low-quality RNA has a severe effect in pPCR quantification [58], transcript estimation [59], differential expression [60] and cDNA synthesis [61] that interferes with gene expression studies.

As it has been discussed above, to obtain high-quality RNA represents a fundamental step for high technology platforms including NGS that have provided many valuable insights into biological systems.

The modified protocol presented in this study demonstrated an increase in RNA yield and quality from all the carbon sources (Fig 2c and Table 1) compared to commercially available kits, which have been reported as the first option for Gram-negative bacteria, because they are rapid, capable of high-throughput analysis and cost-effective [35,62,63]. The use of RNAzol® RT as a single step procedure removed DNA contamination without DNase treatment and reduced RNA time handling and helped diminished sample degradation, as has been tested in similar methods [63].

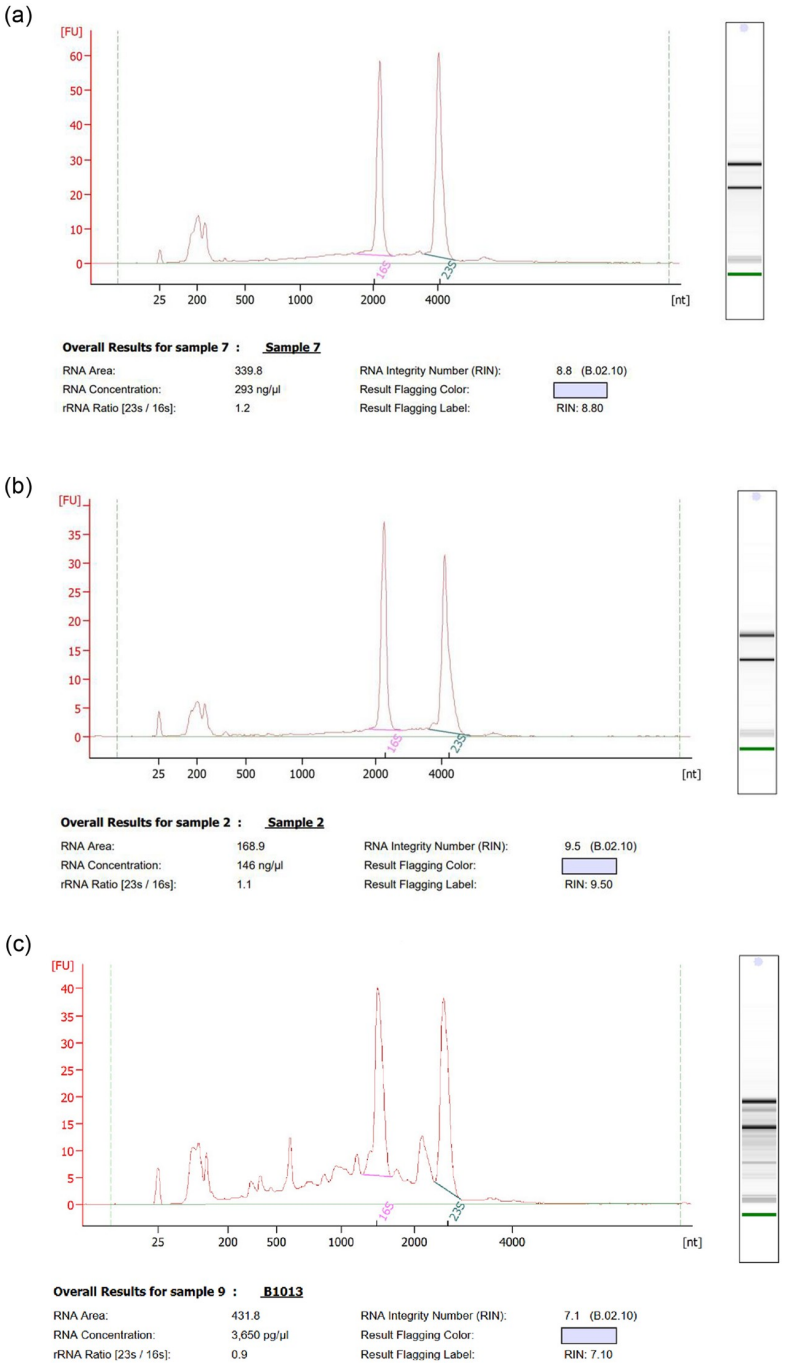

Fig 2. Bioanalyzer results. Electropherograms of total RNA extracted from *Pseudomonas capeferrum* TDA1 grown on: A) phenol (with TriFast method), B) phenol (with modified RNAzol RT method) and C) 2,4-TDA (with modified RNAzol RT method). The main peaks correspond to ribosomal RNA (16S and 23S).

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Regarding the cells grown on 2,4-TDA, pooling was an important key in the modified method. Pooling samples may provide a solution when RNA input is insufficient in a single sample for subsequent analyses [64]. A previous report showed that RNA isolated from pooled *Gyrodactylus salaris* samples was useful for increasing total RNA quality and yield [65]. Moreover, the use of pooling biological samples has been tested for the detection of gene expression changes via microarray [66]. Considering the low RNA or DNA inputs (ng/μL) which cannot be efficiently precipitated, the use of a carrier material has been studied as an effective alternative in some protocols [67]. Glycogen is regularly used in several molecular biology applications precipitating nucleic acids in solution to improve the formation of a visible pellet that simplifies downstream sample processing [67,68]. Finally, the use of a purification and concentration step is highly recommended for removing any phenol trace in RNA extraction involving guanidine-phenol based reagents as RNAzol® RT [69]. This final step has been reported previously in other RNA extraction protocols due to it ensures the recovery of highly concentrated and pure RNA that be used for downstream applications afterwards [70–72].

Conclusions

In the present study, five different methods for RNA isolation from *Pseudomonas capeferrum* TDA1 grown on succinate, phenol and 2,4-TDA were compared. Conventional methods failed to yield high quality RNA from cells grown on 2,4-TDA (Table 1). Therefore, a modified RNAzol RT protocol was developed and demonstrated to be the most efficient to obtain high-quality total RNA from 2,4-TDA grown cells. The modified RNAzol RT method tackles the problem of RNA degradation, its interaction with phenolic compounds and the removal of organic contaminants effectively. Furthermore, the protocol showed to yield high quality RNA for cells grown on phenol, another aromatic carbon source as well as cells grown on succinate. In fact, all bacteria known to aerobically degrade complex aromatic compounds use the same machinery of oxygenation enzymes that release metabolic degradation by-products known to interfere with RNA. Therefore, we are convinced that the present protocol can be used as a guideline as a guideline to improve total RNA extraction from all bacterial on samples from all bacterial cultures growing on complex aromatic carbon sources.

Supporting information

S1 Fig. Bioanalyzer results of total RNA isolated from *P. capeferrum* TDA1 grown on 2–4 TDA using the RNeasy method.
(TIF)

S2 Fig. Bioanalyzer results of total RNA isolated from *P. capeferrum* TDA1 grown on 2–4 TDA using the RNeasy power plant method.
(TIF)

S3 Fig. Bioanalyzer results of total RNA isolated from *P. capeferrum* TDA1 grown on 2–4 TDA using the phenol/chloroform method.
(TIF)

S4 Fig. Bioanalyzer results of total RNA isolated from *P. capeferrum* TDA1 grown on 2–4 TDA using the TriFast method.
(TIF)

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Author Contributions

Conceptualization: Hermann J. Heipieper, Christian Eberlein.

Data curation: María José Cárdenas Espinosa, Tabea Schmidgall, Georg Wagner, Uwe Kappelmeyer, Stephan Schreiber.

Formal analysis: María José Cárdenas Espinosa.

Funding acquisition: Hermann J. Heipieper.

Investigation: María José Cárdenas Espinosa, Uwe Kappelmeyer, Hermann J. Heipieper.

Methodology: Stephan Schreiber, Christian Eberlein.

Supervision: Uwe Kappelmeyer, Stephan Schreiber, Hermann J. Heipieper, Christian Eberlein.

Validation: María José Cárdenas Espinosa, Uwe Kappelmeyer, Hermann J. Heipieper.

Visualization: María José Cárdenas Espinosa.

Writing – original draft: María José Cárdenas Espinosa.

Writing – review & editing: Uwe Kappelmeyer, Hermann J. Heipieper, Christian Eberlein.

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2.3.1 Supplementary information

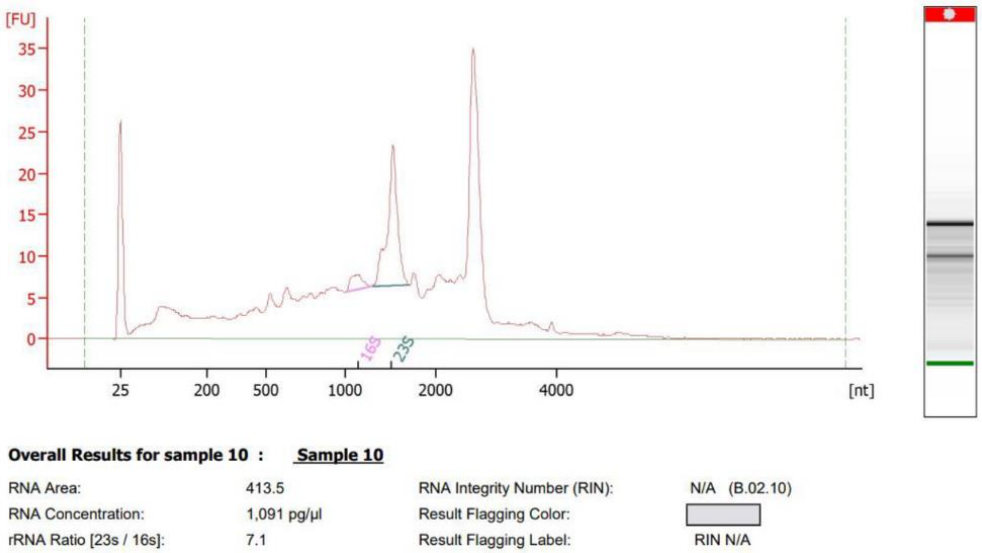


Fig. 1 Bioanalyzer results of total RNA isolated from *P. capeferrum* TDA1 grown on 2–4 TDA using the RNeasy method.

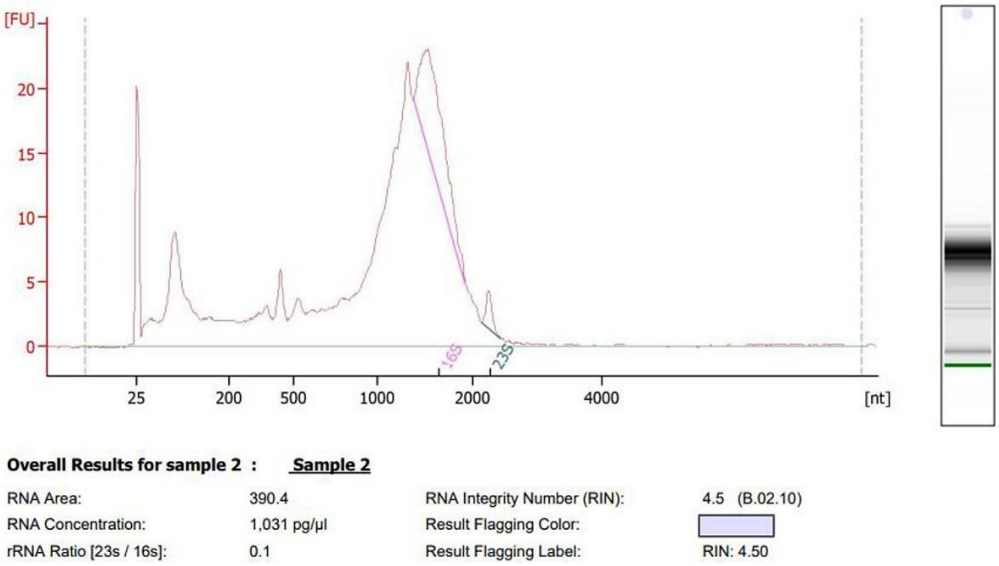
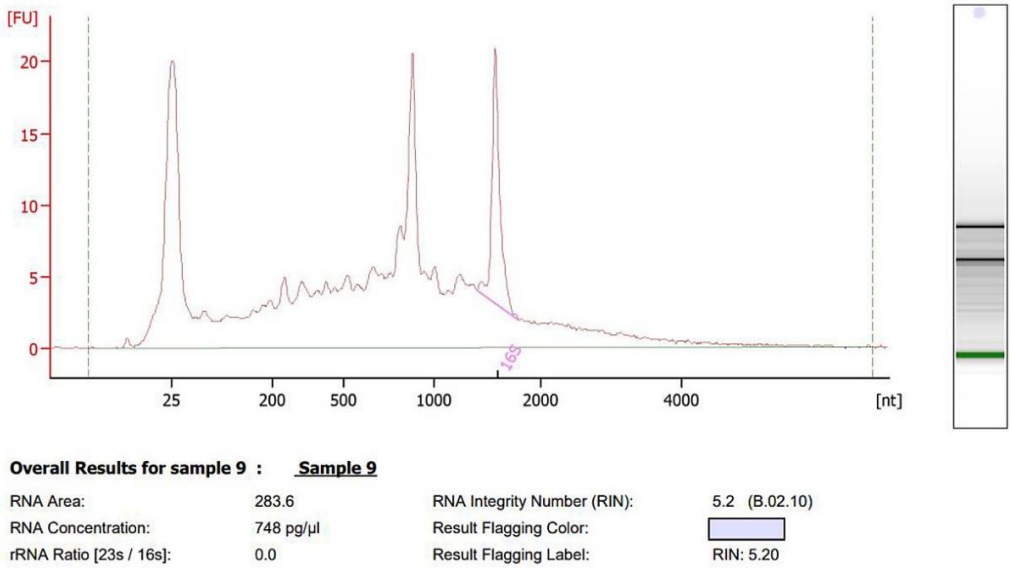


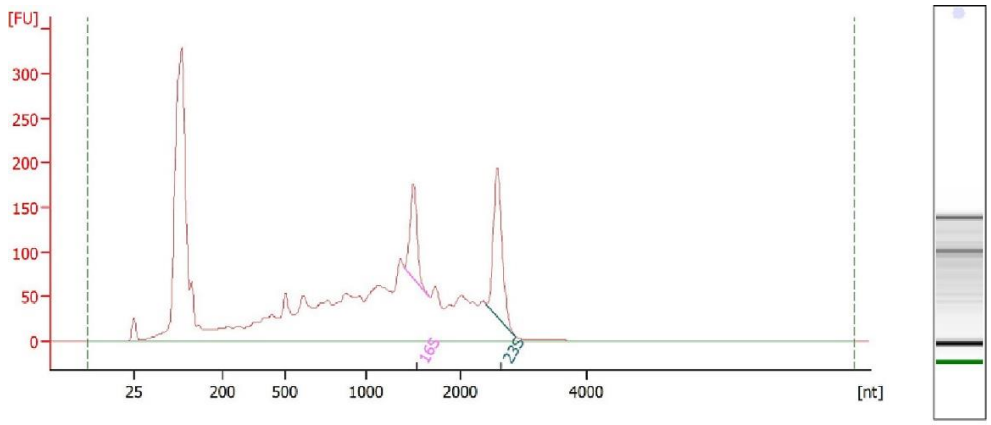
Fig. 2 Bioanalyzer results of total RNA isolated from *P. capeferrum* TDA1 grown on 2–4 TDA using the RNeasy power plant method.



Overall Results for sample 9 : Sample 9

RNA Area:	283.6	RNA Integrity Number (RIN):	5.2 (B.02.10)
RNA Concentration:	748 pg/µl	Result Flagging Color:	
rRNA Ratio [23s / 16s]:	0.0	Result Flagging Label:	RIN: 5.20

Fig. 3 Bioanalyzer results of total RNA isolated from *P. capeferrum* TDA1 grown on 2–4 TDA using the phenol/chloroform method.



Overall Results for sample 4 : Sample 4

RNA Area:	3,659.1	RNA Integrity Number (RIN):	4.5 (B.02.10)
RNA Concentration:	9,659 pg/µl	Result Flagging Color:	
rRNA Ratio [23s / 16s]:	1.7	Result Flagging Label:	RIN: 4.50

Fig. 4 Bioanalyzer results of total RNA isolated from *P. capeferrum* TDA1 grown on 2–4 TDA using the TriFast method.

2.4 Extracellular degradation of a polyurethane oligomer involving outer membrane vesicles and further insights on the degradation of 2,4-diaminotoluene in *Pseudomonas capeferrum* TDA1

Oscar Puiggené, María José Cárdenas Espinosa, Dietmar Schlosser, Stephan Thies, Nico Jehmlich, Uwe Kappelmeyer, Stephan Schreiber, Daniel Wibberg, Joern Kalinowski, Hauke Harms, Hermann J. Heipieper & Christian Eberlein.

Helmholtz Centre for Environmental Research—UFZ, Department of Environmental Biotechnology, Leipzig, Germany

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Extracellular degradation of a polyurethane oligomer involving outer membrane vesicles and further insights on the degradation of 2,4-diaminotoluene in *Pseudomonas capeferrum* TDA1

Òscar Puiggené[1,6,7], María José Cárdenas Espinosa[1,7], Dietmar Schlosser[2], Stephan Thies[3], Nico Jehmlich[4], Uwe Kappelmeyer[1], Stephan Schreiber[4], Daniel Wibberg[5], Joern Kalinowski[5], Hauke Harms[2], Hermann J. Heipieper[1]✉ & Christian Eberlein[1]

The continuing reports of plastic pollution in various ecosystems highlight the threat posed by the ever-increasing consumption of synthetic polymers. Therefore, *Pseudomonas capeferrum* TDA1, a strain recently isolated from a plastic dump site, was examined further regarding its ability to degrade polyurethane (PU) compounds. The previously reported degradation pathway for 2,4-toluene diamine, a precursor and degradation intermediate of PU, could be confirmed by RNA-seq in this organism. In addition, different cell fractions of cells grown on a PU oligomer were tested for extracellular hydrolytic activity using a standard assay. Strikingly, purified outer membrane vesicles (OMV) of *P. capeferrum* TDA1 grown on a PU oligomer showed higher esterase activity than cell pellets. Hydrolases in the OMV fraction possibly involved in extracellular PU degradation were identified by mass spectrometry. On this basis, we propose a model for extracellular degradation of polyester-based PUs by *P. capeferrum* TDA1 involving the role of OMVs in synthetic polymer degradation.

For the past 80 years, polyurethane (PU) has been one of the world's most versatile polymers, rising towards a market value expected to surpass 87 billion USD by 2026 worldwide[1]. Chemically, polyurethane is the condensation product of polyisocyanates and polyols, yielding urethane bonds. Nonetheless, polyurethane structure is rather heterogeneous and depends on the plastic monomers used[2]. For instance, ester or ether bonds may be present in the polyol segment[3]. The resulting broad range of materials has applications in many sectors, such as building and construction, furniture production, automotive or medical devices, due to its excellent mechanical properties, stability and enhanced biocompatibility[4].The high polyurethane demand generates significant amounts of waste globally, of which only 29.7% is recycled, 30.8% is disposed in landfills and 39.5% is recuperated through energy recovery[5,6]. However, each of these methods has several shortcomings including the emergence of toxic by-products (e.g. HCN, NO_x and CO), increasing landfill costs and leakage, high performance equipment, as well as environmental and health concerns[7,8].

[1]Department of Environmental Biotechnology, Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany. [2]Department of Environmental Microbiology, Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany. [3]Institute of Molecular Enzyme Technology, Heinrich Heine University Dusseldorf, Jülich, Germany. [4]Department Molecular Systems Biology, Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany. [5]Microbial Genomics and Biotechnology, Center for Biotechnology (CeBiTec), Bielefeld, Germany. [6]The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2800 Kgs, Lyngby, Denmark. [7]These authors contributed equally: Òscar Puiggené and María José Cárdenas Espinosa. ✉email: hermann.heipieper@ufz.de

In search of sustainable alternatives, biodegradation of PUs has been subject of extensive study[2,9–11]. Due to the presence of urethane bonds in their backbone, polyurethanes are susceptible to hydrolysis by enzymes secreted by microorganisms, thus releasing breakdown products, which may act as a carbon source[12]. Even though fungi (namely *Aspergillus* sp. and *Penicillium* sp.) have been reported as the principal degraders of PU in nature, enzymatic activity has also been associated with different bacterial strains. Among bacteria, some of the well-studied PU-degrading organisms are *Acinetobacter*, *Bacillus subtilis*, *Corynebacterium*, *Comamonas acidovorans*, and members of the genus *Pseudomonas*[3,13]. In the past years, several *Pseudomonas* species have been identified for their degradative potential of various plastic polymers[3,13–16]. Specifically, *Pseudomonas chlororaphis* and *Pseudomonas protegens* (formerly *fluorescens*) Pf-5 and recently the *P. pertucinogena* lineage have been found to degrade polyester-based PUs[14–16].

Initially, biodegradation often requires microbial attachment on the surface of synthetic polymers. The adherence and colonization of bacteria tend to reduce the resistance of the plastic material, which facilities the accessibility of secreted enzymes to modify the physicochemical properties of the polymers. Inside the complex nature of the biofilms, small and non-replicative spherical nanostructures (20–240 nm) called outer membrane vesicles (OMVs) are constantly released by gram-negative bacteria[17]. Furthermore, some reports show that OMVs may contribute to the biofilm formation[18,19]. OMVs harbor active enzymes and extracellular structures were reported to exhibit catabolic activity in different bacteria (*Pseudomonas*, *Rhodoccocus*, *Amycolatopsis* and *Delftia*) grown on aromatic substrates such as phenanthrene and lignin-rich media[20,21].

Resilient plastics such as polyethylene (PE) and polystyrene (PS) strongly depend on the formation of a biofilm in order to increase the surface interactions with bacterial cells[22,23]. For example, *Pseudomonas sp*. AK2 showed an enhanced low-density PE degradation through an adapted biofilm compared to planktonic cells[24]. Other strains such as *Bacillus sp*. grown on PS films presented a reduced polymer mass by 23% after 14 days[25].

Polyester-based PU degrading enzymes in *Pseudomonas* are assumed to be primarily extracellular esterases, lipases and cutinases, which may be membrane-bound or secreted extracellularly[26–28]. These enzymes are involved in a catalytic reaction called hydrolysis, which degrades PU by cleaving the ester bonds. Subsequently, intracellular enzymes and metabolic pathways further mineralize these compounds and use their carbon, nitrogen and energy to grow[29].

As a result of enzymatic degradation, PU waste in landfills may continuously release environmental pollutants into soil or groundwater, such as 4,4′-methylenedianiline (MDA) and 2,4-toluene diamine (2,4-TDA)[30,31], which are considered as possible human carcinogen[32] and pose an environmental risk for species in the aquatic and terrestrial areas[33]. Recently, *Pseudomonas capeferrum* TDA1 was identified as the first bacterial strain capable of degrading 2,4-TDA, as well as a PU oligomer[34]. In that previous report, the enzymes involved in the degradation pathway of 2,4-TDA were proposed and are further confirmed by RNA-seq in this study. Moreover, *Pseudomonas capeferrum* TDA1, its suitability and potential in (bio)technological plastic upcycling has recently acquired significant attention[35,36].

In order to understand different aspects of the biodegradation process, obtaining reliable gene expression data is vital. Therefore, top notch techniques such as advanced genome annotation, RNA-seq transcriptomics and proteomics were applied to study the pathway of 2,4-TDA degradation as well as adaptive responses to environmental changes[37] during growth on different carbon sources. Furthermore, the growth on a PU oligomer was examined and extracellular esterase activity was detected in different cell fractions of *P. capeferrum* TDA1. Hence, a model for extracellular degradation of polyurethane via outer membrane vesicles (including outer membrane-bound and periplasmic hydrolases) was suggested in *Pseudomonas capeferrum* TDA1.

Materials and methods

Chemicals, media and cultivation conditions. *P. capeferrum* TDA1 was grown in mineral media[38] containing the following compounds: 7 g $Na_2HPO_4 \times 2\ H_2O$; 2.8 g KH_2PO_4; 0.5 g NaCl; 0.1 g NH_4Cl; 0.1 g $MgSO_4 \times 7\ H_2O$; 10 mg $FeSO_4$; 5 mg $MnSO_4$; 6.4 mg $ZnCl_2$; 1 mg $CaCl_2 \times 6\ H_2O$; 0.6 mg $BaCl_2$; 0.36 mg $CoSO_4 \times 7\ H_2O$; 0.36 mg $CuSO_4 \times 5\ H_2O$; 6.5 mg H_3BO_3; 10 mg EDTA; 146 µl HCl (37%); per liter of demineralized water. For growth assessement, 3 g/l PU oligomer (Sigma-Aldrich, dihydroxy-functional oligomer, aliphatic urethane of proprietary composition, average $M_n \sim 320$ Dalton) or 2 mM 2,4-TDA or 2 mM 2,4-TDA + succinate were added[34]. Disodium succinate (4 g/l) was used as a control. Growth conditions were implemented as described recently[39].

Esterase/lipase assay. Bacterial cells were grown for 1–2 days, harvested in exponential phase and centrifuged. The pellet was washed with 10 mM KNO_3 and centrifuged once more. Similar to a previous study[40], supernatants, whole-cell samples and OMVs were assayed for esterase/lipase activity. Briefly, in 96-well plates 20–100 µl of sample were incubated with 200 µM of *p*-nitrophenyl esters (*p*-nitrophenyl butyrate, pivalate, valerate and palmitate were added from 5 mM stocks in ethanol, which were always freshly prepared prior to use) in 100 mM Tris–HCl pH 7.5 buffer (final volume 200 µl). Microplates were incubated at 30 °C for 1 h in Tecan GENios Plus Microplate Reader (Tecan, Männedorf, Switzerland) and measurements at 405 nm proceeded every minute. For activity calculation, slopes were normalized as previously described[41] by sample volume (i.e. protein amount) and comparison with the negative controls. *p*-Nitrophenyl extinction coefficient (ε_{410}) and optical path length (d) were considered constant with values of 11.8×10^6 cm^2/mol (at pH 7.5) and 0.5925 cm, respectively.

The normalization equation employed was the following:

$$A = \frac{\Delta E_{405}}{\varepsilon_{405} \cdot d \cdot \frac{V_{sample}}{V_{total}}}$$

where A refers to activity in mol L^{-1} min^{-1} and ΔE_{405} refers to the absorbance increase per min at 405 nm. The molar extinction coefficient (ε_{405}) for *p*-nitrophenol at pH 7.5 and 410 nm was obtained from Kademi et al. (2000)[41] and was assumed to be equal at 405 nm.

Outer membrane vesicles (OMVs) isolation. OMV were isolated from *P. capeferrum* TDA1 samples grown on PU oligomer (Figure S1) or succinate in exponential growth phase. Samples were grown until an OD_{560} 0.4–0.5. Cells were then harvested, and supernatant was filtered through a 0.45-µm pore size membrane (Sartorius AG, Göttingen, Germany). Then, isolation was carried out as detailed in Kadurugamuwa and Beveridge (1995)[42]. OMVs were harvested through ultracentrifugation at 100,000 g for 3 h at 4 °C (L-90 K, Rotor-Type 50.2 Ti, Beckmann, USA). Supernatant was then discarded and OMV pellet was resuspended with the remaining supernatant. Protein concentration was also obtained by Bradford measurement (BioRad). Relative values of OMV release were obtained as previously reported[43] by comparing absolute OMV concentration after isolation to total bacterial protein.

Liquid chromatography-mass spectroscopy (LC–MS). OMVs from cultures with PU oligomer and succinate were harvested and processed by LC–MS/MS analysis. Samples were directly treated with trypsin. Peptide lysates were re-dissolved in water containing 0.1% formic acid (20 µL) and analyzed on a Q Exactive HF instrument (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a TriVersa NanoMate source (Advion, Ithaca, NY, USA) in LC chip coupling mode.

Peptide lysates were injected on a trapping column (Acclaim PepMap 100 C18, 3 µm, nanoViper, 75 µm × 2 cm, Thermo Fisher Scientific) with 5 µL/min by using 98% water/2% ACN 0.5% trifluoroacetic acid, and separated on an analytical column (Acclaim PepMap 100 C18, 3 µm, nanoViper, 75 µm × 25 cm, Thermo Fisher Scientific) with a flow rate of 300 nL/min over 80 min. Mobile phase was 0.1% formic acid in water (A) and 80% ACN/0.08% formic acid in water (B). Raw LC–MS/MS data were processed with Proteome Discoverer (v2.4, Thermo Fisher Scientific). Search settings for the Sequest HT search engine were set to trypsin (Full), max. missed cleavage: 2, precursor mass tolerance: 10 ppm, fragment mass tolerance: 0.02 Da. The LC raw files were searched against the protein-coding sequences of *Pseudomonas* sp. TDA1 (Uniprot Proteome ID UP000476571). The false discovery rates (FDR) were determined with the node Percolator embedded in Proteome Discoverer and was set for the protein FDR (< 1%). Subcellular location of resulting proteins, if not already characterized, was predicted via CELLO v2.5[44].The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[45] partner repository with the dataset identifier PXD029164.

RNA extraction and quantification. Exponentially growing bacterial cells were mixed with in RNA Later solution and centrifuged (5 min at 20,000 g) to collect cells and to discard the supernatant. Total RNA from samples containing succinate or succinate + 2,4-TDA as a carbon source was extracted using the RNeasy kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's protocol including some modifications: First, the cell solution was centrifuged (5 min at 20,000 g) and the supernatant removed. Each sample was mixed with the buffer RLT (700 µl) + β-mercaptoethanol (7 µl), stored on ice for 2 min and transferred to lysing matrix B tubes for homogenization using FastPrep-24 (MP Biomedicals, Inc) during 35 s at 6.5 m/s. Then, the supernatant was removed to a new 1.5-ml low binding micro-centrifuge tube, an equal volume of ethanol (70%) was added, and the samples were centrifuged for 30 s at 8,000 g. To remove DNA contamination, DNA-free DNA removal kit (Thermo- Scientific, Waltham, United States) was added to each sample and the mixture incubated for 1 h at 37 °C.

For cells grown on 2,4 TDA, a modified RNA extraction protocol was applied to account for the presence of polyphenols, polysaccharides and secondary metabolites which interfere with or degrade the RNA[42]. After centrifugation, 0.5 ml of RNAzol® RT (Sigma-Aldrich, St. Louis, USA) were added to the pellets and re-suspended in the reagent. Each solution was transferred to the lysing matrix B tubes and homogenized. After homogenization, the samples were transferred to 1.5-ml micro-centrifuge tubes and 0.2 ml of RNase-free water were added for DNA, protein, and polysaccharide precipitation according to a protocol published earlier[46].

Total RNA samples were quantified using a fluorescent RNA-binding dye Qubit Fluorometer (Thermo Fisher, Waltham, United States) according to the manufacturer's instructions and the RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, United States) following the manufacturer's protocols. Samples with integrity numbers (RIN) above 7.0 were selected for further use.

Ribosomal RNA depletion. Depending on the starting concentration of total RNA, two rRNA depletion methods were used according to the manufacturer's specifications. A Ribominus Transcriptome Isolation Kit for bacteria (Thermo-Scientific, Waltham, United States) was selected for cells grown on succinate or succinate + 2,4-TDA resulting in total RNA concentrations above 50 ng/µl. The enriched mRNA was then purified and concentrated by ethanol precipitation according to the manual with centrifugation at 15,000 g and precipitation at − 80 °C for 45 min. A riboPOOL Kit (siTOOLs Biotech, Martinsried, Germany) was used exclusively for 2,4-TDA-derived pooled samples with low RNA concentrations (at least 5 ng/µl). After rRNA depletion, all the samples were analyzed using the QuantiFluor kit, a Fluorometer (Promega, Wisconsin, USA) and Agilent 2100 Bioanalyzer.

RNA-seq library preparation and sequencing. A RNA-seq library was prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs Inc., Massachusetts, USA), according to the manufacturer's instructions. Briefly, 2.5 ng of total RNA was fragmented using NEBNext First Strand Synthesis Reaction Buffer. After the first and second strand cDNA synthesis, the NEBNext Adaptor was ligated to the

cDNA fragments and the enrichment of the ligated DNA was performed using i5 and i7 (index) primers. Finally, 12 cycles of PCR were used to produce the libraries. The quality of each library was verified using Qubit dsDNA HS assay (Thermo Fisher, Waltham, United States) and Agilent 2100 Bioanalyzer. The libraries were pooled, diluted to 4 nM and sequenced using the MiSeq Reagent Kit v3 600 cycles (Ilumina, California, USA) following the manufacturer's recommendations.

RNA-seq analysis. Treatment with exclusively 2,4-TDA yielded lower bacterial biomass and RNA concentrations and thus, a pooled sample was sequenced, lacking replicates. Pre-alignment quality control (FASTQC), trimmed and mapping reads to the annotated genome (deposited at DDBJ/ENA/GenBank under the accession WOVH00000000) using Bowtie 2 preceded. The analysis of this data was carried out with the DESeq2 package (Bioconductor)[47]. The analysis was fundamentally based on the assessment of the whole transcriptomics profile among treatments, normalization by size factor, filtering of unexpressed genes (≤ 10 reads among all replicates and treatments), and identification of the most overexpressed genes, comparing those treatments containing 2,4-TDA to succinate. Those genes reported on Table S1 represent the top 200 most overexpressed genes in either of the treatments containing 2,4-TDA.

Results and discussion

In 2020, Cárdenas Espinosa and colleagues suggested a degradation pathway for 2,4-TDA as the common precursor and degradation intermediate of polyurethanes in *P. capeferrum* TDA1[31,34,48]. The closest type strain species based on the genome-genome comparison (digital DNA-DNA hybridization) for the organism is *Pseudomonas capeferrum* WCS358[49]. TDA1 and WCS358 can be found in the same species cluster and differ significantly from the *P. putida* type species. The mineralization of 2,4-TDA was proposed to involve an extradiol cleavage of the aromatic compound, as well as two deamination reactions[34]. Here, further insights and proof regarding the mentioned metabolic pathway in *P. capeferrum* TDA1 are provided from transcriptomic data.

The formation of polyphenols and metabolic intermediates as a result of the bacterial activity on the substrate 2,4-TDA, posed a challenge for RNA extraction[46,50,51]. For this reason, an optimized method was established to prevent the interaction of such components with nucleic acids, and to yield RNA of higher quantity and quality than commercial kits and traditional methods[46]. Taking advantage of this new approach, total RNA extraction was conducted from samples of *P. capeferrum* TDA1 grown on 2 mM 2,4-TDA (Figure S2), 2 mM 2,4-TDA supplemented with succinate, or succinate only (as a control). These samples were then complemented with RNA-seq. The presence of the aromatic compound induces a stress response[52] and its degradation, even if it is not the main carbon source. RNA-seq data showed no striking difference between the two 2,4-TDA-containing treatments (Figure S3). PCA clustered each treatment independently, which highlights a particular response associated to the carbon source in the media (Figure S3b). In addition to the PCA results describing a comparable response, 2,4-TDA-containing treatments shared transcriptomics characteristics showing a similar gene expression pattern.

Transcriptional changes in TDA1 upon exposure to the PU monomer 2,4-TDA. Initially, the whole differential expression in *P. capeferrum* TDA1 treated with 2,4-TDA compared to succinate was analyzed. One third of the expressed genes in strain TDA1 grown on 2,4-TDA were overexpressed in comparison to the control (Fig. 1A). From those, 157 genes were expressed more than four-fold. A similar pattern was observed for downregulated genes (Fig. 1B). These results clearly denote the enormous effect that aromatic compounds such as 2,4-TDA have at the cellular transcriptional regulation, including not only their catabolism, but also their transport and defense against the inherent toxicity of these compounds, among many others which are covered subsequently[53–55].

The biochemistry of upper intracellular degradation pathway of 2,4-TDA was extensively discussed earlier[34], and one primary aim of the present work was to validate the degradation pathway in *P. capeferrum* TDA1 and to further elucidate its key players. Several candidates of the previously suggested catabolic pathway were identified to be highly overexpressed in TDA1 cells treated with 2,4-TDA (Fig. 2, Table 1). In Table 1, a candidate list of genes involved in the catabolism of the aromatic compound is presented based on their molecular function, as well as their differential expression when compared to succinate. Interestingly, a complete operon encompassing all the members of the degradation pathway was not detected although in this subset of genes many are located in close vicinity to other genes involved in the pathway (Table 1).

Briefly, the degradation of 2,4-TDA is most probably initiated by adjacent genes *tsaM_1* (candidate gene GNP06_06615) and *pobB_1* (gene GNP06_06620), which could encode the formation of a primary alcohol, given the similarity of 2,4-TDA to toluene 4-sulfonate. 4-aminoanthranilate (2,4-aminobenzoate) could be formed as a result of the function of an alcohol dehydrogenase and subsequently, an aldehyde dehydrogenase. *adhB_1* and *feaB_1* (candidate genes GNP06_06700 and GNP06_05050, respectively) could foster these reactions, given their transcriptional overexpression pattern and their ability to act on a broad range of aromatic substrates[56–58]. Nonetheless, other aldehyde dehydrogenase genes, such as *paoABC* homologs (accession P77324) or *aldH* (candidate gene GNP06_06665) were also significantly overexpressed. Since the substrate specificity of these enzymes is rather broad, the possibility that other proteins could also fulfill this function cannot be excluded[59,60]. Subsequently, a first deamination could be promoted nonspecifically by the action of benzoate 1,2-dioxygenase activity of the highly overexpressed cluster *benABC* (genes GNP06_08305, GNP06_08310 and GNP06_08315), yielding 4-aminocatechol.

It was also proposed that the intracellular degradation pathway of 2,4-TDA encompasses an extradiol ring cleavage of 4-aminocatechol yielding 4-amino-2-hydroxy-muconate semialdehyde; which is most possibly coded by dioxygenase *hpaD* (GNP06_05110). Nonetheless, the dioxygenase *pcaH*, which promotes an *ortho*-cleavage of the 3,4-dihydroxybenzoate aromatic ring, was also notably overexpressed in the presence of 2,4-TDA (*data not*

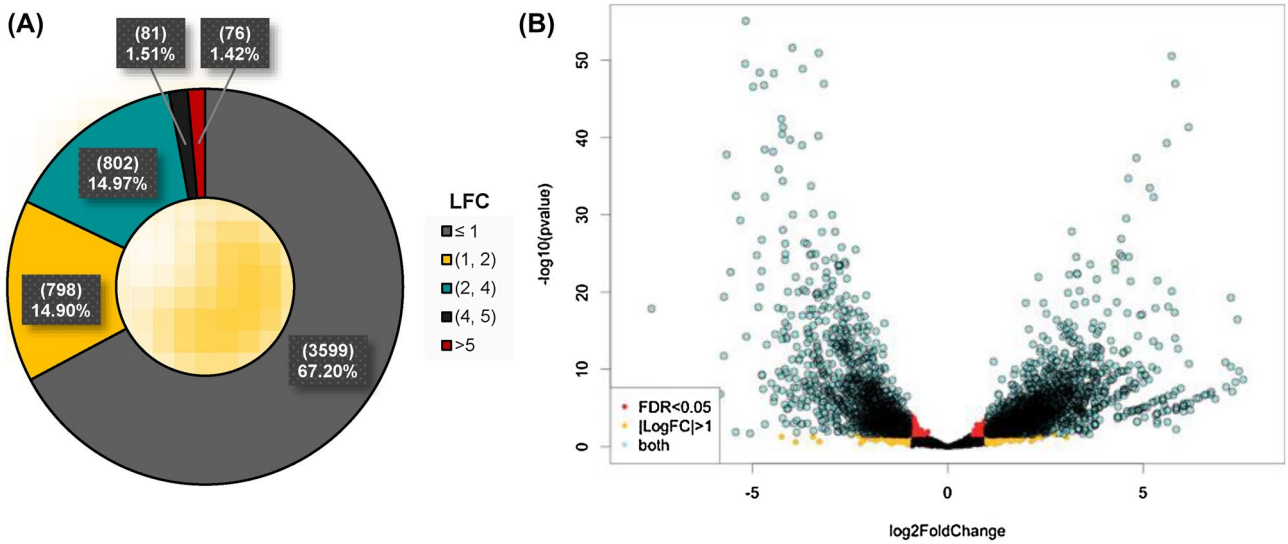

Figure 1. Analysis of differentially expressed genes in 2,4-TDA treated *P. capeferrum* TDA1 compared to a succinate-cultivated control. (**A**) Fractions of differentially expressed genes (DEG) by log2 Fold Change (LFC, logFC). DEGs with LFCs below 2 were mostly not considered for further analysis. (**B**) Volcano plot of the DEG in respect of their p-value (also FDR). *FDR = False Discovery Rate = p-value.* Candidates for significant DEG are characterized by a high p-value and high logFC.

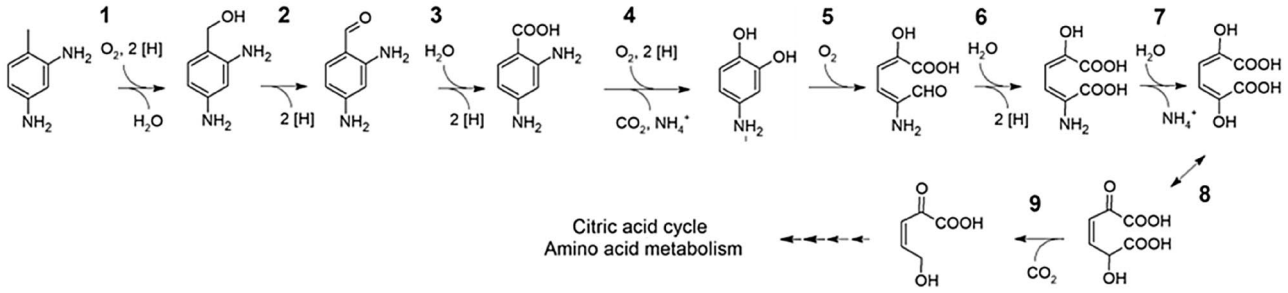

Figure 2. Proposed degradation pathway for 2,4-TDA in *Pseudomonas capeferrum* TDA1, via 4-aminoanthranilate (3), 4-aminocatechol (4), 4-amino-2-hydroxy-muconate semialdehyde (5) and 4-amino-2-hydroxy-muconate (6). The lower degradation pathway of 2,4-TDA needs to be elucidated in further studies.

No.	Gene	2,4-TDA v. Succ		2,4-TDA + Succ		Annotated function	Uniprot[c]
		Log2 fold change[a]	padj[b]	Log2 fold change[a]	padj[b]		
1	*tsaM1_2**	3.40 ± 1.31	0.0545	3.34 ± 0.50	1.16E-10	4-Toluene sulfonate methyl-monooxygenase	GNP06_06615
	*pobB_1**	3.31 ± 1.11	0.0222	2.48 ± 0.42	9.95E-09	4-Toluene sulfonate methyl-reductase subunit	GNP06_06620
2	*adhB_1*	3.40 ± 1.33	0.0586	3.38 ± 0.51	1.77E-10	Alcohol dehydrogenase (quinone)	GNP06_06700
3	*feaB_1*	3.88 ± 1.22	0.0135	3.48 ± 0.52	1.27E-10	Phenylacetaldehyde dehydrogenase	GNP06_05050
4	*benA***	4.16 ± 3.16	0.0428	4.27 ± 0.63	7.61E-11	2-halobenzoate 1,2-dioxygenase large subunit	GNP06_08315
	*benB***	3.71 ± 1.94	0.1751	3.55 ± 0.78	1.61E-05	2-halobenzoate 1,2-dioxygenase small subunit	GNP06_08310
	*benC***	5.83 ± 3.01	0.1700	6.80 ± 1.21	7.00E-08	Benzoate 1,2-dioxygenase electron transfer component	GNP06_08305
5	*hpaD****	2.32 ± 1.05	0.1117	1.97 ± 0.35	4.12E-08	Ring-cleaving dioxygenase	GNP06_05110
6	*hpaE****	2.82 ± 1.52	0.1927	3.31 ± 0.56	1.32E-08	5-carboxymethyl-2-hydroxymuconic semialdehyde dehydrogenase	GNP06_05115
7	*GNP06_05035*	6.02 ± 2.85	0.1295	7.23 ± 1.20	8.51E-09	Aminomuconate deaminase	GNP06_05035
8 + 9	*hpcE_1****	2.76 ± 1.73	0.2721	3.30 ± 0.59	7.69E-08	Homoprotocatechuate catabolism bifunctional isomerase/decarboxylase	GNP06_05120

Table 1. List of gene candidates for the intracellular degradation pathway of 2,4-TDA in *P. capeferrum* TDA1 (*see* Fig. 2). */**/***Adjacent genes (to each other). [a]log2 Fold Change ± standard error (LFC). [b]p-value adjusted (versus control, i.e., succinate). [c]Uniprot refers to the accession number of the TDA1 protein in this database.

shown). Encoded by the adjacent gene *hpaE* (GNP06_05115), 5-carboxymethyl-2-hydroxymuconic semialdehyde dehydrogenase could yield 4-amino-2-hydroxy-muconate.

Finally, the uncharacterized gene GNP06_05035, which encodes a putative aminomuconate deaminase, could enable the second deamination step. This gene holds many homologues in the *Pseudomonas* genus, which have been annotated as RidA family proteins, as well as enamine/imine deaminases. Nonetheless, despite its significant upregulation, it might also be possible that the second deamination step could take place in the lower degradation pathway, as part of the amino acid metabolism, namely given the overexpression of other deaminases, such as the 2-iminobutanoate/2-iminopropanoate deaminase GNP06_17020, among other transaminases and aminotransferases. HpcE_1 (gene GNP06_05120), whose gene is adjacent to *hpaD* and *hpaE*, could further tautomerize and carboxylate the resulting intermediate 2,5-dihydroxy-muconate[34]. The lower degradation pathway of 2,4-TDA needs to be elucidated in further studies.

Induction of general stress response and biofilm formation. To tackle the inherent toxic effects of 2,4-TDA, *P. capeferrum* TDA1 has evolved complicated and sophisticated mechanisms to defend itself against the toxicity of suchlike compounds, yet still being able to use 2,4-TDA as a carbon, nitrogen and energy source[34]. Several genes involved in the general stress response of the cell against a wide range of abiotic environmental stresses showed a significant overexpression in the transcriptomic data set. For instance, genes implicated in the transport, protein misfolding protection and regulation against metal ions stress presented an increased transcriptional expression (Table S1) when 2,4-TDA was present. In the last decades, several reports showcased a similar general stress response in the toluene-tolerant *P. putida* DOT-T1E and *P. putida* S12 in the presence of toluene[61–64].

Furthermore, many genes involved in biofilm formation and bacterial motility were highly expressed in 2,4-TDA treatments when compared to succinate culture. Genes such as diguanylate cyclase GNP06_08145, glutathione transport system permeases *gsiD* and *gsiC* or the *alg* operon were also present in the most overexpressed genes in 2,4-TDA-containing treatments (Table S1). The *alg* operon, formed by *algA, algE, algF*, among many others, is involved in the production and transport of alginate, an exopolysaccharide found in the biofilm matrix, especially produced by the genus *Pseudomonas*[65]. Tripeptide glutathione may impede the formation of bacterial biofilms[66]. Hence the upregulation of glutathione transport, concurred with its non-differentially expressed biosynthetic pathway in the presence of 2,4-TDA (*data not shown*).

It was previously documented that the tricarboxylic (TCA) cycle components were upregulated in the presence of hydrocarbon solvents, in order to modulate the NAD(P)H concentration[63]. It was discussed, that such upregulation could enable the bacteria to cope with the energetic potential loss related to other defense mechanisms, such as solvent efflux pumps and maintenance of redox balance[67]. However, TCA cycle upregulation was not observed in our strain TDA1, similarly to the results obtained in *P. putida* DOT-T1E[68]. Given that DOT-T1E could tolerate higher concentrations and degrade toluene, the authors concluded that the induction of the toluene degradation pathway eclipsed the need to upregulate the TCA cycle[68]. Thus, it is likely that the capability to degrade 2,4-TDA and use this compound as energy, carbon and nitrogen sources prevents the induction of other central metabolism pathways, such as the TCA cycle.

Finally, another defense mechanism used by bacteria to endure in high concentrations of toxic aromatic compounds is actively pumping them out into the extracellular space[54,61]. From a vast subset of enzymes involved in this defense mechanism, efflux pumps, multidrug resistance proteins, RND transporters, as well as specific porins were largely overexpressed in the presence of 2,4-TDA (Table S1). Among these, several proteins of the *ttg (toluene tolerance genes)* efflux systems were found, such as *ttgI* and *ttgC* (Table S1). Nonetheless, the annotated *ttgRABC operon* was not differentially expressed (*data not shown*), which correlates with the downregulation of the TCA cycle: *P. capeferrum* TDA1 endures the inherent toxicity of 2,4-TDA by actively degrading it.

Strategies and enzymes for the extracellular cleavage of PU compounds in P. capeferrum TDA1. Pseudomonads show a promising metabolic potential and high adaptability to a broad range of environmental stresses. Besides, *P. capeferrum* TDA1 can not only tolerate and use a PU monomer as carbon, energy and nitrogen source, but was also observed to degrade a PU oligomer[34]. These features make the strain an interesting candidate for biological recycling. For this sort of applications, the extracellular strategies of the strain to unlock PU compounds are of eminent importance and thus were assessed further in this work.

P. capeferrum TDA1 possesses a membrane-bound esterase activity. PU extracellular degradation occurs via the action of extracellular and membrane-bound hydrolases[13]. Even though *P. capeferrum* TDA1 does not contain any known extracellular polyurethanase homolog (*e.g.* PueA from *P. protegens* Pf-5; *data not shown*), esterase activity has been previously identified to act on polyester-based polyurethane[48,69,70]. Thus, the ability of *P. capeferrum* TDA1 to cleave different *p*-nitrophenyl (*p*NP) esters was assayed[41]. Cultures of TDA1 were grown in PU oligomer or succinate (control) and their supernatant, whole-cell pellets and outer membrane vesicles (OMVs) were isolated and investigated separately. As it can be noticed in Fig. 3A, esterase activity was only detected in whole-cell pellets and OMVs, but not in the supernatant (Fig. 3B), regardless of the carbon source applied. Moreover, the cultivation of TDA1 with the PU oligomer did not induce a significant rise in the esterase activity for the *p*NP substrates analyzed compared to the control. Hence, the obtained results suggest the presence of an outer membrane-bound esterase activity in *P. capeferrum* TDA1. Moreover, the activity was two- and four-fold increased for isolated OMVs compared to the cell pellets.

OMV release in TDA1 was shown to increase significantly in PU oligomer compared to succinate, with relative OMV yields of 0.28 ± 0.05% and 0.09 ± 0.01%, respectively. This three-fold increase shows that the release of OMV is part of the adaptive mechanisms of bacteria to stressful environmental conditions such as the presence

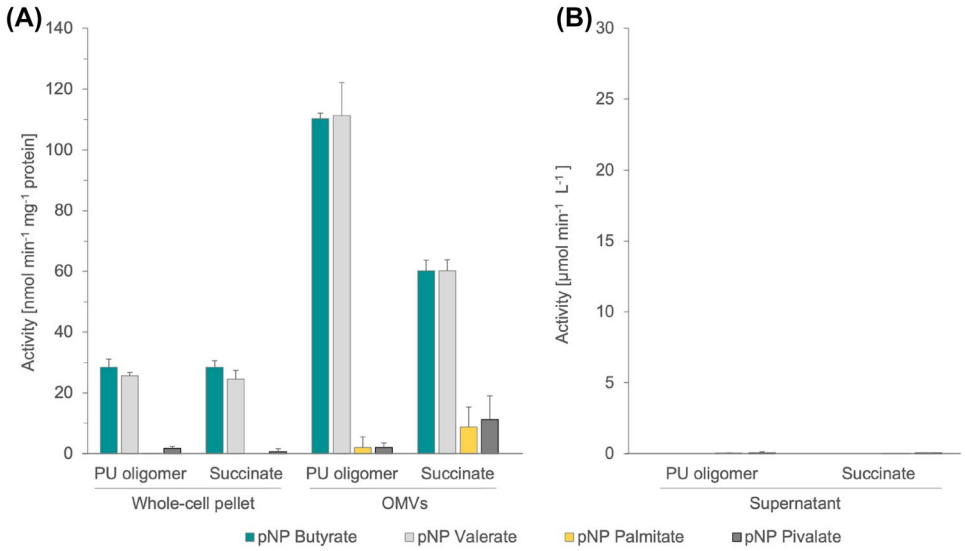

Figure 3. Conversion of *p*-nitrophenyl esters to *p*-nitrophenol by different fractions of *P. capeferrum* TDA1 grown in PU oligomer, or succinate (control). (**A**) Esterase/Lipase activity on whole-cell pellets and outer-membrane vesicles (OMVs) (per mg protein) and (**B**) supernatants (per volume) of *P. capeferrum* TDA1 were assessed. OMV samples were subjected to a protein quantification via BRADFORD. Standard deviations and legend are given.

of toxic PU compounds, as previously discussed for other organic contaminants[71–73]. In addition, OMV may also be used as vehicle to transport the necessary enzyme through extracellular space to unlock new carbon sources like the PU oligomer.

Enzyme candidates identified in OMV fractions. In order to identify the enzymes involved in the cleavage of *p*-nitrophenyl esters and potentially oligomeric PU, OMVs of cells grown on PU oligomer or succinate were analyzed by LC–MS. 318 proteins which are localized –or predicted to be localized– in the periplasm, flagellum, outer membrane or extracellularly were identified. Among those, 95 proteins have not been characterized yet or they possessed domains of unknown function (DUF). Yet, a substantial difference in TDA1's OMV-exoproteome among treatments was perceived (Fig. 4, S4), which suggests that each carbon source induces a distinct and characteristic subset of extracellular proteins and concatenated reactions. However, the esterase activity of both subsets seem to be in the same order of magnitude regardless of the carbon source. Similar results showed that nanopod/OMV formation was induced by growth of *Delftia acidovorans* Cs1-4 on phenanthrene[21]. Furthermore, the study suggested the contribution of extracellular structures as elements supporting metabolic biodegradation processes.

A subset of the hydrolases detected in OMV fractions with annotations that suggest a putative cleaving-function of ester bonds or amid-type bonds was included in Fig. 5. Interestingly, several candidates capable of executing the esterase cleavage were identified. The esterases TesA and EstP were identified, whose homologs have been previously spotted in OMVs of different *Pseudomonas spp.*[74,75] and linked in *P. aeruginosa* and *P. putida* to the cleavage of *p*-nitrophenyl esters of short acyl chains[76,77]. Notably, these characterized esterases were exclusively detected in OMV of the PU culture (TesA) *or* in the OMV fraction of the succinate culture (EstP), which could indicate a regulation of each esterase in a substrate-specific manner. Nevertheless, such substrate-specific effect on the exo-esterase expression has not yet been investigated and further research will be needed to prove these hypotheses.

Other proteins such as, amidases, peptidases, proteases, or Lipid A deacylase could also contribute to the cleavage of *p*-nitrophenyl esters of short acyl chain, although only one or a few of them may be dominant. The mentioned hydrolases and thus the extracellular degradation pathway could be conserved among *Pseudomonas* spp. and potentially Proteobacteria (Fig. 5).

A possible strategy to enhance the degradation of a polyurethane oligomer by the action of hydrolases present in OMVs in P. capeferrum TDA1. Microbial plastic degradation has been extensively studied in the last decades, yet a relation with OMVs harboring hydrolytic enzymes has never been presented. Nonetheless, OMVs have been identified recently to induce lignin-derived aromatic compound degradation in *P. putida* KT2440[20]. Interestingly, the authors suggested a model through which hydrolytic periplasmic enzymes in secreted vesicles could also promote degradation via OMV lysis[20]. Thus, macromolecular degradation may occur through the action of both periplasmic and membrane-bound hydrolases harbored in OMVs. According to the results presented in this work, OMVs can be considered as a supporting mechanism for biodegradation (Fig. 6). This proposed method would function alongside free extracellular enzymes and membrane-

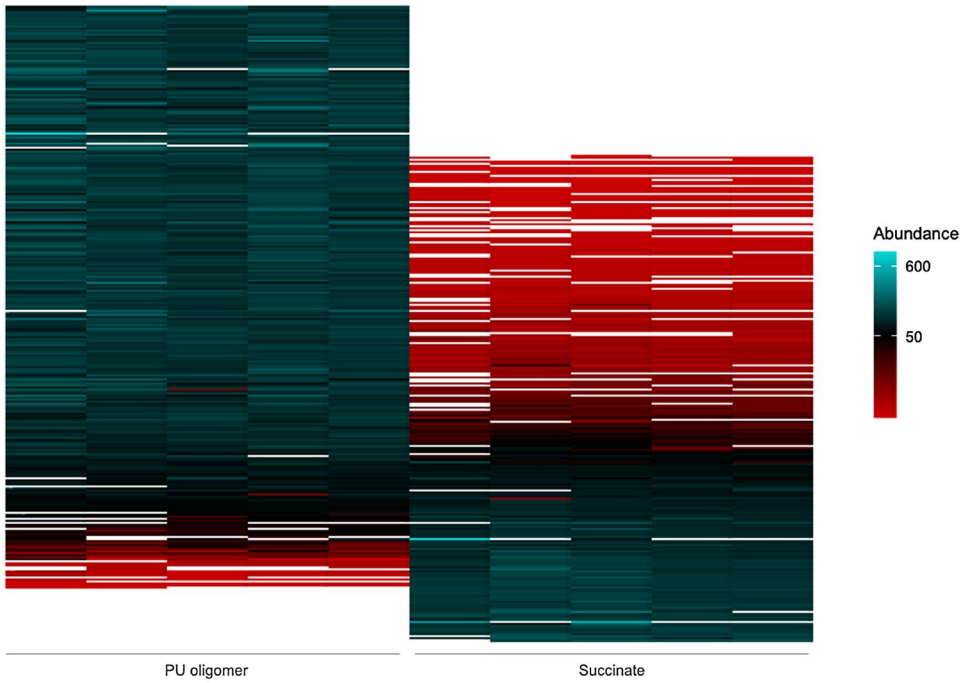

Figure 4. Heatmap of extracellular proteins detected by mass spectrometry on OMV fractions of TDA1 in PU oligomer or succinate showing the difference of the samples. Abundance for each of the five replicates of each treatment is given as a color scale, being *white* non detected enzymes.

Protein	Average Abundance (scaled)	Subcellular Location[a]	Homology (*P. putida* KT2440)	Accession
Alpha/beta fold hydrolase cpo		Periplasm	93 %	A0A6L6T9R6
Arylesterase TesA		Periplasm	93 %	A0A6L6TDG0
Predicted Zn-dependent peptidases		Periplasm	100 %	Q88CR7
Putative protease tldD		**Outer membrane**	100 %	Q88PB0
Putative Peptidase		Cell membrane	100 %	Q88LM2
ACP phosphodiesterase		Periplasm, **Outer membrane**	93 %	A0A6L6TIK5
Metallopeptidase		**Outer membrane**, Extracellular, Periplasm	41 %	A0A6L6TL79
Serine-type D-Ala-D-Ala carboxypeptidase		Periplasm	97 %	A0A6L6TND5
Dienelactone hydrolase family protein		Periplasm	94 %	A0A6L6THG0
N-acetylmuramoyl-L-alanine amidase		**Outer membrane**, Periplasm	100 %	Q88DD0
PepSY domain-containing protein		Periplasm	-	A0A6L6TC05
Lipid A deacylase		**Outer membrane**	100 %	Q88I47
Autotransporter esterase EstP		**Outer membrane**	91 %	A0A6L6TH84

Figure 5. Candidate list from mass spectrometry of OMV samples in both PU oligomer and succinate. Candidates were selected due to their abundance in the OMV samples, their subcellular location, and their homology to proteins in *P. putida* KT22440. [a] *In the case that subcellular location was not annotated for the respective protein, prediction* via *CELLO v.2.5.* The proteomes of OMVs from cells grown on succinate and OMVs from cells on the PU oligomer clearly differ from each other and esterases were detected in both conditions.

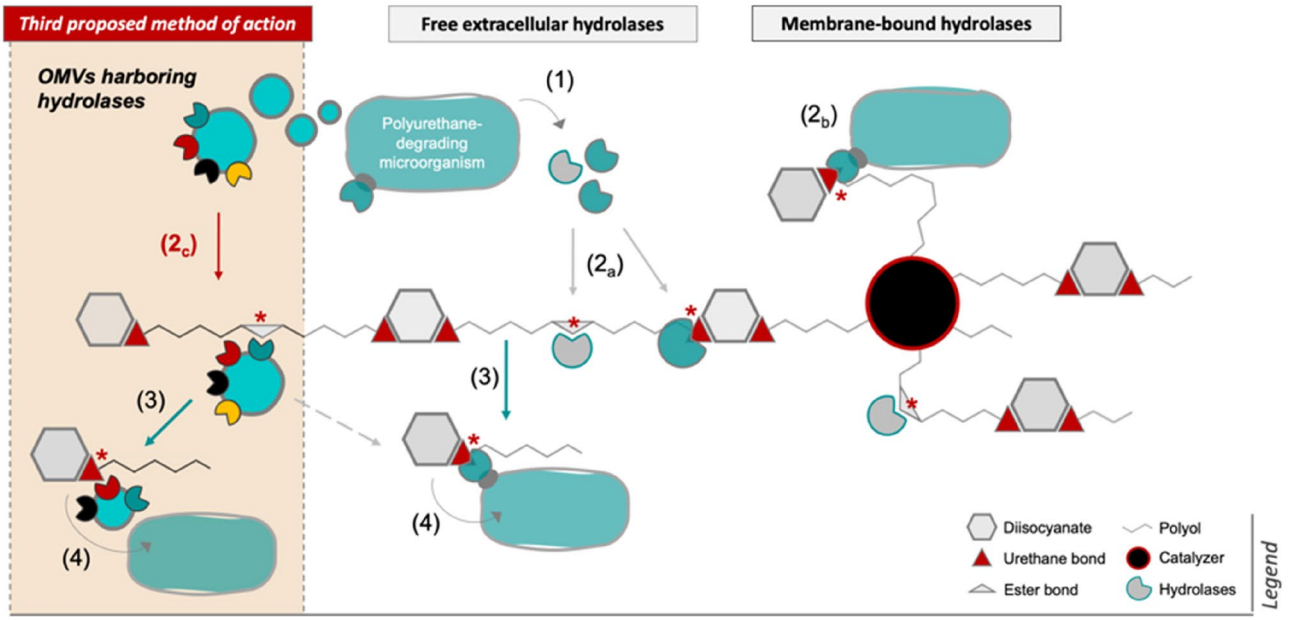

Figure 6. Model for extracellular degradation of polyester-based polyurethane via OMV function by *P. capeferrum* TDA1. A new mechanism to biodegrade extracellular polyurethane through the release of outer membrane vesicles (OMVs) by the bacterial cell is suggested. These OMVs could harbor outer membrane-bound or periplasmic hydrolases to promote degradation (2_c). In the case of the latter, their function would occur through the lysis of the OMVs (*not depicted*). Procedure: (1) Degradative microorganisms express free and membrane-bound hydrolytic enzymes, and release OMVs. Enzymatic degradation (*) occurs through (2_a) extracellular enzymes and (2_b) outer membrane-bound hydrolases, which can also be present in (2_c) OMVs. As a result, (3) smaller PU fragments may be released and further cleaved, (4) which can be finally transported in the cell and mineralized.

bound hydrolases, as is widely recognized[3,13]. The uptake of catabolic products would occur subsequently by the bacterial cell, which would promote the mineralization of the compound. Moreover, we surmise that such model could be conserved among *Pseudomonas* spp. and potentially *Proteobacteria,* given that OMV release is an ubiquitous defense mechanism among Gram-negative bacteria[72,78,79].

Conclusion and future perspectives

Biodegradation of aromatic substrates by *Pseudomonas* contributes significantly to pollutant removal in various ecosystems. Understanding of the biochemical pathways may provide vital information for enhancing catabolic efficiency. The results presented in this work demonstrate that *P. capeferrum* TDA1 degrades PU monomers efficiently. Thus, TDA1 could be preferentially employed in a two-step degradation process, in which enzymatic catalysis of the macromolecular polymer initially yielded plastic monomers. Furthermore, *P. capeferrum* TDA1 could use these monomers to synthesize novel value-added products in a new circular plastic economy, as recently studied[80].

OMVs and external cell structures have been proven important in numerous microbial activities including biodegradation[20,21]. It can be expected that free and membrane-bound hydrolases alongside play a role in the extracellular degradation of PU monomers and oligomers, as described in our proposed model (Fig. 6).

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Author contributions

O.P., M.J.C.E., H.J.H, C.E. conceived and designed the analysis. O.P., M.J.C.E. collected the data. D.S., S.T., S.S., N.J., D.W., J.K., H.J.H. contributed data or analysis tools. O.P., M.J.C.E., U.K., S.S.; N.J. performed the analysis. O.P. wrote the paper. M.J.C.E. revised the paper. H.J.H., C.E., M.J.C.E., S.T., N.J., H.J.H. critically reviewed the article.

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Correspondence and requests for materials should be addressed to H.J.H.

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2.4.1 Supplementary Information

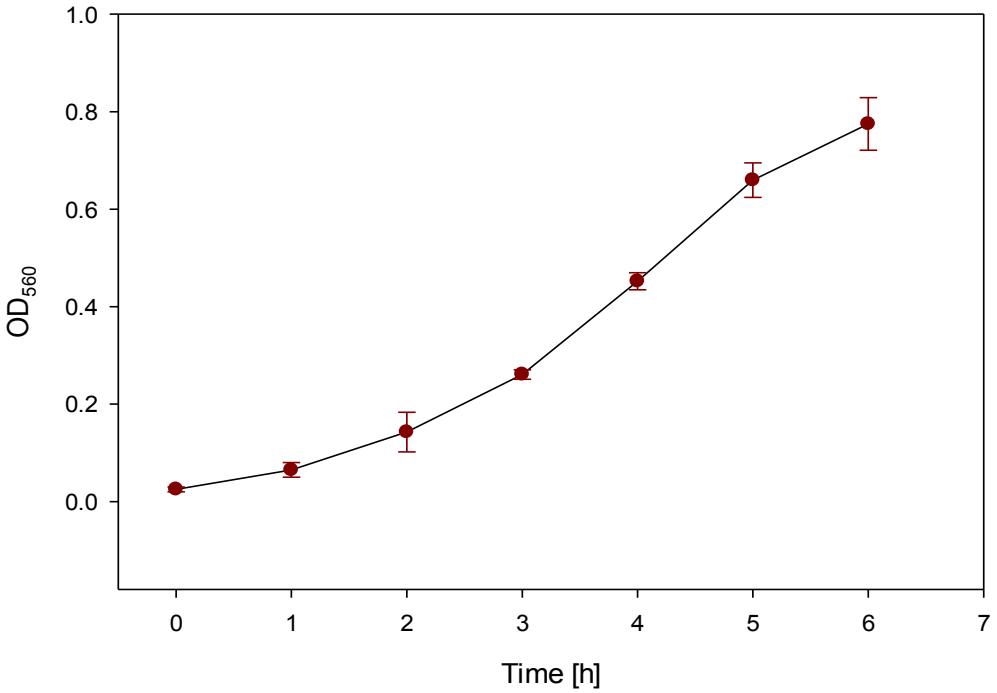

Figure S1. Growth kinetics of *P. capeferrum* TDA1 grown on PU oligomer.

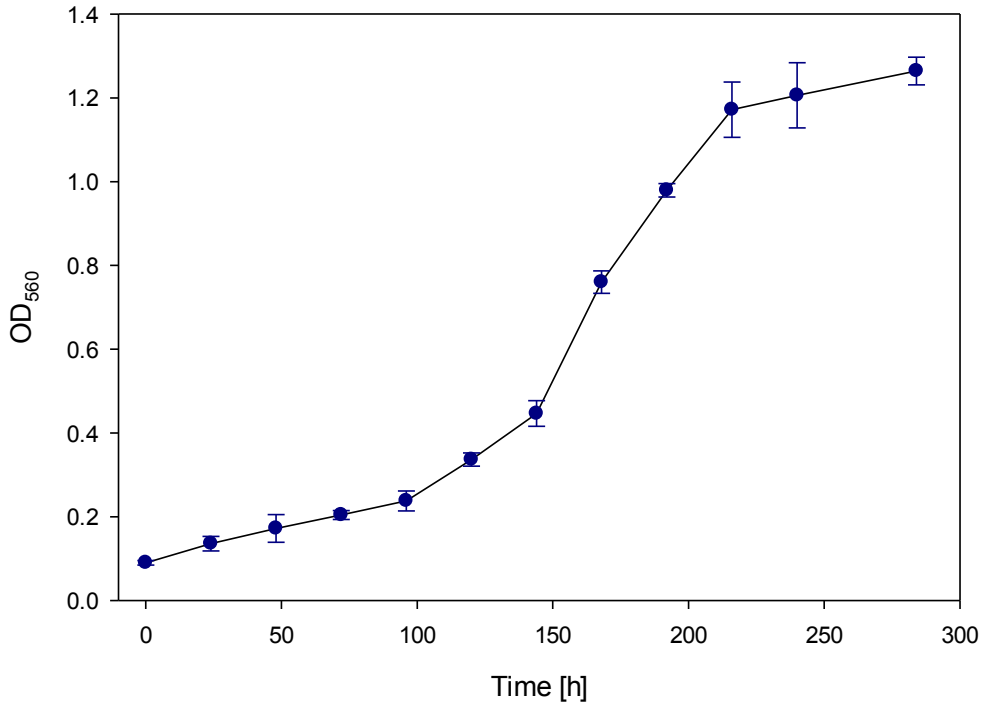

Figure S2. Growth kinetics of *P. capeferrum* TDA1 grown on 2,4-TDA.

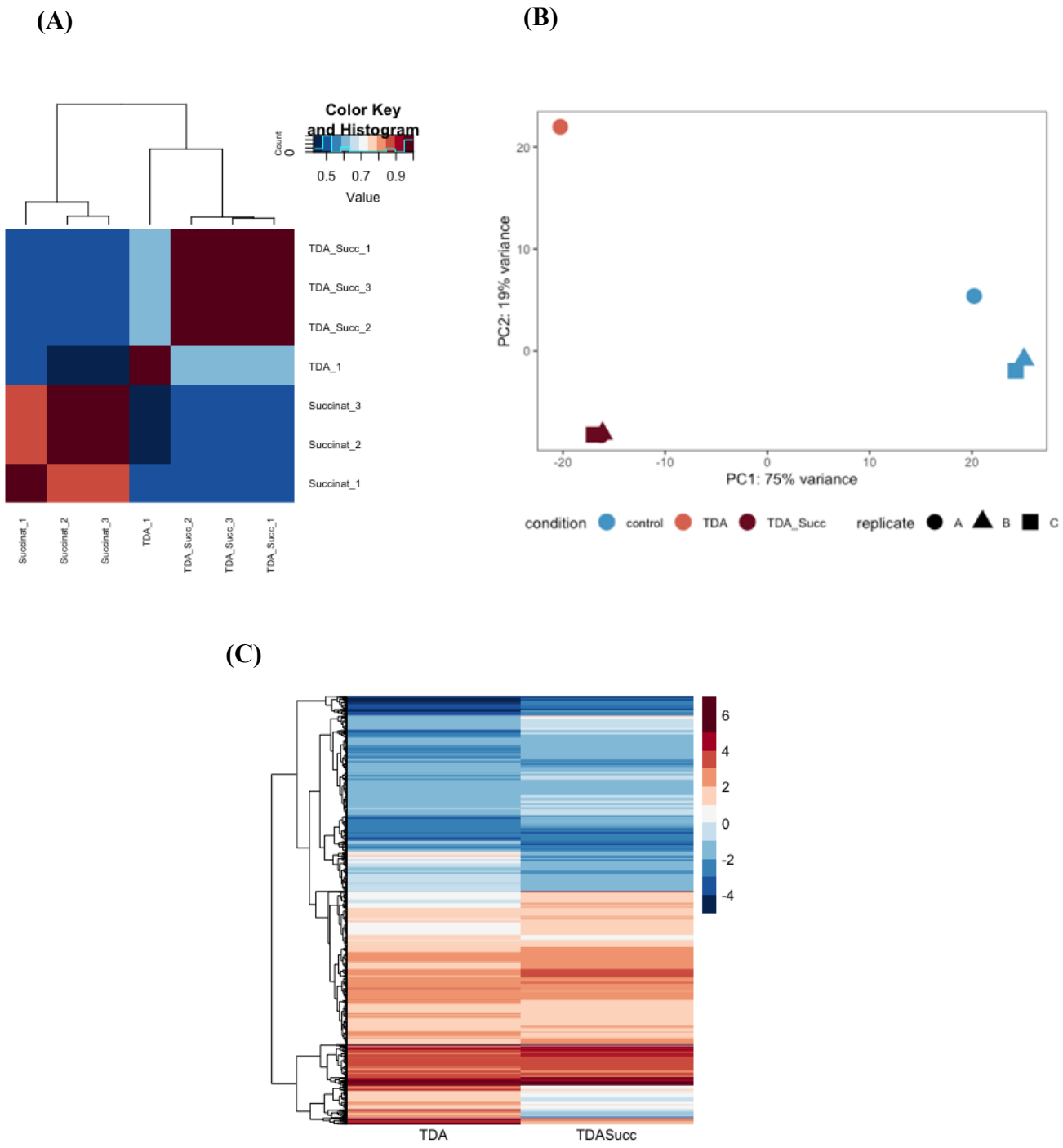

Figure S3. Comparison of treatments 2,4-TDA (*TDA*) and 2,4-TDA supplemented with succinate (*TDASucc*). (A) Pair-wise correlation heatmap (using spearman's correlation method) per replicate. (B) PCA of normalized RNAseq read counts. *(A, B) also include succinate, control samples*. C) Heatmap of differentially expressed genes of each treatment when compared to control samples.

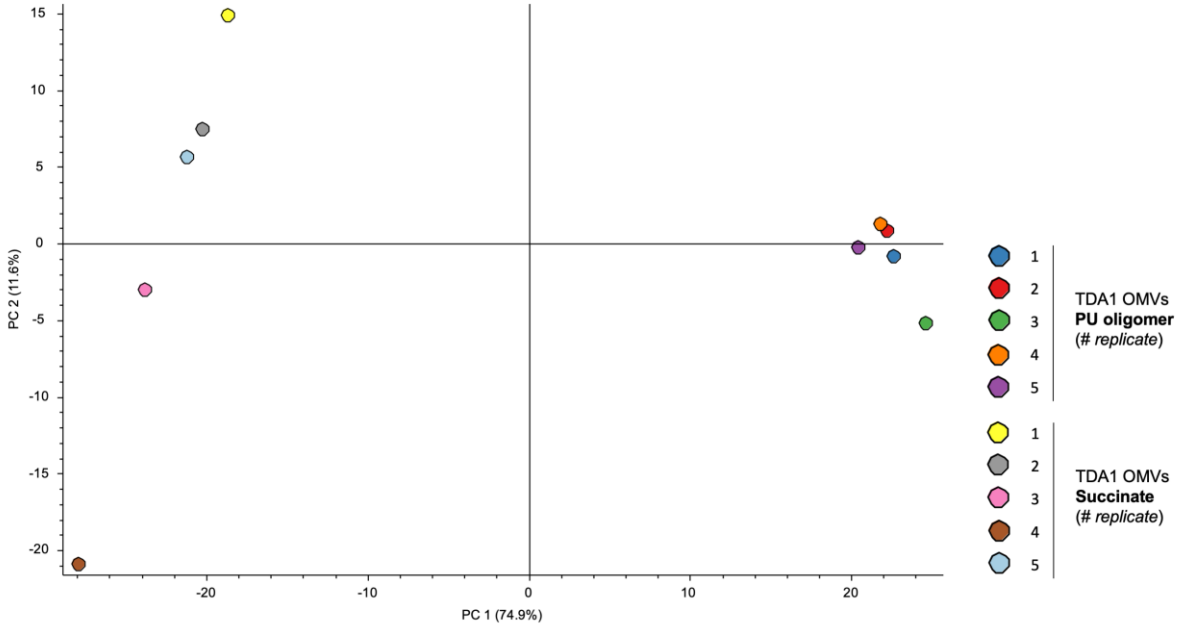

Figure S4. PCA Analysis of *P. capeferrum* TDA1 exoproteome of OMVs grown in PU oligomer or succinate. This analysis, though, also includes cytosolic proteins in low concentrations yet considered as contamination of the OMV sample. Legend is given.

Table S1. List of genes with a high overexpression pattern grouped by function retrieved from the RNA-Seq data of *P. capeferrum* TDA1 cells grown on 2,4-TDA or 2,4-TDA supplemented with succinate (Succ) compared to the control (succinate). p-value adjusted (padj), as well as the presented fold change values of the correlation between treatments 2,4-TDA and succinate displayed considerably higher numbers compared to the treatment 2,4-TDA supplemented with succinate due to the existence of a single 2,4-TDA replicate, which is heavily penalized by the normalization algorithm. Nonetheless, such replicate was composed by a pool of cultures – for biomass reasons.

gene	2,4-TDA v. Succ		2,4-TDA + Succ v. Succ		Gene Function	Uniprot[c] (homology)
	LFC[a]	padj[b]	LFC	padj		
Genes involved in aromatic compound degradation						
paaE	7,1096	1,01E-02	7,1870	1,28E-08	1,2-phenylacetyl-CoA epoxidase, subunit E	P76081
ahpF	6,1665	5,04E-11	1,5914	2,81E-02	Alkyl hydroperoxide reductase subunit	P35340
Hgd	6,1336	4,31E-13	1,8883	9,24E-06	2-(hydroxymethyl)glutarate dehydrogenase	Q0QLF5
paaI_2	6,1095	1,13E-01	5,9584	2,98E-06	Acyl-coenzyme A thioesterase PaaI	P76084
paaB	6,0221	1,30E-01	5,8149	7,51E-06	1,2-phenylacetyl-CoA epoxidase, subunit B	P76078
gudD	5,9329	7,18E-03	6,6207	8,94E-10	Glucarate dehydratase	P42206
hcaD	5,8580	1,38E-04	4,7354	2,04E-09	3-phenylpropionate/cinnamic acid dioxygenase ferredoxin-NAD(+) reductase component	J7R3Y9
mdlC	5,3875	2,73E-08	2,2273	2,52E-04	Benzoylformate decarboxylase	P20906
aroH	5,3535	1,76E-13	-0,4249	3,52E-01	Phospho-2-dehydro-3-deoxyheptonate aldolase	P80574
paaH	5,2235	3,94E-01	4,3773	9,89E-08	3-hydroxyadipyl-CoA dehydrogenase	P76083
glaH	5,0320	8,07E-03	4,4507	1,66E-08	Glutarate 2-hydroxylase	P76621
GNP06_17000	5,0204	8,04E-03	4,4466	2,22E-08	4-sulfomuconolactone hydrolase	A6XIG7
cntA	4,7977	1,89E-02	4,7430	2,35E-09	Carnitine monooxygenase oxygenase subunit	D0C9N6
paaA	4,4554	4,52E-02	4,4715	1,32E-08	1,2-phenylacetyl-CoA epoxidase, subunit A	P76077
gudP_1	4,3711	2,47E-02	4,6211	1,26E-12	putative glucarate transporter	Q46916
hipO	4,3548	6,02E-02	4,3707	7,33E-07	Hippurate hydrolase	P45493
aroE_1	3,5101	2,32E-01	4,0876	5,54E-07	Shikimate dehydrogenase (NADP(+))	Q8Y9N5
quiA_1	3,0324	4,49E-02	4,0234	3,01E-17	Quinate/shikimate dehydrogenase (quinone)	Q59086
aroQ	2,9849	3,81E-01	3,9177	1,52E-06	3-dehydroquinate dehydratase	P43877
Genes regulated by general stress response involved in metal ions-related functions						
czcC_1	7,3440	4,21E-03	7,4661	2,91E-09	Cobalt-zinc-cadmium resistance protein CzcC	P13509
zupT_2	6,1680	1,90E-17	1,8562	1,79E-08	Zinc Transporter ZupT	–
yciC	5,8081	1,07E-13	2,5025	5,75E-13	Putative metal chaperone YciC	P94400
cueR_1	4,8645	1,10E-01	4,9975	6,75E-06	HTH-type transcriptional regulator CueR	Q93CH6
Genes involved in the pump out of aromatic compounds						
*bepF_1**	7,2169	4,40E-09	3,6703	5,18E-06	Efflux pump periplasmic linker BepF	Q8FWV8

bepF_2	7,1753	1,17E-01	2,9350	1,30E-02	Efflux pump periplasmic linker BepF	Q8FWV8
mdtA_4	6,8294	2,42E-02	*not signif. expressed* [d]		Multidrug resistance protein MdtA	P76397
mdtC_3	6,1920	9,86E-02	*not signif. expressed* [d]		Multidrug resistance protein MdtC	–
bepE_1	5,7354	1,41E-02	3,7828	8,47E-04	Efflux pump membrane transporter BepE	Q8G2M6
oqxB17	5,7895	4,51E-06	3,6505	2,92E-08	multidrug efflux RND transporter permease subunit	–
ttgI_1	5,3507	2,13E-03	2,9696	3,56E-04	Toluene efflux pump outer membrane protein TtgI	Q93PU3
*oqxB7**	4,6525	1,89E-05	2,5363	1,08E-08	multidrug efflux RND transporter permease subunit	A0A5E7AVI6
*ttgC_3**	3,8718	3,53E-02	0,7146	5,01E-01	putative efflux pump outer membrane protein TtgC	Q88N32
aaeB_3	3,6700	1,90E-02	4,3776	1,15E-20	p-hydroxybenzoic acid efflux pump subunit AaeB	–
ttgI_4	3,6487	8,09E-02	3,7932	7,72E-11	Toluene efflux pump outer membrane protein TtgI	Q93PU3
Genes involved in Biofilm induction and production						
GNP06_08145	4,1307	2,33E-02	4,4085	8,13E-15	diguanylate cyclase	A0A6L6TCN3
algF	5,1916	5,59E-02	5,5937	2,84E-07	Alginate biosynthesis protein AlgF	Q06062
algE	4,8917	1,33E-02	5,4815	1,44E-12	Alginate production protein AlgE	P18895
algJ	3,9323	6,08E-01	4,7296	2,47E-12	putative alginate O-acetylase AlgJ	Q88ND3
algX	3,6863	5,37E-02	4,1352	2,13E-15	Alginate biosynthesis protein AlgX	Q51372
algA	3,1104	7,20E-02	3,7643	3,38E-17	Alginate biosynthesis protein AlgA	P07874
gsiC	5,6091	2,03E-02	5,3806	8,85E-07	Glutathione transport system permease protein GsiC	P75798
gsiD	4,5606	3,54E-02	4,2416	1,06E-07	Glutathione transport system permease protein GsiD	P75799
gloB_1	3,7321	9,31E-03	2,7045	7,68E-09	Hydroxyacylglutathione hydrolase	–
Enzymes with deaminase, transaminase and aminotransferase activity						
phnW	5,2254	2,03E-04	4,0055	2,29E-10	2-aminoethylphosphonate-pyruvate transaminase	Q9I434
kce_1	4,6059	5,32E-01	4,7619	1,67E-11	3-keto-5-aminohexanoate cleavage enzyme	Q8RHX2
GNP06_17020	4,3980	2,08E-01	4,5934	3,45E-05	2-iminobutanoate/2-iminopropanoate deaminase	–
argD_1	4,1445	1,37E-01	5,2993	1,26E-11	Acetylornithine aminotransferase	Q9X2A5
MPHCKDGE_04608	3,7016	3,75E-01	6,0630	2,42E-08	Aminotransferase · Pyridoxal phosphate-dependent transferase, major domain	–

[a]*LFC: log2 Fold Change.*
[b]*padj: p-value adjusted when compared to the control.*
[c]*Uniprot accession number of the closest homolog annotated and characterized in the database (if given).*
[d]*Genes which were not significantly expressed (≤ 10 counts considering all replicates and treatments).*
**Genes clustered together, possibly as a part of the same operon.*

3. Discussion

3.1 Characterization of a bacterial strain able to grow on building blocks of polyurethane and identification of degrading enzymes

The increasing plastic production has become a global concern. While mechanical and chemical recycling represent the first lines of intervention to solve this problem, upcycling options based on catalytic transformations will eventually be necessary to reconvert enormous quantities of such material [74]. During the last years, bacteria have become an important source of new degradable enzymes due to their high level of biodiversity. Nevertheless, biodegradation is a complex process that depends on another factors, including molecular weight of the polymers, surface characteristics, morphology, availability of a substrate, etc., which leads to the continuous search for new, highly efficient enzymes [75, 76].

The biocatalytic upcycling of plastic waste has been used as a "green strategy" to recover chemical monomers and dimers in order to transform them into high-value products for industrial applications[77]. The most detailed studies on biodegradation of synthetic compounds have been conducted on PET, PU, PE and PS. However, PET and PU are more susceptible to biodegradation due to the presence of hydrolysable ester bonds compared to carbon backbone polymers, such as PE, PS, and PP, which degradation mechanisms are still unknown[75].

In 2016, Yoshida et al[75] reported that *Ideonella sakaiensis* 201-F6 was able to depolymerize PET polymers and utilize the terephthalate subunits as a carbon and energy source degrading almost completely PET films in six weeks. In addition, two versatile cutinases (PETase and MHETase) converted the plastic polymer into its two monomers: terephthalic acid (TA) and ethylene glycol (EG), which are used as carbon and energy feedstock for different microorganism to produce bioplastics afterwards. *Bacillus subtilis* MZA-75 degraded PU by extracellular esterases into 1,4-butanediol (BDO) and adipic acid (AA) and utilized these monomers as carbon source to mineralize the polymer[78].

In this thesis, *Pseudomonas capeferrum* TDA1 was able to degrade a PU oligomer and use 2,4-TDA as carbon, nitrogen and energy source. In a previous study, 2,4-TDA (0.55 mM) was consumed as sole carbon source or co-metabolized with ethanol by an unknown culture (7), which highlight the catalytic performance of *P.*

capeferrum TDA1. In addition, this strain was tested in a mixed culture, containing AA, BDO, ethylene glycol and 2,4-TDA as substrates. Despite that 2,4-TDA was removed due to the inhibition of substrate utilization, the defined microbial consortium could transform the PU hydrolysate into rhamnolipids[79].

The use of fuel-based plastic monomers and oligomers as feedstock for the synthesis of high-value products represents a significant improvement in the transition to a circular economy. Despite the fact that plastic upcycling is still considered a new approach, it had shown that plays an essential role in reducing dependency on fossil resources, valorizating waste and creating low-cost monomer for plastic production compared to traditional processes. However, in order to reduce the carbon footprint of the chemical industry, this technology should be extended to other synthetic polymers. The production of new sustainable products using plastics monomers and dimers as carbon sources valorizes waste stream enormously and exploit the metabolic versatility of microorganisms[75, 77]. *Rhodococcus rhodochrous* can convert styrene, a PS monomer, into 3-vinylcathecol (used in the synthesis of dyes and pharmaceutical products). TA and EG were converted by a modified *Pseudomonas sp*. GO16 into hydroxyalkanoyloxyalkanoate (HAA), which can be copolymerized with diisocyanate and butanediol to yield a novel bio-PUR[77, 80]. *Burkholderia sacchari* IPT189 was able to produce PHA using propionic acid as a sole carbon and energy source[81]. In spite of the growing research on plastic depolymerization and bio-upcycling, these processes still need to be economically feasible and reach higher production rates.

In recent years, valorization of PUR waste via the biotechnological production of value-added products have attracted an increasing interest as a promising up-cycling strategy. Unlike PET, which contains only TA and EG in its main chain, PUR polymers have a more complex backbone yielding a broader spectrum of degradation products, including amines, alcohols, acids, aromatics, etc[59]. For instance, carboxylic acids and alcohols can be used for the synthesis of virgin PUR and other polyesters, such as poly(butylene succinate) (PBS)[77, 82]. Amines are utilized for the polyamides and virgin PUR synthesis[83, 84]. EG is used as a substrate

for the production of glyoxylic acid, which have a wide range of applications in chemical industry[85].

This variety of monomers produced by PU degradation have led to several metabolic pathways to use them as a carbon and energy source. The precise identification of catalytic activities and structural mechanism of polymer hydrolysis could be useful to develop industrial processes at large scale[86].

Briefly, monooxygenation of the aromatic ring was followed by dioxygenases responsible for double hydroxylation of the substrate to form the central intermediate (catechol), then, extradiol dioxygenases cleaved the aromatic ring using the *meta*-pathway and, finally this compound was introduced to the central metabolism.

Despite the fact that catechol degradation tends to proceed via intradiol cleavage pathway in most *Pseudomonas* strains [87, 88], biodegradation of mono-aromatic substrates by meta-cleavage pathway seems to be more effective. For instance, enhanced catabolic activity in *P. fluorescens* PU1 grown on phenol was detected by higher activity of catechol 2,3-dioxygenase, a meta cleavage enzyme, compared to *ortho*-pathway[89]. *P. pseudoalcaligenes* strain C70 harbors extradiol dioxygenases that triggers two catechol *meta*-pathways and thus, higher degradation efficiency of phenol [90]. However, both pathways can also be inducted by high substrate concentrations in order to avoid the accumulation of toxic intermediates [90, 91].

Subsequently, RNA-sequencing (RNA-seq) was applied to bacterial cells grown on different carbon sources in order to analyze differential gene expression. This technique has been widely used due to its high sensitivity, excellent gene coverage and good reproducibility for highly complex data sets [92]. To identify and fully comprehend the metabolic pathways used to degrade environmental pollutants and plastic polymers represents a key factor to scale up microbial degradation and fulfil the requirements of the industry[86].

3.2 Optimization of a RNA extraction protocol for *Pseudomonas capeferrum* TDA1 growing on aromatic substrates

Nowadays, RNA-seq is considered the most powerful, robust and adaptable technique for measuring gene expression and transcription activation at genome-wide level [93, 94]. However, obtaining good sequencing coverage and depth depends on extract high quality RNA [95]. During microbial degradation of aromatic compounds, central intermediates such as phenols and catechols tend to interact with polyphenol oxidases, which are responsible for the browning effect in plants, fruits and cereals [96, 97].

In this thesis, brown precipitates were observed during the degradation of 2,4-TDA by *Pseudomonas capeferrum* TDA1, which represented a hurdle in RNA isolation due to the low quality and yield obtained by traditional and commercial methods. Tunali-Boz et al (2015)[98] analyzed the capability of hydrocarbon compounds as potential carbon sources by yeast isolates. Brown precipitates were observed in the media and were identified as a positive result of degradation. In a similar study, bacterial genera such as *Pseudomonas, Serratia* and *Acinetobacter* degraded crude oil samples and brown precipitates were also present in experimental plates[99].

Considering the complexity of the media, some "strategies" were implemented to improve the quality and yield. For instance, this procedure included a mixture of guanidine thiocyanate and phenol (RNAzol RT) in a monophasic solution, which is exclusively used for RNA extraction. Glycogen contributed to the formation of a visible pellet and the cleaning up step removed any phenol trace in RNA extraction (Figure 1, Chapter 2.3). Given the low yield of RNA obtained by single samples (2 – 3 ng/µL), pooling was implemented to increase RNA input for rRNA depletion. Sample pooling has been successfully validated in numerous RNA isolation protocols including SARS-CoV-2 [100, 101], microbial communities [102], phytoplankton [103] and bacteria and yeasts [104].

Moreover, in this thesis, the presence of the gene *yfiH* encoding for a polyphenol oxidoreductase laccase (EC 1.10.3.2) was detected in the genome of *Pseudomonas capeferrum* TDA1. This enzyme has previously reported as phenolic and non-phenolic compounds oxidizer and it has been isolated from several *Pseudomonas* species [105].

Lastly, the quality and yield of the total RNA extracted were measured by A_{260}/A_{280}, $A_{260}/_{230}$ ratios and RNA integrity. Both assays are independent and must be evaluated in order to assure reliable and reproducible results. Using the modified protocol, both absorbance ratios (2.02 ± 0.16, 1.95 ± 0.01, respectively) (Table 1, Paper 3) demonstrated the purity of the total RNA (32, 33). Moreover, RIN (RNA integrity number) values were analyzed and samples with a RIN higher than 7.0 were selected for downstream applications, confirming the high-quality total RNA [106] (Table 1, Chapter 2.3).

3.3 Transcriptional changes in *Pseudomonas capeferrum* TDA1 grown on 2,4-TDA and characterization of outer membrane vesicles (OMVs) with hydrolytic activity

Profound knowledge of transcriptional gene regulatory mechanisms is not only fundamental for biological purposes, but also can lead to economically or industrially relevant applications such as diagnostics, therapeutics and bioremediation [69]. The whole differential gene expression in *P. capeferrum* TDA1 treated with 2,4-TDA and succinate + 2,4-TDA were compared to succinate (control). The genes previously reported as candidates for 2,4-TDA degradation were confirmed as highly expressed compared to the control (Table 1, Chapter 2.4).

Simultaneously, aromatic compounds can serve as nutrients to be metabolized by bacteria but also as cellular stressors. When bacterial cells are exposed to these compounds, they exhibit a multifactorial stress-response: i) novel degradation pathways, ii) changes in lipid metabolism and efflux pumps for adaptation to sub-optimal growth conditions, iii) reorganization of the cell envelope, biofilm formation, and cell-to-cell interactions [107]. Transcriptomic analyses upon toluene exposition in *P. putida* S12 showed that differentially expressed genes increased immediately after toluene addition. From these genes, only 5% corresponds to genes involved directly in the catabolism of toluene while the main cellular response was focused on proteins assisting the cell toward the toxic effects of toluene [107, 108]. In this thesis, similar response was observed in bacterial cells grown on 2,4-TDA and 2,4-TDA + succinate.

In this thesis, several genes related not only to the metabolism of the aromatic compound, but also involved in biofilm formation, solvent extrusion and general

stress response (Table S1, Chapter 2.4). Bacterial cells grown on 2,4-TDA revealed 157 genes upregulated over a four-fold compared to the control. Regularly, degradation of aromatic substrates exhibits an increased expression of genes involved in stress response [109, 110], which tends to generate dynamic changes at transcriptional levels.

In bacterial cells containing 2,4-TDA and 2,4-TDA + succinate, 712 shared genes were upregulated at least two-fold (compared with succinate), demonstrating their particular effect of 2,4-TDA on catabolic systems and solvent tolerance. The presence of the aromatic substrate activated degrading genes to metabolize the aromatic compound. In this study, either succinate was used as a carbon source for growth support and other main functions whereas 2,4-TDA was degraded to reduce its toxicity or co-metabolism could take place in the presence of succinate and 2,4-TDA. Co-metabolism of glucose and aromatics has been evaluated in different *Pseudomonas* strains previously, revealing the presence of active genes involved in the degradation of phenylpropanoid compounds (e.g, ferulic acid) into vanillin, [111, 112] and showed its potential use for biotechnological applications.

In a similar study, *Pseudomonas putida* CSV86 was capable of degrading aromatics substrates in the presence of organic acids (succinate), which suggest its efficiency for bioremediation of aromatics even in the presence of a simple carbon source, thus evading carbon catabolite repression [113].

Polymer cleavage by extracellular enzymes called hydrolases (e.g., esterases, lipases, cutinases, oxidases, ureases, proteases, etc.) are secreted by bacterial colonies capable to break down long-chain plastics into low molecular monomers and oligomers. These compounds can be either assimilated inside the cells or metabolized into CO_2 [10]. This initial step is associated to molecular weight reduction and loss of mechanical properties [10, 75]. In the recent years, best performing enzymes have been found in several microorganisms including the genera *Thermobifida, Rhodococcus, Pseudomonas, Acinetobacter, Comamonas, Corynebacterium, Bacillus* and *Ideonella*. However, enzymatic hydrolysis has been reported exclusively in amorphous regions (disordered domains) from different plastic materials, whereas crystalline regions (ordered regions) are highly resistant to enzymatic degradation. The identification of novel enzymes able to degrade

high-crystallinity regions should be considered a priority in order to enhance depolymerization efficiency.

In Gram-negative bacteria, hydrolytic enzymes have been identified inside spherical membranous structures called outer membrane vesicles (OMVs). These vesicles play key roles in numerous functions such as cell-cell communication, biofilm formation and horizontal gene transfer [114, 115]. OMVs harboring active enzymes and extracellular structures were reported to exhibit catabolic activity in different bacteria (*Pseudomonas, Rhodoccocus, Amycolatopsis* and *Delftia*) grown on aromatic substrates such as phenanthrene and lignin-rich media [46, 47].

In order to identify hydrolytic activity in extracellular enzymes from *P. capeferrum* TDA1, supernatant, whole-cell pellets and outer membrane vesicles (OMVs) were assessed from bacterial cultures containing PU oligomer or succinate (control) as a carbon source. Esterase activity was detected in whole-cell pellets, but not in the supernatant. Despite the presence of the PU oligomer, a significant rise in the hydrolytic activity compared to the control was not observed (Figure 3, Chapter 2. 4). In the case of OMVs, esterase activity was highly enriched in isolated OMVs compared to the cell pellets (Fig 3, Chapter 2.4).

Relative OMV yields in TDA1 raised significantly in PU oligomer ($0.28 \pm 0.05\%$) compared to succinate ($0.09 \pm 0.01\%$). This three-fold increased activity could demonstrate that the release of OMV is part of the adaptive mechanisms of bacteria to stressful environmental conditions. In *P. putida*, hydrolases and oxidoreductases packaged into outer membrane vesicles were detected for samples cultivated on lignin-rich media. Furthermore, *in vivo* and *in vitro* experiments demonstrated that these enzymes are active for catabolizing aromatic compounds[46]. Similar results were described in other microorganisms such as *Delftia acidovorans Cs1-4* grown on phenanthrene, which induced OMV and extracellular structure formation[47] acting as promoting elements in biodegradation.

According to the results obtained in this chapter, the subset of the detected hydrolases in OMV fractions can be considered as supporting elements for biodegradation. (Figure 6, Chapter 2.4). This new approach offers potential insights in the extracellular degradation of PU monomers and oligomers. Additionally, *P.*

capeferrum TDA1 can be considered as an efficient PU monomer degrader and could be further employed in "bio-upcycling" processes.

3.4 Current and future perspectives of plastic bio-upcycling

Nowadays, the new vision of circular economy mandates to minimize waste production and CO_2 release. Post-consumer plastics depolymerization and subsequently use of monomers and oligomers represents a key target for the next generation of sustainable plastics. Recent studies show promising perspectives and new opportunities to degrade plastic waste and use it as an important feedstock for the production of value-added materials, becoming the most sustainable option to treat plastic so far[116]. Although several enzymes have contributed to PET, ester-based PU and PA oligomers depolymerization, the knowledge about the metabolic degradation pathways and molecular mechanisms is still sparse[86, 98]. Thus, biocatalytic degradation of synthetic polymers such as PVC, PP, PS and PE has not been completely demonstrated yet[86].

The identification of plastic-degrading enzymes with desirable properties and functionalities is an urgent task, but also it is important to enhance catalytic efficiency for industrial applications[76]. The fully understanding of polymer-degrading enzymes and metabolic pathways will provide new insights about which polymers can be degraded and how to design new plastics that could be efficiently bio-recycled.

Some recent reports show that protein engineering can be used in order to improve enzymatic efficiency, specificity and thermostability[77, 117], nevertheless, design optimization is largely empirical because of limited understanding of the enzyme structure– function relationships[76]. Emerging computational approaches, such as machine learning and artificial intelligence, represent a great promise in deciphering protein features and functions and may guide protein engineering in the future[76].

In order to improve bio-recycling and upcycling processes, physicochemical treatments have been included to tackle some limitations, including low degradation rates and inefficient depolymerization[116].

Mechanical pre-treatments have a significant impact on polymeric structures, properties, composition and reactivity. For instance, Tournier et al (2020)[118], successfully demonstrated that pre-treated PET waste (extrusion and micronization) can contributed to an efficiently depolymerization (~ 90%) to its monomeric building blocks by hydrolases at an industry-relevant scale. Highly amorphous PET powder was obtaining by mechanical milling after 20 hours of treatment[119]. UV and thermo-oxidative degradation were performed on commercial polymers such as PE, PP and polyamide-6, resulting in weight loss of polymer and reduction of molecular weight[120].

On the other hand, chemical treatments at mild temperatures (< 200 °C) can recover monomers and oligomers from different plastic polymers that could be used by microorganisms as potential carbon sources[25].*Pseudomonas putida* KT2440 and *Pseudomonas umsongensis* GO16 are able to metabolize EG from PET depolymerization and produce bioplastics (PHA)[77, 120].

The combination of mechanical, chemical and biochemical techniques could replace conventional recycling methods (Figure 1) using a two-step process where plastic waste can be depolymerized enzymatically and the resulting building blocks could be considered as an important feedstock for the synthesis of added-value materials, which accomplish plastic waste circularity.

Treatment	Incineration Energy Conversion	Mechanical Recycling	Chemical Recycling	Organic Composting	Mechano-green chemical	Bio-Catalytic	mechano-green-chemical-biocatalytic
Plastic-type							
HDPE		✓					✓
LDPE	✓	✓					✓
Polypropylene		✓					✓
PVC	✓		✓				✓
Polyurethane			✓				✓
Polystyrene			✓				✓
ABS		✓	✓				✓
PMMA			✓				✓
PET		✓	✓		✓	✓	✓
Polylactic acid		✓	✓	✓	✓	✓	✓
PHA			✓	✓	✓	✓	✓
PBS		✓	✓	✓	✓		✓
Starch				✓			✓
Mixed Plastic waste	✓					✓	✓

Figure 1.8 Mechanical, chemical and bio-catalytic methods can be implemented to treat plastic waste efficiently (Adapted from Nikolaivits *et al*, 2021).

Nowadays, microbial degradation of PUR is considered a highly attractive and eco-friendly alternative for solid waste treatment. Nevertheless, some challenges still need to be addressed to develop an efficient biodegradation and upcycling process. First, more studies should be focused on the screening of urethane-degrading enzymes, which can be mixed in an optimal cocktail with the known polyester hydrolases, improving degradation rates. Secondly, molecular and structural mechanisms of polymer hydrolysis should be fully understood to scale up the bioprocesses at industrial level. Lastly, it is important to establish highly efficient and cost-effective methods for the separation of degradation products from PU waste, especially for small molecules, including alcohols and acids.

Multilevel strategies to reduce plastic waste and stimulate sustainable alternatives should be part of a rethinking towards a circular economy. New plastics should be designed in order to be used, reused, repaired and recycled (mechanically, chemically and biologically) keeping high value without posing risk for human health and environment.

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5.1 Curriculum Vitae

5.2 Co-author contributions

Author contribution statement, María José Cárdenas Espinosa

Towards biorecycling of plastics: Isolation and characterization of *Pseudomonas capeferrum* TDA1, a bacterium capable to degrade polyurethane mono- and oligomers.

Author contribution statement:

Title: **Screening and cultivating microbial strains able to grow on building blocks of polyurethane**

Journal: Methods in Enzymology

Author: María Jose Cárdenas Espinosa, Andrea Colina Blanco, Hermann J. Heipieper, and Christian Eberlein

María José Cárdenas Espinosa:

- Study and experimental design
- Perform the experiments
- Data interpretation
- Writing of manuscript

Andrea Colina Blanco:

- Study and experimental design
- Perform the experiments

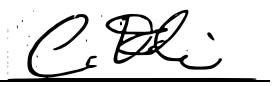
Hermann J. Heipieper:

- Writing of manuscript - review & editing
- Data interpretation

Christian Eberlein (senior author) :

- Study and experimental design
- Data interpretation
- Writing of manuscript - review & editing

María José Cárdenas Espinosa



Christian Eberlein

Author contribution statement, María José Cárdenas Espinosa

Towards biorecycling of plastics: Isolation and characterization of *Pseudomonas capeferrum* TDA1, a bacterium capable to degrade polyurethane mono- and oligomers.

Author contribution statement:

Title: **Toward Biorecycling: Isolation of a Soil Bacterium That Grows on a Polyurethane Oligomer and Monomer**

Journal: Frontiers in Microbiology

Author: María Jose Cárdenas Espinosa, Andrea Colina Blanco, Tabea Schmidgall, Anna Katharina Atanasoff-Kardjalieff, Uwe Kappelmeyer, Dirk Tischler, Dietmar H. Pieper, Hermann J. Heipieper and Christian Eberlein

María José Cárdenas Espinosa:

- Experimental design
- Perform the experiments
- Writing of manuscript
- Data analysis and interpretation

Andrea Colina Blanco:

- Perform the experiments
- Data Analysis and interpretation

Tabea Schmidgall:

- Perform the experiments
- Data Analysis and interpretation

Anna Katharina Atanasoff-Kardjalieff:

- Perform the experiments

Uwe Kappelmeyer:

- Experimental design

Dirk Tischler:

- Genome sequencing and annotation

Dietmar H. Pieper:

- Data Analysis and interpretation
- Writing of manuscript
-

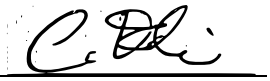
Hermann J. Heipieper:

- Experimental design
- Perform the experiments
- Contribution of reagents, materials and analysis tools
- Writing of manuscript - review & editing

Christian Eberlein (senior author):

- Experimental design
- Perform the experiments
- Contribution of reagents, materials and analysis tools
- Writing of manuscript

María José Cárdenas Espinosa



Christian Eberlein

Author contribution statement, María José Cárdenas Espinosa

Towards biorecycling of plastics: Isolation and characterization of *Pseudomonas capeferrum* TDA1, a bacterium capable to degrade polyurethane mono- and oligomers.

Author contribution statement:

Title: **An optimized method for RNA extraction from the polyurethane oligomer degrading strain *Pseudomonas capeferrum* TDA1 growing on aromatic substrates such as phenol and 2,4- diaminotoluene**

Journal: Plos One

Author: María José Cárdenas Espinosa, Tabea Schmidgall, Georg Wagner, Uwe Kappelmeyer, Stephan Schreiber, Hermann J. Heipieper, Christian Eberlein

María José Cárdenas Espinosa:

- Study and experimental design
- Data curation
- Data validation
- Writing of manuscript

Tabea Schmidgall:

- Data curation

Georg Wagner:

- Data curation

Uwe Kappelmeyer:

- Experimental design
- Data curation
- Supervision
- Data validation
- Writing of manuscript - review & editing

Stephan Schreiber:

- Data curation
- Conceptualization

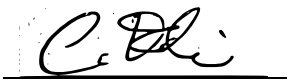
Hermann J. Heipieper:

- Conceptualization
- Funding acquisition
- Investigation
- Supervision
- Data validation
- Writing – review & editing

Christian Eberlein (senior author):

- Conceptualization
- Methodology
- Supervision
- Writing – review & editing

María José Cárdenas Espinosa



Christian Eberlein

Author contribution statement, María José Cárdenas Espinosa

Towards biorecycling of plastics: Isolation and characterization of *Pseudomonas capeferrum* TDA1, a bacterium capable to degrade polyurethane mono- and oligomers.

Author contribution statement:

Title: **Extracellular degradation of a polyurethane oligomer involving outer membrane vesicles and further insights on the degradation of 2,4-diaminotoluene in *Pseudomonas capeferrum* TDA1**

Journal: Scientific reports

Author: Oscar Puiggené, María José Cárdenas Espinosa, Dietmar Schlosser, Stephan Thies, Nico Jehmlich, Uwe Kappelmeyer, Stephan Schreiber, Daniel Wibberg, Joern Kalinowski, Hauke Harms, Hermann J. Heipieper & Christian Eberlein.

Oscar Puiggené:

- Study and experimental design
- Data collection
- Data analysis
- Writing of manuscript

María José Cárdenas Espinosa:

- Study and experimental design
- Data collection
- Data analysis
- Writing of manuscript – review & editing

Dietmar Schlosser:

- Data contribution

Stephan Thies:

- Data contribution
- Writing of manuscript - review & editing

Nico Jehmlich:

- Data contribution
- Data curation
- Data analysis
- Data validation
- Writing of manuscript - review & editing

Stephan Schreiber:

- Data contribution
- Data analysis

Uwe Kappelmeyer:

- Data analysis

Daniel Wibberg:

- Data contribution
- Writing – review & editing

Joern Kalinowski:

- Data contribution

Hauke Harms:

- Writing – review & editing

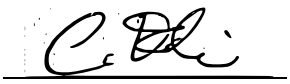
Hermann J. Heipieper:

- Study and experimental design
- Writing – review & editing

Christian Eberlein (senior author):

- Study and experimental design
- Writing – review & editing

María José Cárdenas Espinosa



Christian Eberlein

5.3 List of publications and conference contributions

Publications:

- **Cárdenas Espinosa MJ**, Colina Blanco A, Heipieper HJ, Eberlein C. Screening and cultivating microbial strains able to grow on building blocks of polyurethane. *Methods Enzymol*. 2021;648:423-434. https://doi.org/10.1016/bs.mie.2020.12.008

- **Cárdenas Espinosa MJ**, Blanco Colina A, Schmidgall T, Atanasoff-Kardjalieff Anna AK, Kappelmeyer U, Tischler D, Pieper Dietmar H., Heipieper HJ., Eberlein C. Toward Biorecycling: Isolation of a Soil Bacterium That Grows on a Polyurethane Oligomer and Monomer. *Frontiers in Microbiology*.2020;11:1664-302X. https://doi.org/10.3389/fmicb.2020.00404**.**

- **Cárdenas Espinosa MJ**, Schmidgall T, Wagner G, Kappelmeyer U, Schreiber S, Heipieper HJ, et al. (2021) An optimized method for RNA extraction from the polyurethane oligomer degrading strain Pseudomonas capeferrum TDA1 growing on aromatic substrates such as phenol and 2,4-diaminotoluene. *PLoS ONE* 16(11): e0260002. https://doi.org/10.1371/journal.pone.0260002

- Puiggené, Ò., **Espinosa, M.J.C**., Schlosser, D. *et al*. Extracellular degradation of a polyurethane oligomer involving outer membrane vesicles and further insights on the degradation of 2,4-diaminotoluene in Pseudomonas capeferrum TDA1. *Sci Rep* 12, 2666 (2022). https://doi.org/10.1038/s41598-022-06558-0

Conference contributions:

- **05.2021**. "Plastic-eating bacteria: Isolation and Identification of a soil strain that grows on a polyurethane oligomer and monomer". *European Federation of Biotechnology*, 2021. Poster. On-line.

- **09.2021**. "Towards plastic-biodegradation: isolation and characterization of a soil bacterium growing on polyurethane oligo- and monomers". 11th *International Conference on Environmental Engineering and Management*, 2021. Muttenz, Switzerland. Oral presentation.

- **10.2021.** "Plastic-eating bacteria: Isolation and Identification of a soil strain that grows on a polyurethane oligomer and monomer". *HIGRADE Conference* 2021, UFZ – PhD Conference, Leipzig, Germany. Poster. On-line.

- **11.2021**. "Plastic - Degradation: A Model for Extracellular Degradation of Polyurethane by *P. capeferrum* TDA1". *EMBO-workshop: Bacterial membrane vesicles: Biogenesis, functions and medical applications*, 2021. Poster. On-line.

5.4 Declaration of authorship

I, María José Cárdenas Espinosa, hereby declare that:

• I have written this thesis autonomously with my own ideas and judgements. No other sources than the stated quotations from other works were used. Full reference of the source has been provided in the proper way.

• All persons involved as co-authors, who supported me for the selection of the materials for this thesis are stated including their respective contribution. No other persons than stated above were involved in the preparation of the thesis, in particular, no PhD consultants were used, and no third party has received direct or indirect financial benefits in goods and services for a contribution to this thesis.

• This thesis in an equal or similar form has not been submitted to an academic institution to obtain a doctoral degree or any other academic degree, and has not been published yet.

• No further doctoral examination process has taken or is taking place.

María José Cárdenas Espinosa

Leipzig, 22. August .2022

5.5 Acknowledgements

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