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SYNTHETIC MICROBIAL COMMUNITIES FOR PLASTIC UPCYCLING

Isabel B. A. Valencia
Michigan Technological University, ibvalenc@mtu.edu

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By

Isabel B. A. Valencia

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Department of Biological Sciences

Thesis Advisor:	*Dr. Steve Techtmann*
Committee Member:	*Dr. Rebecca Ong*
Committee Member:	*Dr. Rupali Datta*
Department Chair:	*Dr. Casey Huckins*

Table of Contents

List of Figures

List of Tables

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Abstract

Plastic waste and human hunger pose major threats to the health and wellbeing of populations world-wide. Using microbial methods to upcycle polyethylene terephthalate (PET) plastic into value-added compounds such as single cell protein (SCP) for human consumption is a unique solution to both these issues. Two monomers of chemically deconstructed PET (DCPET), terephthalate (TPA) and ethylene glycol (EG), have been previously shown to be biodegradable by enriched microbial communities. Using this top-down knowledge to inform the reconstruction of a minimal microbial community constructed from isolated members of these communities is a novel way to efficiently process these monomers via a synthetic microbial community. This study combines *Rhodococcus* sp. TE21C, shown to be a generalist capable of degrading TPA but not ethylene glycol, and *Paracoccus* sp. RL32C, previously identified as an ethylene glycol specialist that can also degrade TPA. *Rhodococcus* sp. TE21C has been shown to have a sensitivity to ethylene glycol. Combining these two isolates in a co-culture grown under high ethylene glycol conditions, *Paracoccus* sp. RL32C dominates the community with a minor contribution from *Rhodococcus* sp. TE21C. *Paracoccus* sp. RL32C appeared to benefit from the co-culture and grow to a higher density under these conditions. When grown under conditions that replicate the composition of DCPET, *Paracoccus* sp. RL32C supports the growth of *Rhodococcus* sp. TE21C in media containing only EG. In conditions where TPA is present, however, *Rhodococcus* sp. TE21C outcompetes *Paracoccus* sp. RL32C. These minimal synthetic communities can identify microbial interactions and help inform the reconstruction of efficient synthetic communities to effectively degrade PET plastic.

1 Synthetic Microbial Communities for Plastic Upcycling

Global food insecurity and plastic pollution

Human hunger and plastic pollution are two major issues affecting the world today. In 2021, the World Health Organization estimated that approximately 2.3 billion people were considered moderately or severely food insecure. Moderate food insecurity is characterized by not having consistent access to food, while severe food insecurity is defined as an individual going without food for a day or longer (World Health Organization, 2021). The USDA estimates that in low- and middle- income countries, a third of the population may not have consistent access to food resources (USDA). Combating food insecurity is one of the United Nations Sustainable Development Goals (Goal #2). This goal aims to "end hunger, achieve food security and improved nutrition, and promise sustainable agriculture" (United Nations, 2022).

Another current global issue is the accumulation of plastic waste in the environment. It is estimated that by the year 2050, cumulative plastic waste will reach over 250 billion tons. This affects over 800 animal species, and continues to increase (Urbanek et al., 2021). Only approximately 9% of plastic waste in 2015 was recycled, with 79% being landfilled and thus accumulating in the environment (Geyer et al., 2017). A solution to alleviate both food insecurity and plastic pollution is to utilize plastic-degrading bacteria to metabolize plastic waste and produce single cell protein, which can then be consumed by humans. Yeasts and spirulina are both examples of SCPs that are already utilized by many people (Bratosin et al., 2021). SCP production is currently used for animal feed applications as well as food additives (Bratosin et al., 2021), and the existing systems provide a starting point for the industrial production of SCP using novel substrates such as PET plastic waste. Bacteria have a protein content between 30% and 70% dry mass, making them an accessible and concentrated source of nutrition (Garimella et al., 2017). While SCP is appealing for the ability to produce nutritional products from waste streams, there are some unique challenges to using microorganisms as a complete food source. While quickly multiplying bacteria is attractive from a production standpoint, it also has the potential to create an excess of ribonucleic acid (RNA) that can detract from the nutrition of the end product (Bratosin et al., 2021). Ingestion of high quantities of RNA may increase uric acid concentration in blood plasma, resulting in kidney stones and gout. Therefore, the high nucleic acid content of microbial cells compared to conventional protein sources presents a challenge for the use of microbial cells for SCP. Several methods have been adapted to reduce the RNA content, including enzyme and chemical treatments (Ritala et al., 2017). Despite some of the challenges, single cell protein is an appealing alternative food source due to the ability to produce protein from waste streams, such as inedible plant material or plastic waste.

Microbial degradation of PET

Polyethylene terephthalate (PET) is the most commonly used plastic in the world, with the majority of manufactured PET being produced as single-use packaging material (Soong et al., 2022). PET plastic is composed of two monomers, terephthalate (TPA) and ethylene glycol, and bacteria capable of degrading PET have been identified (Taniguchi et al., 2019). By coupling microbial plastic waste biodegradation and the production of single cell protein as a food source, this work aims to alleviate both food insecurity and plastic waste accumulation at once. PET degrading bacteria offer an exciting prospect for turning harmful plastic waste into a useful product for human consumption.

Using microbial degradation as an alternative to landfilling waste plastics supports the aims of the 2023 Bold Goals for U.S. Biotechnology and Biomanufacturing, specifically Goal 2.2, focused on improving the end-of-life of plastics through biological and chemical methods (The United States Government, 2023). The degradation pathway of PET plastic has been characterized with several microorganisms shown to be capable of degradation. The degradation of PET plastic results in an intermediate, mono(2-hydroxyethyl) terephthalic acid (MHET) and bis(2-hydroxyethyl) terephthalate (BHET). MHET can also be degraded to terephthalate (TPA) and ethylene glycol.

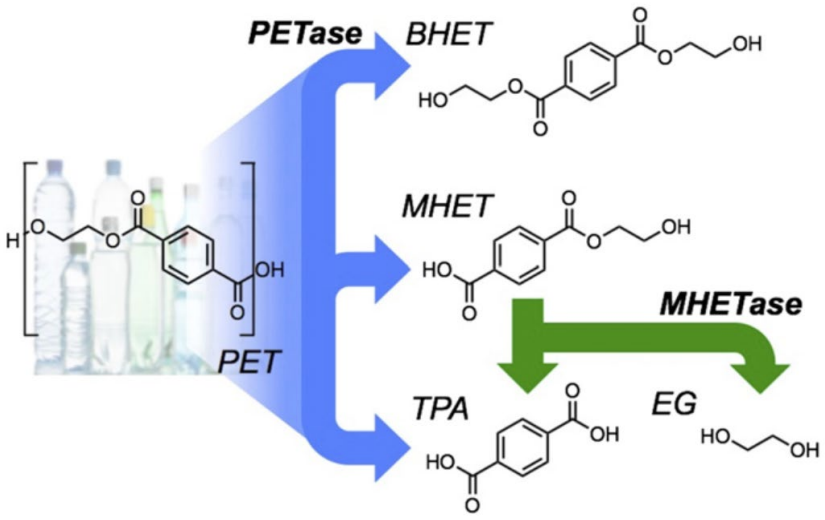

Figure 1. Biological degradation pathway for the degradation of PET plastic into BHET, MHET, TPA and ethylene glycol, aided by enzymes PETase and MHETase (Austin et al., 2018).

Several microorganisms have been shown to degrade PET, one of which is *Ideonella sakaiensis* (Yoshida et al., 2016). The degradation of PET to MHET in *I. sakaiensis* is facilitated by an enzyme known as PETase, and MHET is degraded to TPA and ethylene glycol by the MHETase enzyme. PET is generally recognized as resistant to degradation, however success has been achieved in the biodegradation of PET films (Palm et al., 2019). In addition to PET plastic, PETase is able to degrade varying semi-aromatic

polyesters, making it a candidate for use in the degradation of other types of plastics (Jerves et al., 2021).

Due to the discovery of PETase from *I. sakaiensis*, other potential PET hydrolases have been examined. *Thermobifida fusca* was shown to produce poly(butylene terephthalate-co-adipate) (BTA-1) hydrolase, and was successful in degrading PET film. Many of these enzymes are cutinase enzymes, capable of degrading the cell walls of plants. In addition, a gene similar to that which creates PETase was identified from the genome of a *Streptomyces* species (Soong et al., 2022). The majority of the bacteria with the potential for PET degradation are of the phylum *Actinobacteria*, which includes the *Thermobifida* species mentioned above. These bacteria possess cutinase, lipase, and carboxylesterase enzymes thought to be useful for breaking down PET (Danso et al., 2019). However, these identified PETases and other enzymes act relatively slowly, so they are not currently considered an efficient method of biodegradation. A worldwide genome and metagenome search was conducted in 2018 to identify enzymes similar in structure to PETase; over 800 total enzymes and genes were found distributed across both terrestrial and marine habitats. This indicates that there is a large potential for bacteria other than those mentioned above to possess the ability for PET biodegradation (Danso et al., 2018).

Although there is potential for degradation in this pathway, it is believed that the microbial breakdown of solid plastic occurs slowly (Danso et al., 2019). Coupling chemical and biological methods for plastic waste processing is an advantageous strategy for efficient upcycling. The treatment of PET plastic with ammonium hydroxide produces terephthalate, terephthalic monoamide, and ethylene glycol (Schaerer et al., 2023c). The monomers produced during chemical deconstruction are more accessible for bacterial metabolism and make the process of degradation considerably quicker (Schaerer et al., 2023b). Similarly, heat treatment is a potential method for deconstructing PET as well, with added catalysts such as zinc acetate and aluminum isopropoxide speeding up the process (Lee et al., 2021). Combining chemical and biological methods for the deconstruction and degradation of PET plastic is an efficient method for the treatment and upcycling of this otherwise discarded product.

PET degradation products

Ethylene glycol is a building block and breakdown product of PET. It is a chemical that is used in many applications, such as antifreeze and brake fluid. It is considered to be "readily biodegradable"; however, this was determined through studies using complex microbial communities such as those in soil and wastewater treatment environments. Individual species may be more susceptible to the toxic effects of ethylene glycol, including some green algae species in aquatic environments (Staples et al., 2001). Potential sensitivities to ethylene glycol must be considered when assembling a synthetic microbial community to degrade PET, as it has the potential to accumulate in media as a harmful waste product of degradation. A study of ethylene glycol degradation in bioreactors indicated that it may be degraded completely after 21 days under anaerobic conditions with added phosphate (Mrklas et al., 2003). Hence, a community of bacteria that can degrade PET and ethylene glycol may be an effective combination. Despite its potential toxicity, there are some organisms capable of metabolizing ethylene glycol such

as *Pseudomonas putida* (Mückschel et al., 2012), *Acetobacterium woodii* (Trifunović et al., 2016) and *Yarrowia lypolytica* (Carniel et al., 2023). A *Paracoccus* strain from a consortium enriched to metabolize deconstructed PET has been identified as an ethylene glycol specialist, verified through metatranscriptomic analysis (Schaerer et al., 2023a). Isolates with this ability would be valuable additions to synthetic communities used to degrade deconstructed PET plastic as they could potentially alleviate any toxicity from ethylene glycol.

Terephthalic acid is another monomer of PET. It is considered an environmental pollutant, primarily resulting from wastewater discharge. However, some microbes are able to degrade it. With an optimal nitrogen source, a *Pseudomonas* species isolated from wastewater was able to degrade it with a half-life of approximately 12 hours (Zhi Jiang et al., 2011). Similarly, a test was conducted in 1999 to determine the effects of various microorganisms and cultures on the degradation of terephthalic acid; while the *Pseudomonas* isolates tested were not able to degrade it, communities from both compost and garden soil yielded positive results (Lefèvre et al., 1999). This offers a positive outlook on the potential of a microbial community to degrade both PET and its breakdown products.

Synthetic microbial communities

The examples covered above represent the abilities of individual bacteria to degrade PET as isolates. However, there is also discourse around selecting or building a microbial community to more efficiently achieve a given task. Selecting and combining microbes to achieve a desired result is often a complicated process, as the factors influencing microbial community composition and activities are still not entirely understood. A phenomenon known as community succession has been observed in microbial communities; this refers to the changing of community composition over time as the substrate is altered due to the byproducts and activities of the previous phase of microbial growth (Wright et al., 2019). A distinguishing feature of microbial communities is this ability for rapid changes in the community composition. While this community structure can be monitored at any given time with methods such as DNA sequencing, this approach may not offer complete insight into the relationships between microbes in a community. DNA extraction methods are not consistently effective for all organisms, and the sequencing techniques can only offer a glimpse into who is there, not how they are interacting (Nemergut et al., 2013).

The complexity of natural microbial community interactions provides a roadblock for adapting natural communities for PET degradation. However, the potential to combine known species to create synthetic communities is a potential solution to this issue. When bacterial species are combined, they engage in at least one ecological interaction. This may include a commensal reaction such as a food chain, where one species feeds off the metabolic byproducts of another. It may also include competition, where both species are competing for the same resource, or a cooperative interaction. In a cooperative interaction, a given species may be negatively impacted by its own metabolic byproduct, which can be consumed and therefore reduced by another species (Großkopf and Soyer,

2014). Cooperative interactions may be the key to successful PET degradation; the breakdown products of PET such as ethylene glycol may be harmful to one species while acting as a carbon source for another. This deterministic relationship requires an understanding of available resources for a community to predict structure and function (Schafer et al., 2023). Division of labor in synthetic communities is a fundamental concept for the effective combination of species for a desired output (Teng et al., 2023). By designing communities with respect to each species' naturally selected functions, complex substrates can be effectively processed by consortia that would not be usable by a single isolate (Rafieenia et al., 2022).

While pairwise or three-way combinations of microbial species are convenient on an experimental level, the lack of biodiversity may result in issues down the road. Studies have shown that a decrease in biodiversity may make the community more susceptible to stress factors and that this may result in a decrease in functionality (De Roy et al., 2013). This creates a case for combining a large number of species with known functions and metabolisms to most effectively degrade PET, instead of simply combining 2 or 3 species. The ability to compose microbial communities using species that may not naturally exist in the same environment is another attractive prospect. (Jagmann & Philipp, 2014).

There are two main approaches for generating microbial communities for industrial purposes: top-down and bottom-up. Top-down approaches involve using evolutionary pressure to fine-tune an existing community to perform a certain task, while bottom-up approaches involve building communities from isolates to achieve a prescribed function (San León and Nogales, 2022). Guided by existing microbial communities with already established interactions, top-down approaches use pressures such as growth on selective media or antibiotic treatment. Schaerer et al. investigated a top-down approach for enriching microbial communities on varying plastic-derived substrates, including deconstructed PET (DCPET), TPA, and ethylene glycol. Two environmental communities were grown on these substrates and examined using both metagenomic and metatranscriptomic reads. *Rhodococcus* was shown to be the most active organism in both enriched communities when grown on DCPET and TPA and in one of the two communities, *Paracoccus* was the most active when grown on ethylene glycol. With *Rhodococcus* being present in all communities, it is suggested that *Rhodococcus* may be a generalist capable of degrading TPA. However, *Rhodococcus* showed decreased expression in ethylene glycol treatments, indicating a potential sensitivity. In contrast, ethylene glycol treatment resulted in a high abundance of *Paracoccus*, suggesting that it may be an ethylene glycol specialist (Schaerer et al., 2023a). While these communities are often resilient, the interactions are often complex and hard to predict due to the number of members (Gilmore et al., 2019). However, this complexity also provides stability and resilience to these communities, with the potential for redundant functions between species reinforcing the community function overall (Peng et al., 2016). In contrast, bottom-up approaches are generally composed of 2 or more populations that are carefully combined in a controlled environment, resulting in lower complexity but more control over the end product (De Roy et al., 2013). The construction of these

communities depends on the concept that cooperation can be prevalent in microbial interactions (Rodríguez Amor and Dal Bello, 2019). Manipulating existing cooperative interactions creates a case for using top-down knowledge to inform the construction of bottom-up communities.

Study objectives

The objectives of this study are to determine the validity of a two-part synthetic community for the degradation of PET plastic, using top-down enrichment work to inform bottom-up reconstruction of a minimal community. This study combines *Rhodococcus sp. TE21C*, an identified TPA degrader that is sensitive to ethylene glycol, with *Paracoccus sp. RL32C*, an ethylene glycol specialist that is also able to degrade TPA. Both isolates are derived from environmental communities enriched on deconstructed PET (DCPET) plastic (Schaerer et al., 2023b), and were identified as key members. By combining these two strains, we hypothesize that *Paracoccus* sp. RL32C will ease the inhibitory effects of ethylene glycol on *Rhodococcus* sp. TE21C and encourage cooperative interactions, allowing for improved growth of the co-culture under varying ethylene glycol conditions.

1.1 Methods and Materials

Isolations

To isolate bacteria from enriched communities, individual diluted communities (EB2, LS1, LS2, LS3) at 5% and 10% dilution were spread on Bushnell Haas with 10 g/L TPA and R2A agar plates and incubated at 30°C. Isolated colonies were selected and re-streaked on Bushnell Haas with TPA, and R2A agar. Colonies were selected from each plate in quadruplicate and grown in corresponding liquid media (Bushnell Haas with TPA and LB broth, respectively) in glass tubes with shaking at 30°C. Growth curves were collected by measuring optical density daily with a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific) at 600nm (OD_{600}). Samples were collected to make freezer stocks and archived at -80°C. *Rhodococcus* sp. TE21C underwent two additional rounds of restreaking before being grown in liquid media to achieve isolation.

16S sequencing

To determine isolate identity and status, Sanger sequencing was performed. Each sample was prepared for sequencing by DNA extraction, performed with a Zymo Quick DNA Mini-Prep kit (Zymo Research). Extracted DNA was prepared for quantification with a Qubit dsDNA HS assay kit (Invitrogen) and quantified with a Qubit 3.0 Fluorometer (Invitrogen). The 16S rRNA gene was sequenced using 27F and 1492R primers to identify taxonomy. Samples were sent to Arizona State University's Genomics Facility for 16S rRNA sequencing using their Sanger sequencing instrument.

16S rRNA reads analysis

The sequencing information returned for each sample contained a sequencing text file, a sequencing chromatogram, and a summary of sequencing results. The samples listed as

"Results available" on the sequencing summary were further examined using the ApE software (Davis and Jorgensen, 2022) to view sequencing chromatograms. Samples that exhibited single peaks at a majority of positions were further examined for isolate identity with NCBI BLAST using blastn against the non-redundant database.

Utilization of DCPET

Isolate utilization of deconstructed PET (DCPET) was determined through growth curve generation. DCPET was prepared using the protocol described in Schaerer et al. (Schaerer et al., 2023b). A solution of 10% DCPET by volume in sterile water was used as the growth media and sole carbon source for this experiment. Each isolate was combined with the stock solution in a 96-well plate, which was incubated at 30°C with shaking. OD_{600} measurements were collected daily with a Synergy LX multi-mode reader (BioTEK). Isolates with a final growth measurement greater than 0.5 were considered to use DCPET as a carbon source.

Genome sequencing

The previously extracted DNA was sent for full genome sequencing at SeqCenter. Isolates were chosen for sequencing based on taxonomy suggested by 16S rRNA gene sequencing results, as well as the utilization of DCPET as a carbon source. Using Unix, the quality of the returned genome sequencing data was checked with fastQC (Chen et al., 2018), and raw reads were trimmed by Trimmomatic (Bolger et al., 2014). The trimmed genomes were assembled using SPAdes (Prjibelski et al., 2020) and annotated with Prokka (Seeman, 2014). CheckM (Parks et al., 2014) was used to generate assembled genome statistics and check completeness and contamination. The 16S rRNA gene sequences were extracted from the annotated genomes and used to identify the isolate identity through NCBI BLAST using blastn (U.S. National Library of Medicine). The annotated genomes were examined for the following TPA and ethylene degradation genes: terephthalate 1,2 dioxygenase reductase component, terephthalate 1,2 dioxygenase oxygenase component (α and β subunits), lactaldehyde reductase and NADH-dependent aldehyde reductase.

Phylogenomic tree generation

Phylogenomic trees were constructed using the program GToTree (Lee 2019). Complete genomes of type strains for members of the genera Rhodococcus and Paracoccus were used from RefSeq. GToTree was used to identify single copy genes from the reference sequences as well as from the MAGs from terephthalate enriched communities LS1 and EB2 (Schaerer et al., 2023a) (Schaerer et al., 2023b). These single copy genes were aligned using muscle (Edgar 2004). The alignments were trimmed and concatenated. Finally, a tree was constructed from the concatenated alignment FastTree (Price et al., 2010). Trees were visualized using TreeDyn (Chevenet et al., 2006).

Ethylene glycol inhibitory concentrations

After genome sequencing results confirmed that both *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C were isolates, they were tested to determine the inhibitory concentration of ethylene glycol. An initial test was performed on *Rhodococcus* sp.

TE21C in Bushnell Haas with 10 g/L TPA (BHTPA) with 0% (v/v), 1% (v/v), 2% (v/v), 5% (v/v), and 10% (v/v) ethylene glycol, tested in triplicate. After inoculation, the *Rhodococcus* sp. TE21C samples were grown at 30°C with shaking in glass tubes. OD_{600} was measured daily for 6 days. This procedure was repeated for *Paracoccus* sp. RL32C in LB broth with the same concentrations of ethylene glycol. As the inhibitory concentration for *Rhodococcus* sp. TE21C appeared to fall between 2% and 5%, the above experiment was repeated with 2.5% (v/v), 3% (v/v), 3.5% (v/v), 4% (v/v), and 4.5% (v/v) ethylene glycol.

Mixing experiment 1: inhibitory ethylene glycol conditions
Rhodococcus sp. TE21C and *Paracoccus* sp. RL32C were grown from freezer stocks in Bushnell Haas with 10 g/L TPA and LB broth, respectively. At experiment t=0, each culture was rinsed twice in BHTPA and resuspended in this media before equalizing the OD values, measured with a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific). The experimental setup was completed in triplicate as shown below.

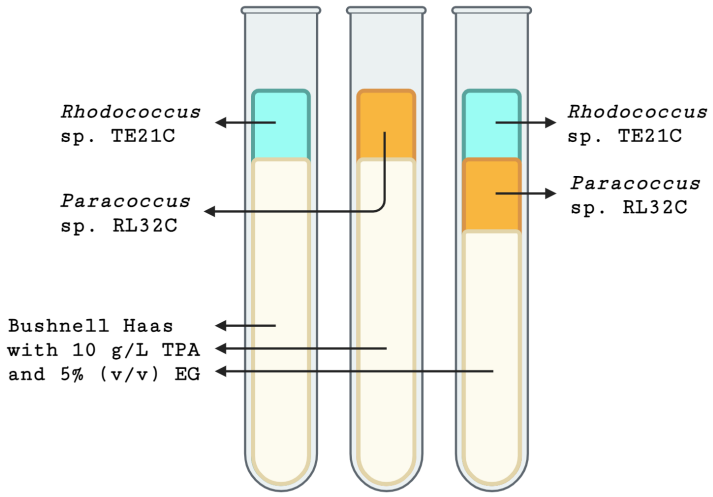

Figure 2. Mixing experiment 1 setup. *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C were each added to individual tubes containing Bushnell Haas with 10 g/L terephthalate (TPA) and 5% (v/v) ethylene glycol (EG). The co-culture tubes contained equal amounts of both *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C.

The cultures were grown in 15mL glass vials at 30°C with shaking at 200rpm for 6 days, with OD_{600} measured daily and a 1 mL sample collected from each tube for post-experiment HPLC analysis. After 6 days, DNA was extracted from each sample using an adapted 96-well plate rapid DNA extraction method. In each well, 100 µL of bacterial culture was added to 50 µL 20% IGEPAL. Five freeze-thaw cycles were performed with 15 minutes at room temperature and 15 minutes at -80°C for each cycle. After this, 25 µL of diluted Proteinase K (made by adding 87.5 µL of 20 µg/µL Proteinase K to 2412 µL ultrapure water). The plate was then put in a thermocycler for 1 cycle at 60°C for 1 hour and 95°C for 15 minutes before being centrifuged for 5 minutes. The supernatant containing extracted DNA with no cellular debris was pipetted into Eppendorf tubes and

used for subsequent analyses. Extracted DNA was prepared for quantification with a Qubit dsDNA HS assay kit (Invitrogen) and quantified with a Qubit 3.0 Fluorometer (Invitrogen). The extracted DNA was then sent to SeqCenter for 16S rRNA sequencing to determine community composition. Samples collected for HPLC analysis were filtered through a 0.2 µm cellulose acetate spin filter into an Eppendorf microcentrifuge tube using a Sorvall Legend Micro 21R Centrifuge (Thermo Scientific) at 15,000xg for 3 minutes. For samples with high biomass, the supernatant was pipetted into a second spin filter and the process was repeated. Samples were transferred to HPLC vials, and a 10x dilution was created for each sample with sterile water before analysis.

Mixing experiment 2: non-inhibitory ethylene glycol conditions
Rhodococcus sp. TE21C and *Paracoccus* sp. RL32C were grown from freezer stocks. Three types of Bushnell Haas media were prepared: TPA (10 g/L TPA), EG (3 g/L ethylene glycol), and EG+TPA (10 g/L TPA and 3g/L ethylene glycol) to replicate the component ratios of DCPET (Schaerer et al., 2023). At t=0, each isolate was rinsed twice in Bushnell Haas media and OD measurements were equalized. Co-culture duet plates (Cerillo) were used, with each well separated by a 0.2 µm polycarbonate plastic filter to allow the exchange of media while keeping isolates physically separated. Each combination of isolate and media was examined in triplicate in the layout shown below. *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C were tested as isolates, as well as a 1:1 mixture of both. Continuous growth curves were collected for the duration of the experiment using a Stratus kinetic multiplate reader (Cerillo), incubated at 30°C with shaking.

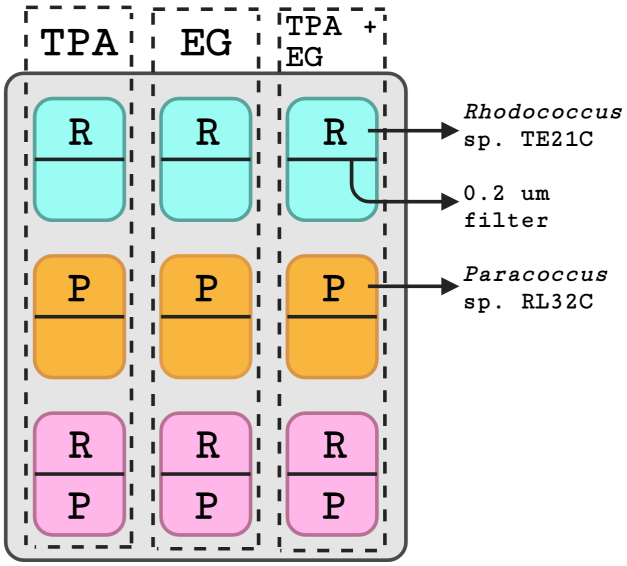

Figure 3. Mixing experiment 2 setup. Each culture (*Rhodococcus* sp. TE21C alone, *Paracoccus* sp. RL32, and a co-culture of the two) was prepared in three media types (terephthalate (TPA) alone, ethylene glycol (EG) alone, and TPA+ethylene glycol together). Each condition was completed in triplicate in wells with halves separated by a 0.2 µm polycarbonate plastic filter.

At the end of the experiment, liquid media was collected from each well and filtered through a 0.2 μm cellulose acetate spin filter with a centrifuge at 15,000xg for 5 minutes to prepare samples for HPLC analysis.

HPLC analysis
An Agilent 1200 HPLC system equipped with a G1311A quaternary pump, G1322A degasser, G1329A autosampler, G1315B DAD detector, G1362A RID detector, and G1316A temperature column controller was used for TPA and ethylene glycol quantification. A standard solution of terephthalic acid at a concentration of 1 mg/mL in N, N-dimethylformamide was created and analyzed at 300nm using a diode-array detector (DAD). This solution was further diluted to four concentrations to create a calibration curve. A standard solution of ethylene glycol at a concentration of 10g/L was prepared in ultrapure water, diluted to four concentrations, and analyzed with a refractive index detector (RID) to create a standard curve. A Waters μBondapak C18 column (3.9 mm × 300 mm, 10 μm) with a column temperature of 45°C was used for the required separations. The mobile phase was a 0.2% formic acid-water solution (A) and 0.1% formic acid-acetonitrile solution (B) with a flow rate of 0.4 mL/min. The DAD separations were analyzed at an injection volume of 10 μL for 25 min and the RID separation was analyzed at an injection volume of 20 μL for 20 min.

Statistical analysis
RStudio (RStudio Team, 2020) was used for statistical analysis. The zoo package in R (Zeileis and Grothendieck, 2005) was used to perform an ANOVA on the growth data from each experiment to compare the final ODs of each sample and determine significance, as well as to compare the initial ODs to final growth. An ANOVA was used for both TPA and ethylene glycol HPLC results, to compare each final concentration to the initial to determine if there was significant degradation. All ANOVA outputs were used with a TukeyHSD post hoc test for pairwise comparisons. Finally, the community composition data from co-culture experiment 1 was analyzed with a student's T-test. The composition data was normalized to reflect the final OD measurements, and then the isolate abundance was compared to the mixture to determine whether the co-culture significantly affected the growth of each isolate.

1.2 Results

Isolations and genome sequencing
Growth curves were generated for isolates grown initially in BHTPA and LB broth as well as with DCPET as the sole carbon source. Samples showing substantial growth to an OD greater than 0.5 on DCPET were considered for further work. To identify the sample taxonomy of the potential isolates, 16S rRNA sequencing was used. Of the 54 samples sent for 16S sequencing, 32 were indicated to be isolates. From these, 16 isolates were selected for genome sequencing to further confirm taxonomic identity. Both *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C were verified to be isolates and used

for subsequent studies. Initial growth curves of *Rhodococcus* sp. TE21C in BHTPA and *Paracoccus* sp. RL32C in LB broth along with both isolate's growth on DCPET is shown below in Figure 4.

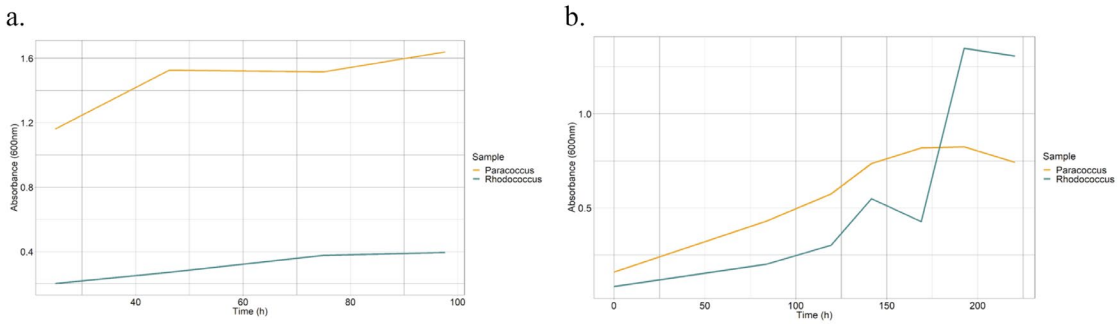

Figure 4. (a) Initial growth curves of *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C, grown in BHTPA and LB broth, respectively. (b) Initial growth curves of *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C, grown on DCPET as the sole carbon source.

Isolate	16S Sequencing Taxonomy	Genome Sequencing Taxonomy	Contamination	Completeness	Quality
TE21C	*Rhodococcus* sp. ARG_BN062	*Rhodococcus*	7.67	99.81	High
RE23C	*Cellulosimicrobium funkei* strain SZN21	*Cellulosimicrobium composti*	1.83	99.13	High
RL23A	*Cellulosimicrobium funkei* strain SZN21	*Shinella, Chelatococcus*	100	100	Low
RL24B	*Cellulosimicrobium cellulans*	*Cellulosimicrobium composti*	1.83	99.13	High
RL21B	*Chelatococcus* sp. CO-6	*Chelatococcus, Shinella*	71.05	100	Low
RL21C	*Uncultured Chelatococcus* sp. clone 4	*Shinella, Chelatococcus*	100	100	Low
RL22C	*Uncultured Chelatococcus* sp. clone 4	*Shinella, Chelatococcus*	100.38	100	Low
RL22A	*Shinella zoogloeoides* strain RTC	*Shinella sp. HZN7*	0.58	99.87	High
RL22B	*Shinella zoogloeoides* strain RTC	*Shinella sp. HZN7*	0.58	99.87	High
RL23C	*Shinella yambaruensis* strain A9	*Shinella sp. HZN7*	1.75	99.87	High
RL32C	*Paracoccus pantotrophus* strain ACCC10489	*Paracoccus pantotrophus*	0.45	99.39	High
TL11C	*Pseudomonas* sp. NF-2	*Chelatococcus, Rhodococcus, Pseudomonas*	179.48	85.24	Low
TL13A	*Pseudomonas* sp. NF-2	*Pseudomonas, Rhodococcus*	41.43	96.55	Low
TL13B	*Pseudomonas hydrolytica* strain KHPS2	*Rhodococcus, Pseudomonas, Paracoccus*	156.84	100	Low

TL13C	*Pseudomonas toyotomiensis* strain SM2	*Rhodococcus, Pseudomonas, Paracoccus*	121.11	98.28	Low
TL32A	*Pseudomonas alcaliphila* strain CLSI12	*Pseudomonas, Chelatococcus*	214.3	100	Low
TL32B	*Pseudomonas chengduensis* strain T1624	*Hyphomonas, Rhodococcus, Pseudomonas, Chelatococcus, Uncultured bacteria*	284.3	100	Low

Table 1. Samples selected for genome sequencing, comparing 16S rRNA sequencing taxonomy to genome sequencing taxonomy. Contamination and completeness for each sample were calculated using CheckM and were used to estimate genome assembly quality with the quality assessment criteria from Bowers et al., 2017.

Isolate	GC content (%)	Assembly length (bp)	Length of longest contig (bp)	No. of contigs	N50 (bp)	L50 (bp)
Rhodococcus sp. TE21C	67.63	7582378	2372772	42	829318	3
Paracoccus sp. RL32C	67.53	4372680	299557	153	89595	14

Table 2. Genome statistics for *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C, generated with Prokka.

The assembled and annotated genomes for *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C were each examined for the following genes identified in TPA and ethylene glycol degradation pathways: terephthalate 1,2 dioxygenase reductase component, terephthalate 1,2 dioxygenase oxygenase component (α and β subunits), lactaldehyde reductase and NADH-dependent aldehyde reductase. The results are summarized below in Table 3.

Substrate	Gene	***Rhodococcus* sp. TE21C**	***Paracoccus* sp. RL32C**
Terephthalate	tphA1 (terephthalate 1,2 dioxygenase reductase component)[1]	Yes	No
	tphA2 (terephthalate 1,2 dioxygenase reductase component a subunit)[1]	Yes	No

	tphA3 (terephthalate 1,2 dioxygenase reductase component b subunit)[1]	Yes	No
Ethylene glycol	fucO (glycoaldehyde reductase)[1]	No	No
	aldA (glycoaldehyde dehydrogenase)[2]	No	No
	EgaA (mycofactin-associated dehydrogenase)[3]	No	No

Table 3. Genes involved in TPA and ethylene glycol degradation and presence within sequenced genomes of *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C. Genes identified from MetaCyc[1] (https://metacyc.org/), Shimizu et al., 2024[2], and Bordel et al., 2024[3]

As shown above, the sequenced *Rhodococcus* sp. TE21C genome included the 3 genes involved in TPA degradation and did not possess the genes involved in ethylene glycol degradation. The EgaA gene, identified by Shimizu et al., is a gene involved in ethylene glycol assimilation by *Rhodococcus jostii* but was not identified in the genome of *Rhodococcus* sp. TE21C. The genome of *Paracoccus* sp. RL32C was not shown to contain any genes involved in TPA or ethylene glycol degradation. Average nucleotide identity (ANI) was used to identify the closest relative of each isolate, comparing the sequenced metagenomes to *Rhodococcus* and *Paracoccus* metagenome bins from EB2 and LS1 enriched communities, discussed in Schaerer et al. *Rhodococcus* sp. TE21C had a 100.00% ANI similarity with the EB2 *Rhodococcus* bin. Phylogenetic analysis suggests that *Rhodococcus* sp. TE21C may be a close relative of *Rhodococcus pyridinivorans* (95.92% ANI) or *Rhodococcus rhodochrous* (96.38% ANI). *Paracoccus* sp. RL32C also showed a 99.99% ANI similarity to the EB2 *Paracoccus* bin. Phylogenetic analysis suggests this may be a close relative of *Paracoccus pantotrophus* (99.29% ANI). *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C along with EB2 bins and genera type strains are shown below in Figure 5. *Paracoccus pantotrophus* has been identified as an ethylene glycol degrader (Bachmann et al., 2023), and is being examined for potential use as a biological chassis for degradation of PET monomers (Pal et al., 2024).

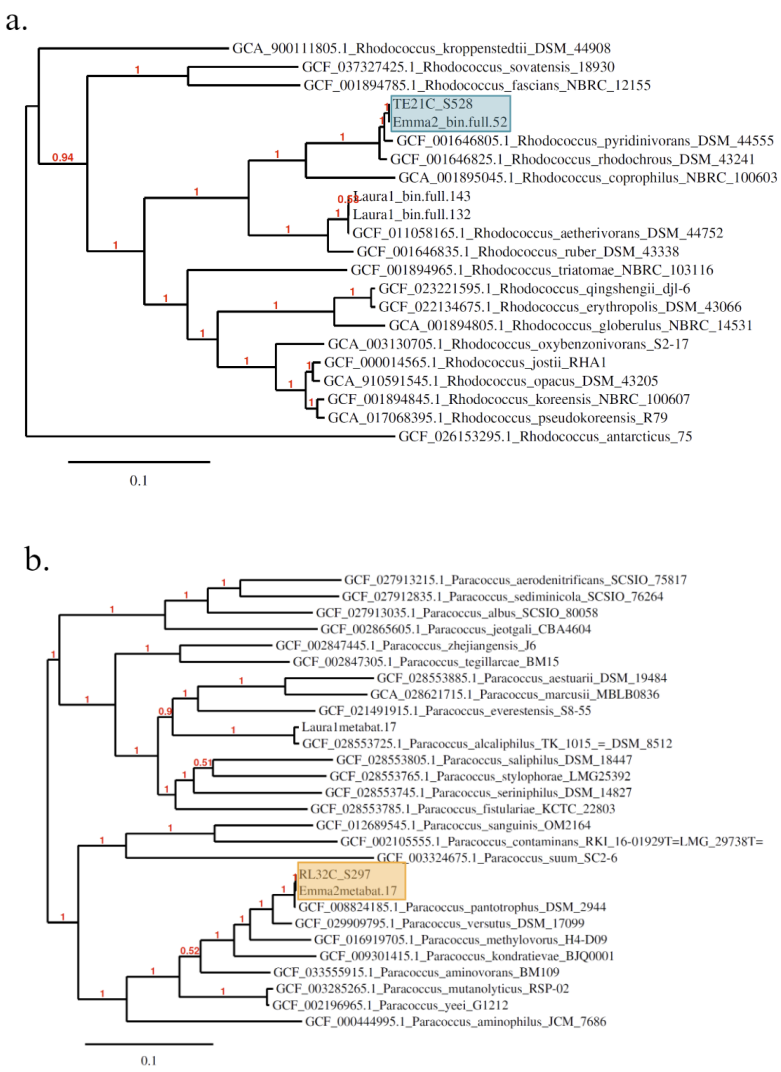

Figure 5. Phylogenomic trees showing *Rhodococcus* sp. TE21C and EB2 bin, highlighted in blue (a.) and *Paracoccus* sp. RL32C and EB2 bin highlighted in orange (b.), including type strains of each genera

Ethylene glycol: inhibitory concentration

Growth curves were generated for *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C to determine the minimum inhibitory concentration of ethylene glycol. Figure 6 shows that the inhibitory concentration for *Rhodococcus* sp. TE21C falls between 2% (v/v) and 5% (v/v) ethylene glycol in BHTPA, identified by a cessation of growth between these concentrations. *Rhodococcus* sp. TE21C was again tested with ethylene glycol concentrations between 2.5% (v/v) and 4.5% (v/v). These concentrations appeared to be equally inhibitory to all samples but still allowed some growth, so 5% (v/v) ethylene glycol was assumed to represent the minimum inhibitory concentration for *Rhodococcus* sp. TE21C. *Paracoccus* sp. RL32C was tested in LB broth and appeared to have an inhibitory concentration between 5% (v/v) and 10% (v/v), with a reduction of growth occurring between these concentrations (Figure 7). An ethylene glycol concentration of

5%, equivalent to 55.65 g/L, was used in subsequent experiments to inhibit *Rhodococcus* sp. TE21C growth without negatively affecting *Paracoccus* sp. RL32C.

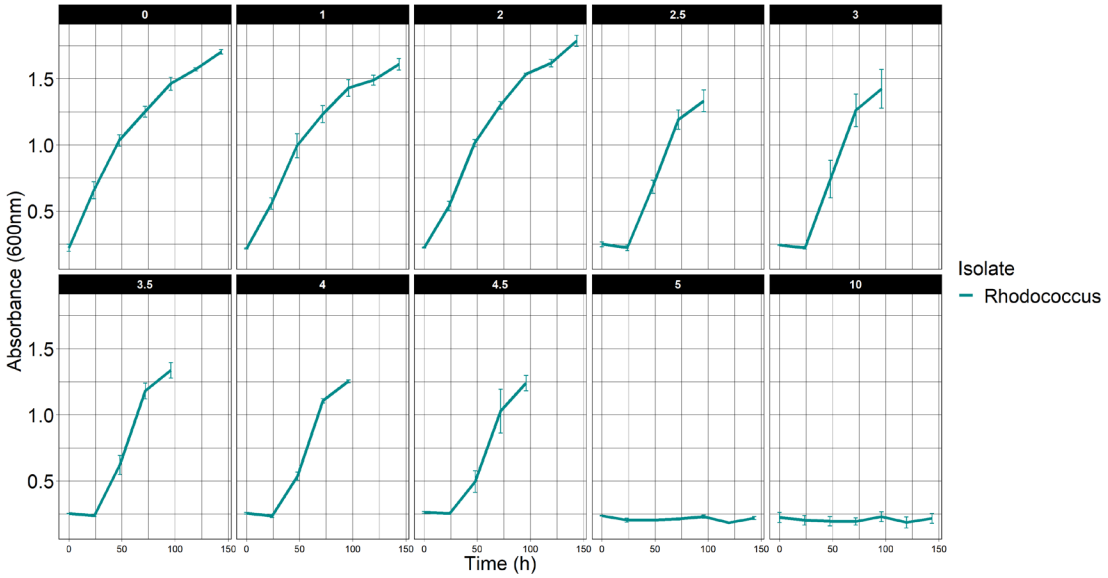

Figure 6. Growth of *Rhodococcus* sp. TE21C in varying concentrations of ethylene glycol between 0% (v/v) and 10% (v/v) in BHTPA.

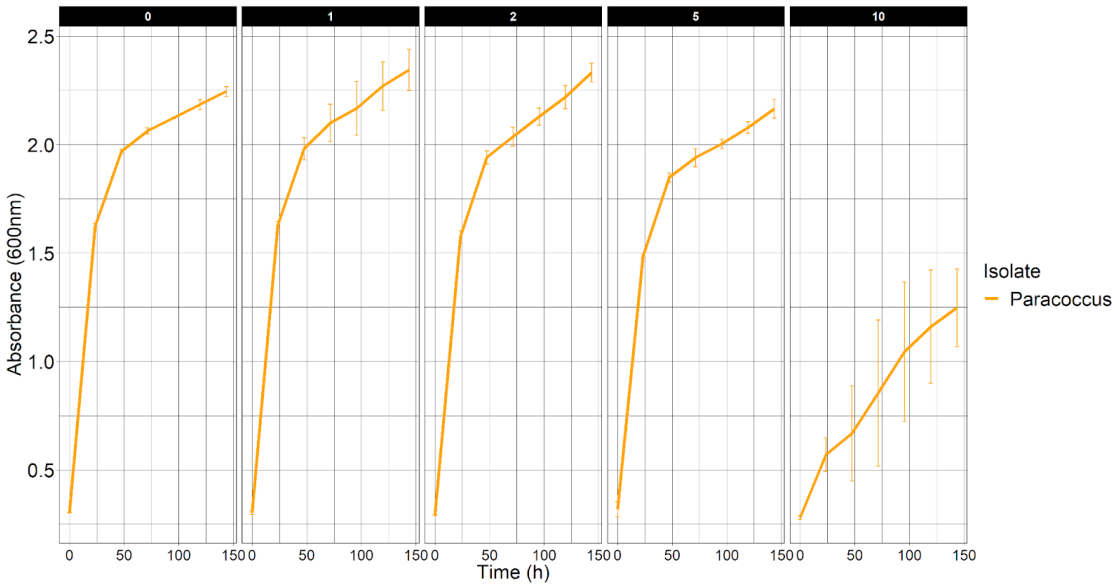

Figure 7. Growth of *Paracoccus* sp. RL32C in varying concentrations of ethylene glycol between 0% (v/v) and 10% (v/v) in LB broth.

Mixing experiment 1: inhibitory ethylene glycol conditions

We hypothesized that the ability of *Paracoccus* sp. RL32C to tolerate higher concentrations of ethylene glycol and its ability to grow on ethylene glycol would allow *Paracoccus* sp. RL32C to positively affect the growth of *Rhodococcus* sp. TE21C in non-permissive conditions of high ethylene glycol. We expected that in the co-culture, growth of *Rhodococcus* sp. TE21C would be stimulated and would positively benefit from the *Paracoccus* sp. RL32C metabolism of ethylene glycol.

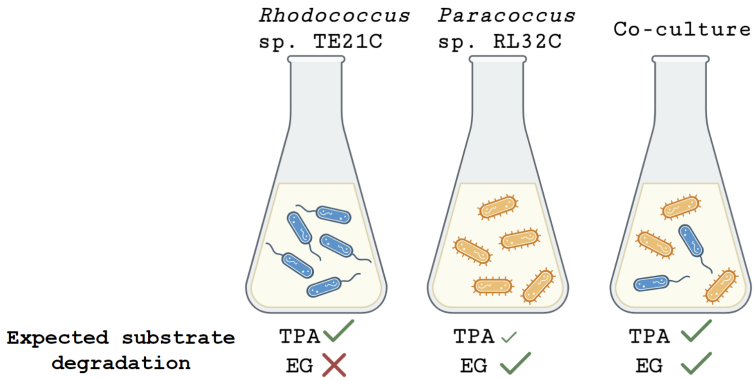

Figure 8. Conceptual model of mixing experiment 1. *Rhodococcus* sp. TE21C is capable of metabolizing terephthalate (TPA), but is inhibited by ethylene glycol (EG). *Paracoccus* sp. RL32C is capable of metabolizing both TPA and EG, but preferentially degrades EG. By combining these strains in a co-culture, *Rhodococcus* sp. TE21C will not be inhibited by EG, as *Paracoccus* sp. RL32C will degrade it and both TPA and EG will be metabolized.

Growth curves

Growth curves were used to compare isolate and co-culture growth under stressful conditions, characterized by 10 g/L TPA and 55.65 g/L ethylene glycol in Bushnell-Haas media. *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C were both tested as isolates, as well as a co-culture composed of the two isolates together in a 50:50 ratio based on initial OD. Growth was determined by OD measurements collected daily for the duration of the experiment (Figure 9). The co-culture grew to a higher density more quickly than either isolate, with the *Rhodococcus* sp. TE21C isolate exhibiting no growth as was expected. In contrast, the *Paracoccus* sp. RL32C isolate showed substantial growth to an OD of 1.46 (±0.072) after 144 hours. Log phase for *Paracoccus* sp. RL32C was reached after 24 hours, whereas the co-culture appeared to exhibit no lag phase. Similarly, the final density of *Paracoccus* sp. RL32C was lower than the co-culture final density of 1.55 (±0.060). However, although the final densities differed, there was determined to be no significant difference between the samples (Table A1).

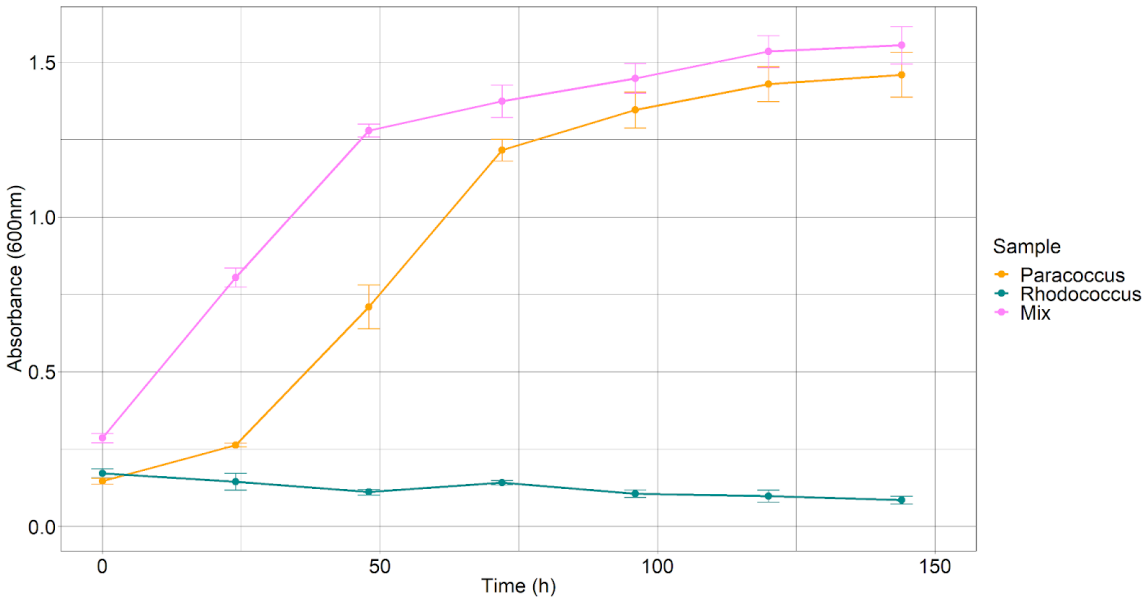

Figure 9. Isolate and community growth curves under stressful conditions, measured at 600nm with a spectrophotometer. Measurements were taken at 24-hour intervals.

Community composition

16S rRNA sequencing was performed to determine the community composition for each sample at the final time point. The relative abundance of *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C was scaled to the final optical density to reflect the growth of each sample (Figure 10). As expected, the *Paracoccus* sp. RL32C isolate grew successfully while the *Rhodococcus* sp. TE21C isolate did not. The co-culture was still dominated by *Paracoccus* sp. RL32C, which suggests that while there was an improvement in growth in the co-culture, *Paracoccus* sp. RL32C was still able to outcompete *Rhodococcus* sp. TE21C. A small percentage of the co-culture growth was represented by *Rhodococcus* sp. TE21C (4.81 % ± 1.90 %), indicating that *Rhodococcus* sp. TE21C may have been inhibited to the point of little growth even in the co-culture. There was no significant difference between the growth of *Paracoccus* sp. RL32C nor *Rhodococcus* sp. TE21C as isolates compared to the co-culture (Table A4). Despite the limited growth of *Rhodococcus* sp. TE21C in the co-culture, the presence of *Rhodococcus* sp. TE21C appeared to in a positive effect on the growth of *Paracoccus* sp. RL32C.

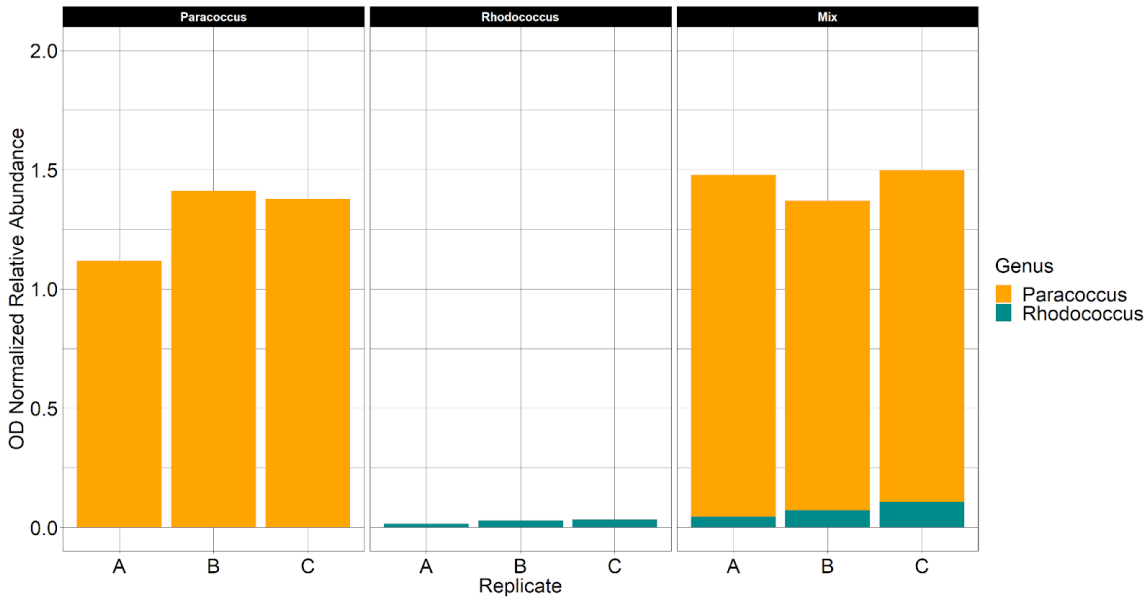

Figure 10. Community composition determined by 16S rRNA sequencing at final time point. Relative abundance was normalized to final optical density measurement to scale abundance to sample growth.

Degradation of terephthalate and ethylene glycol

HPLC (high-performance liquid chromatography) analysis was performed to determine the TPA and ethylene glycol concentration in the media over the course of the experiment. Samples for HPLC were collected at the same time as absorbance was measured. The HPLC results showing TPA concentration are below in Figure 11. The concentration of TPA varied greatly between replicates, but the overall trend showed a decrease in TPA from the approximate starting concentration of 10 g/L, with the *Rhodococcus* sp. TE21C isolate showing the most decrease to 7.86 (± 0.513) g/L. Overall, despite the high growth observed in both the *Paracoccus* sp. RL32C alone and the co-culture, there was limited terephthalate degradation. This could suggest that the observed growth was due to preferential degradation of ethylene glycol by *Paracoccus* sp. RL32C.

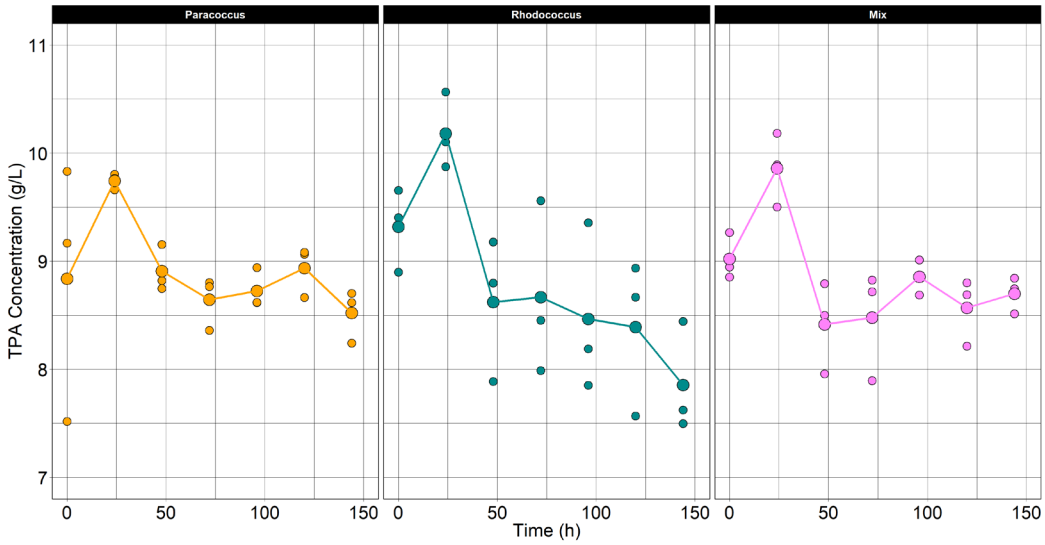

Figure 11. HPLC results for TPA degradation under stressful conditions. The samples were diluted 10X and resulting concentrations were used to calculate the original concentrations.

HPLC analysis was also performed to measure ethylene glycol concentration, shown in Figure 12 below. The ethylene glycol was added at 5 % by volume. The measured ethylene glycol concentration (approximately 70 g/L) was higher than the expected value of approximately 55 g/L. Regardless, similar to the TPA degradation data, concentrations varied greatly by replicate for each sample with no clear degradation pattern occurring.

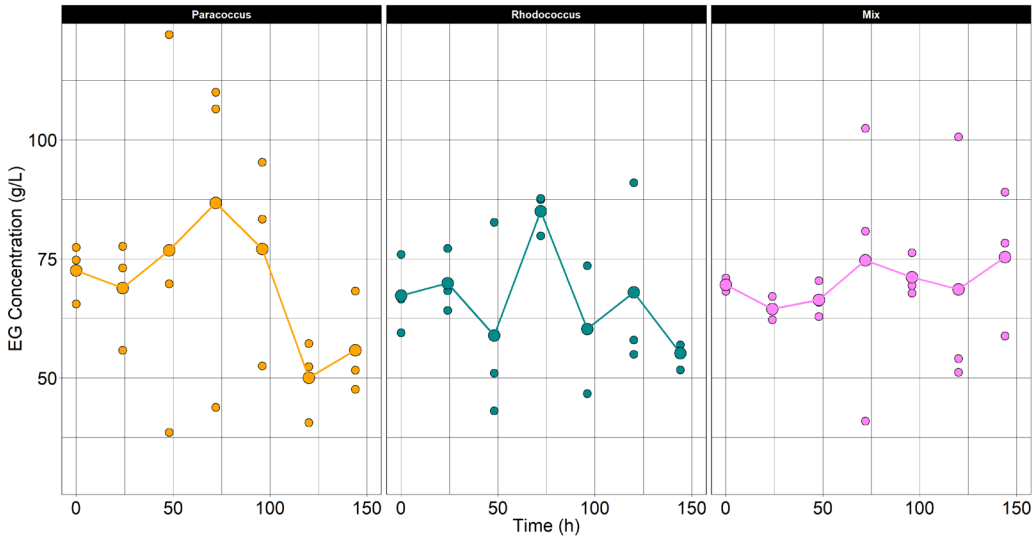

Figure 12. HPLC results for ethylene glycol degradation under stressful conditions.

Mixing experiment 2: non-inhibitory ethylene glycol conditions

We hypothesized that the ability of *Paracoccus* sp. RL32C to grow on ethylene glycol would allow *Paracoccus* sp. RL32C to continue to positively affect the growth of *Rhodococcus* sp. TE21C even in conditions with a non-inhibitory concentration of ethylene glycol. We expected that in the co-culture, growth of *Rhodococcus* sp. TE21C would be stimulated and would positively benefit from *Paracoccus* sp. RL32C*'* ability to metabolize ethylene glycol.

Growth curves

Continuous growth curves were collected for the *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C isolates under non-stressful conditions, as well as for each component in the co-culture. Non-stressful conditions were characterized by 10 g/L TPA and 3 g/L ethylene glycol to replicate the chemical composition of deconstructed PET. The 3 g/L of ethylene glycol is not a sufficient concentration to prevent *Rhodococcus* sp. TE21C growth. Through the use of co-culture plates and a continuous-read plate reader, the co-culture components were separated by a filter but capable of sharing metabolites, allowing for the measurement of individual growth curves. Figure 13 below shows the growth curves of each isolate in Bushnell-Haas media with TPA alone, ethylene glycol alone, and a combination of the two. The *Paracoccus* sp. RL32C appeared to experience some inhibition when in the co-culture, not growing to as high a density as the *Paracoccus* sp. RL32C isolate alone in any sample. However, the *Rhodococcus* sp. TE21C isolate appeared to benefit from the co-culture, growing to a higher density in the ethylene glycol media and performing very similarly in the co-culture as the isolate. In the media with 3 g/L of ethylene glycol alone, the *Paracoccus* sp. RL32C grew to a higher density than *Rhodococcus* sp. TE21C in both isolate and co-culture scenarios; however, in the media containing TPA, *Rhodococcus* sp. TE21C isolate grew to a higher density at a faster rate than the *Paracoccus* sp. RL32C isolate. The final OD for each sample is shown below in Table 4. However, when statistically comparing co-culture components to their isolate counterparts, only the *Paracoccus* sp. RL32C in TPA alone and EG+TPA showed a significant difference in final OD (Table A5).

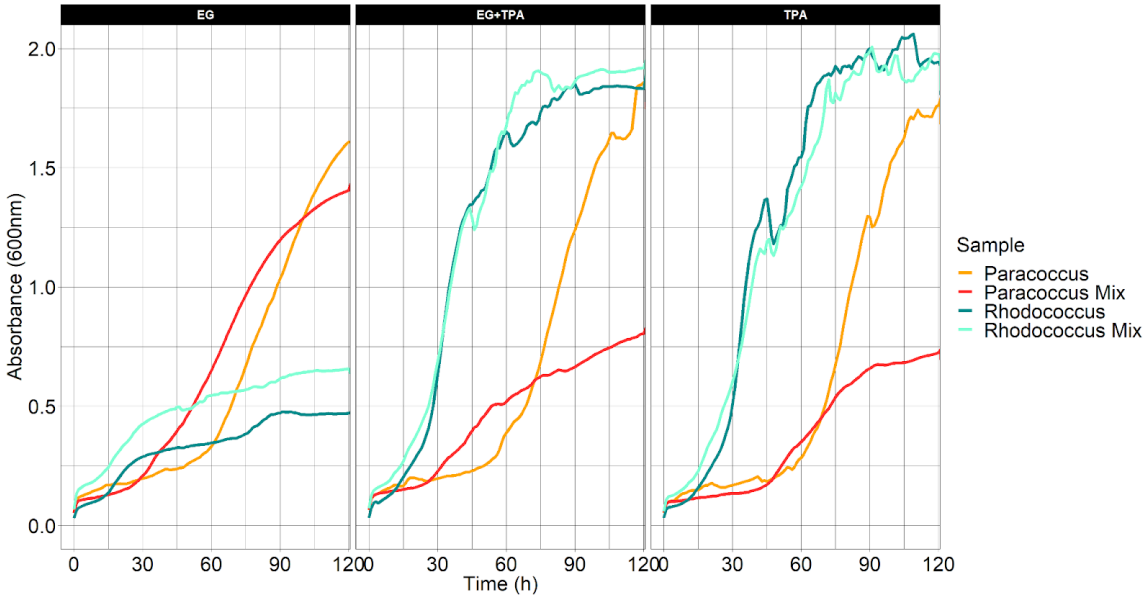

Figure 13. Continuous growth curves for *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C isolates and each component of the co-culture in media containing ethylene glycol alone, TPA alone, and a combination of the two.

		OD600		
		EG	**TPA**	**EG+TPA**
Isolate	***Rhodococcus* sp. TE21C**	0.47±0.02	1.95±0.25	1.83±0.23
	***Paracoccus* sp. RL32C**	1.6±0.10	1.76±0.06	1.86±0.15
Co-culture	***Rhodococcus* sp. TE21C**	0.66±0.08	1.97±0.12	1.92±0.12
	***Paracoccus* sp. RL32C**	1.41±0.11	0.73±0.06	0.85±0.17

Table 4. Final OD for each isolate and co-culture component in three treatment types (ethylene glycol alone (EG), terephthalate alone (TPA), and ethylene glycol and terephthalate together (EG+TPA)).

Rhodococcus sp. TE21C grew similarly as an isolate and in the co-culture in all media types (Figure 13). When in the co-culture, the *Rhodococcus* sp. TE21C grew to a slightly higher density in media containing only ethylene glycol, suggesting beneficial metabolite sharing between *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C in those conditions. The *Paracoccus* sp. RL32C isolate grew very consistently between each media type (Figure 13). However, this was considerably different compared to the co-culture in the media containing TPA. *Paracoccus* sp. RL32C appeared to be inhibited in the co-culture, with the samples in TPA media growing to a much lower density than the isolate alone.

However, this did not hold true for the media containing only ethylene glycol with both the *Paracoccus* sp. RL32C isolate and co-culture performing very similarly in each condition.

HPLC analysis

HPLC analysis was conducted to determine the degradation of both TPA and ethylene glycol in each experimental condition. Due to the small volume of the plate readers used to collect continuous data, media samples were only collected at the beginning and final timepoints for analysis. TPA degradation (Figure 14) occurred in all samples, with the co-culture in TPA/ethylene glycol media showing the most degradation followed by the *Rhodococcus* sp. TE21C isolate in the same media. The *Paracoccus* sp. RL32C isolate showed the least TPA degradation in both TPA and TPA/ ethylene glycol media. Ethylene glycol degradation was also measured (Figure 14). The highest amount of degradation was shown in the co-culture in media containing only ethylene glycol. The *Rhodococcus* sp. TE21C isolate in ethylene glycol media showed no degradation; in contrast, the final concentration of ethylene glycol was measured to be more than the starting concentration, possibly due to pipetting calibration or error in the dilution process. All other samples showed significant degradation, with *Paracoccus* sp. RL32C isolates showing relatively equal ethylene glycol degradation in both media with ethylene glycol alone as well as the TPA+ethylene glycol mixture.

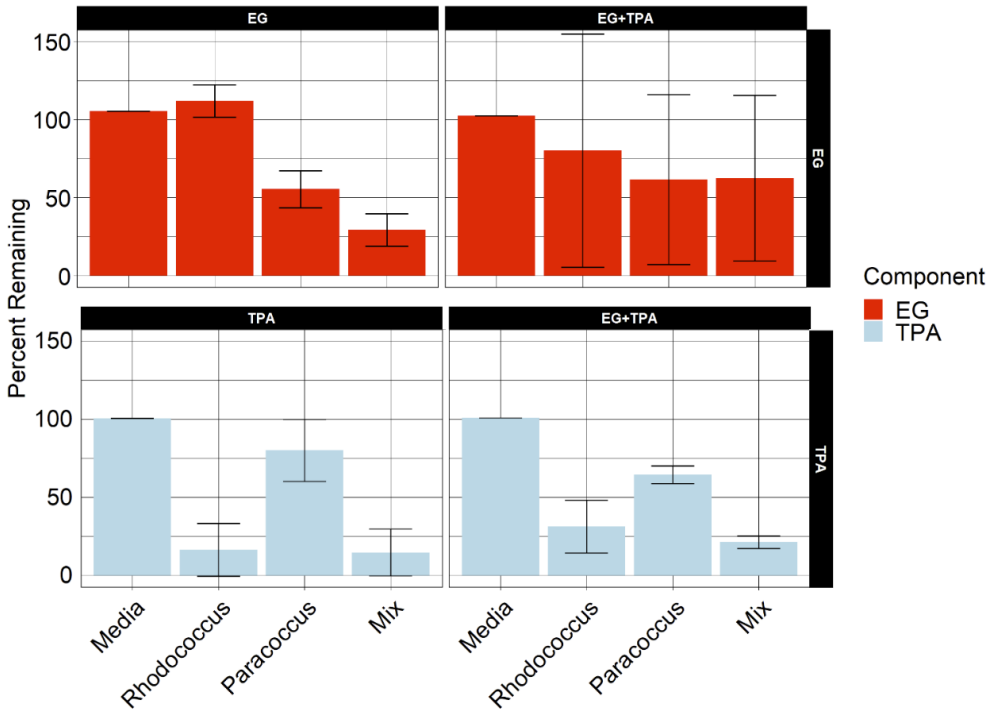

Figure 14. Percent of TPA and ethylene glycol (EG) remaining in each experimental condition (TPA alone, EG alone, and TPA+EG) at the final time point, measured by HPLC analysis. Media samples represent the original concentrations in Bushnell-Haas and were collected at the experiment start.

1.3 Discussion

Mixing Experiment 1

Under stressful conditions, there appeared to be some cooperation between *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C occurring in the co-culture, seen in the higher final density observed in the co-culture than the isolates alone (Figure 9). We originally hypothesized that the isolates would divide labor in the co-culture, with *Paracoccus* sp. RL32C degrading the ethylene glycol and limiting the stress on *Rhodococcus* sp. TE21C. *Rhodococcus* sp. TE21C would then degrade the TPA in the media without inhibition. This hypothesis is consistent with previous work indicating *Paracoccus* sp. RL32C is an ethylene glycol specialist and *Rhodococcus* sp. TE21C is a TPA degrading generalist (Schaerer et al., 2023a). Division of labor in this scenario would be characterized by the successful growth of both isolates in the co-culture. This would result in clear degradation of both TPA and ethylene glycol in the media, as both isolates would be engaged in a cooperative interaction allowing them both to metabolize their preferred substrates. Experimentally, the final OD of the co-culture was higher than the *Paracoccus* sp. RL32C, indicating that there may have been cooperation occurring to allow for higher growth. However, the difference in final OD between the *Paracoccus* sp. RL32C and co-culture was not statistically significant, so the hypothesis of division of labor was not entirely supported by the growth curves. The co-culture did not appear to experience a lag phase before reaching exponential growth as compared to the *Paracoccus* sp. RL32C isolate, indicating that the co-culture may have had an advantage in starting growth.

The HPLC data showed no clear degradation of either substrate. The TPA data (Figure 11) showed little degradation, with only 1 to 2 g/L being degraded, indicating that neither bacterium was utilizing TPA as a main carbon source under these conditions. Similarly, no degradation trend was observed in the HPLC analysis of ethylene glycol (Figure 12). One possible explanation for this could be that ethylene glycol was being degraded first before the TPA, and with approximately 70 g/L of ethylene glycol in the media there was far more present than the *Paracoccus* sp. RL32C could degrade over the duration of the experiment Preferential degradation of ethylene glycol over TPA is suggested in Bao et al.'s work on the co-degradation of TPA and ethylene glycol. Their work showed that a co-culture of two engineered *Pseudomonas putida* strains (one to degrade TPA and another to degrade EG) worked more efficiently as a co-culture compared to one single strain engineered to degrade both. In the case of their co-culture, EG and TPA were degraded almost simultaneously, whereas in the monoculture, ethylene glycol was degraded first (Bao et al., 2023).

Genomic analysis

Examining the genomes of *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C, genes expected to be involved in TPA degradation were all found in the *Rhodococcus* sp. TE21C genome as expected. However, no genes found in either TPA or ethylene glycol degradation were identified in the genome of *Paracoccus* sp. RL32C. This may be due to missing part of the genome, as the CheckM completeness score was 99.39% and the genomes were still fragmented with 153 contigs. Another explanation could be that *Paracoccus* sp. RL32C utilized a different pathway for ethylene glycol and does not

possess the fucO or YLL056C genes identified in ethylene glycol degradation (SRI International, 2022). Despite not being able to detect known homologs of genes involved in TPA and EG degradation, *Paracoccus* sp. RL32C was still able to grow using TPA and EG as the sole carbon source. The community composition data also does little to support the original hypothesis. While the *Paracoccus* sp. RL32C isolate grew and the *Rhodococcus* sp. TE21C isolate did not, the final community composition (Figure 10) also shows that *Rhodococcus* sp. TE21C did not grow in the co-culture either. This indicates that the concentration of ethylene glycol may have been so high that *Paracoccus* sp. RL32C did not degrade enough to successfully ease the inhibition and allow *Rhodococcus* sp. TE21C to grow. This indicates that *Rhodococcus* sp. TE21C growth was not significantly different between the *Rhodococcus* sp. TE21C isolate and the *Rhodococcus* sp. TE21C in the co-culture (Table A1), however slightly higher ODs were observed for the *Rhodococcus* sp. TE21C in the co-culture. Similarly, *Paracoccus* sp. RL32C did not appear to significantly benefit from the co-culture either (Table A1). This suggests that while each co-culture member may have benefitted slightly, *Paracoccus* sp. RL32C still outcompeted *Rhodococcus* sp. TE21C in co-culture and the division of labor hypothesis was not entirely supported. Although there is not clear support for a mutualistic relationship with both parties benefitting, there appears to be a commensal relationship with *Paracoccus* sp. RL32C showing a decreased lag phase in the presence of *Rhodococcus* sp. TE21C and growing to a slightly higher density. For future work, a longer experiment with this high level of ethylene glycol could better examine the degradation processes and allow for examination of the microbial interactions and degradation after some of the ethylene glycol is removed.

Mixing Experiment 2

The original division of labor hypothesis was expected to hold true for non-stressful conditions as well, with *Paracoccus* sp. RL32C and *Rhodococcus* sp. TE21C mutually benefitting from co-culture conditions. The growth curves showed that the interactions in co-culture varied based on media type (Figure 13). In media containing ethylene glycol alone, the co-culture appeared to benefit *Rhodococcus* sp. TE21C, with a higher final OD in the co-culture compared to the isolate alone. However, *Rhodococcus* sp. TE21C as an isolate did show some growth in media containing ethylene glycol alone, due to the ethylene glycol concentration not being entirely inhibitory. *Paracoccus* sp. RL32C in this media acted very similarly as an isolate and within the co-culture, indicating that when ethylene glycol alone is present, *Paracoccus* sp. RL32C is unaffected but *Rhodococcus* sp. TE21C may benefit from co-culture and reduction of inhibition. However, in both media containing TPA alone and TPA+ethylene glycol, the *Rhodococcus* sp. TE21C acted similarly as an isolate and in the co-culture. However, *Paracoccus* sp. RL32C showed inhibition in these scenarios. In both cases, *Paracoccus* sp. RL32C grew to a considerably lower density in the co-culture, indicating that when *Rhodococcus* sp. TE21C is in suitable conditions in media containing TPA, division of labor does not occur. Instead, *Rhodococcus* sp. TE21C outcompetes *Paracoccus* sp. RL32C when presented with a suitable growth substrate. It was observed that *Paracoccus* sp. RL32C exhibits a considerably longer lag phase than *Rhodococcus* sp. TE21C and in further work, this could be adjusted by mixing the co-culture with a higher percentage of

Paracoccus sp. RL32C, or continuing the duration of the experiment to allow all isolates and co-culture to reach stationary phase. Similarly, due to the membrane separation of each component of the co-culture for this experiment, no physical interactions could occur and this may have affected the growth and interactions of the isolates in co-culture.

The HPLC data also shows that co-culture degradation varies by media type. *Rhodococcus* sp. TE21C and the co-culture degraded TPA most effectively, with the highest amount of TPA degradation occurring when ethylene glycol was also present in both scenarios. This suggests that when the ethylene glycol concentration is not inhibitory to growth, *Rhodococcus* sp. TE21C can degrade TPA to the same extent whether *Paracoccus* sp. RL32C is present or not. Interestingly, *Rhodococcus* sp. TE21C and the co-culture degraded more TPA in the media containing TPA+ethylene glycol compared to media with TPA alone. The *Paracoccus* sp. RL32C isolate degraded more TPA in media containing TPA alone rather than in media with TPA+ethylene glycol. This suggests that while *Paracoccus* sp. RL32C may be able to degrade TPA, when ethylene glycol is present, it will degrade that first. The HPLC analysis of ethylene glycol degradation reaffirms *Paracoccus* sp. RL32C is an ethylene glycol specialist, effectively degrading ethylene glycol as an isolate whereas *Rhodococcus* sp. TE21C did not. In the co-culture, media with ethylene glycol alone showed more ethylene glycol degradation than in the media with both ethylene glycol and TPA together. This reaffirms the concept that *Paracoccus* sp. RL32C experiences inhibition when *Rhodococcus* sp. TE21C is in suitable conditions.

Experiment Comparison
From Mixing Experiment 1, it appears that under stressful conditions, *Paracoccus* sp. RL32C will outcompete *Rhodococcus* sp. TE21C even in co-culture and that the division of labor concept does not hold true. This indicates that with a high level of ethylene glycol in the media, *Paracoccus* sp. RL32C does not ease the inhibition on *Rhodococcus* sp. TE21C enough for it to effectively grow or degrade TPA. A comparison of ethylene glycol degradation between experiments was not calculated due to the inconclusive HPLC results from Mixing Experiment 1. However, a commensal relationship may exist under these conditions, with *Paracoccus* sp. RL32C benefitting with no effect on *Rhodococcus* sp. TE21C. However, Experiment 2 shows that the interactions between *Rhodococcus* sp. TE21C and *Paracoccus* sp. RL32C are dependent on media type, with both cooperation and competition having the potential to occur. With the apparent cooperation happening in media containing only ethylene glycol, *Rhodococcus* sp. TE21C may have benefitted from the reduction of ethylene glycol inhibition as a result of *Paracoccus* sp. RL32C' metabolism.

1.4 Conclusion

In this study we examined the interactions between *Rhodococcus* sp. TE21C, a TPA generalist, and *Paracoccus* sp. RL32C, an ethylene glycol specialist under stressful and non-stressful conditions. When ethylene glycol is at an inhibitory concentration to *Rhodococcus* sp. TE21C, *Paracoccus* sp. RL32C outcompetes it even in co-culture, with a slight commensal benefit to *Paracoccus* sp. RL32C. In non-stressful conditions, we

observed that when only ethylene glycol is present, *Paracoccus* sp. RL32C will outcompete *Rhodococcus* sp. TE21C but will support the growth of *Rhodococcus* sp. TE21C. When only TPA is present, *Rhodococcus* sp. TE21C will outcompete and negatively impact the growth of *Paracoccus* sp. RL32C. However, when both TPA and ethylene glycol are present, *Rhodococcus* sp. TE21C will still outcompete *Paracoccus* sp. RL32C but will not affect the amount of TPA or ethylene glycol that is degraded in the mixture. A valuable addition to this research would be to collect transcriptomic data to determine the gene activity in degrading both TPA and ethylene glycol. This may offer important insight into the pathways used to degrade these substrates and how mixing these isolates together affect the gene expression. Altering the ratios of each isolate in the co-culture may also affect the outcome of these experiments through equalizing lag phases between isolates and allow for creating the most effective combination of isolates. Regardless, the results shown here serve as a valuable basis for understanding interspecies interactions and using top-down enrichments to inform the bottom-up reconstruction of a minimal synthetic community to degrade PET plastics.

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A Appendix: Statistical analyses

Abbreviations

Rhodo: *Rhodococcus* sp. TE21C

Para: *Paracoccus* sp. RL32C

TPA: terephthalate

EG: ethylene glycol

Table A1. Statistical comparison (ANOVA and Tukey HSD post hoc test) of final OD_{600} values for Mixing Experiment 1

ANOVA	Degrees of freedom	F value	P value
	2	670.3	8.85e-08

Tukey HSD: P values	*Rhodococcus* sp. TE21C	*Paracoccus* sp. RL32C	Mixture
Rhodo		3e-07	2e-07
Para			0.167
Mixture			

Table A2. Statistical comparison (ANOVA and Tukey HSD post hoc test) of initial and final terephthalate concentrations determined by HPLC analysis for Mixing Experiment 1

ANOVA	Degrees of freedom	F value	P value
	2	1.31	0.1782

Tukey HSD: P values	Rhodo final	Para final	Mixture final
Rhodo initial	0.073		
Para initial		0.98	
Mixture initial			0.98

Table A3. Statistical comparison (ANOVA and Tukey HSD post hoc test) of initial and final HPLC ethylene glycol concentrations for Mixing Experiment 1

ANOVA	Degrees of freedom	F value	P value
	2	2.71	0.107

Tukey HSD: P values	Rhodo final	Para final	Mixture final
Rhodo initial	0.57		
Para initial		0.26	
Mixture initial			0.96

Table A4. Statistical comparison (T-test) of community composition data for Mixing Experiment 1

T-Test	t	Degrees of freedom	P value
Rhodo	-2.66	2.32	0.10
Para	-0.71	2.72	0.53

Table A5. Statistical comparison (ANOVA and Tukey HSD post hoc test) of final OD_{600} values for Mixing Experiment 2

ANOVA	Degrees of freedom	F value	P value
	6	52.05	1.39e-12

TPA alone	Rhodo final	Rhodo mix final	Para final	Para mix final
Rhodo final		1.00	0.88	0.00
Rhodo mix final			0.75	0.00
Para final				1e-07
Para mix final				

EG alone	Rhodo final	Rhodo mix final	Para final	Para mix final
Rhodo final		0.857	0.00	6e-07
Rhodo mix final			5e-07	2.78e-05
Para final				0.78
Para mix final				

TPA+ EG	Rhodo final	Rhodo mix final	Para final	Para mix final
Rhodo final		1.00	1.00	1e-07
Rhodo mix final			1.00	0.00
Para final				1e-07
Para mix final				

Table A6. Statistical comparison (ANOVA and Tukey HSD post hoc test) of terephthalate degradation for Mixing Experiment 2

ANOVA	Degrees of freedom	F value	P value
	4	7.65	0.00088

TPA alone	Rhodo	Para	Mix
Rhodo		0.058	0.98
Para			0.0074
Mix			

TPA + EG	Rhodo	Para	Mix
Rhodo		9.73e-05	1.00
Para			6.9e-05
Mix			

Table A7. Statistical comparison (ANOVA and Tukey HSD post hoc test) of ethylene glycol degradation for Mixing Experiment 2

ANOVA	Degrees of freedom	F value	P value
	4	1.08	0.39

EG alone	Rhodo	Para	Mix
Rhodo		0.64	0.20
Para			0.99
Mix			

TPA + EG	Rhodo	Para	Mix
Rhodo		0.54	1.00
Para			1.00
Mix			