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RESEARCH ARTICLE

Degradation of polyethylene terephthalate (PET) plastics by wastewater bacteria engineered via conjugation

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Abstract

Wastewater treatment plants are one of the major pathways for microplastics to enter the environment. In general, microplastics are contaminants of global concern that pose risks to ecosystems and human health. Here, we present a proof-of-concept for reduction of microplastic pollution emitted from wastewater treatment plants: delivery of recombinant DNA to bacteria in wastewater to enable degradation of polyethylene terephthalate (PET). Using a broad-host-range conjugative plasmid, we enabled various bacterial species from a municipal wastewater sample to express FAST-PETase, which was released into the extracellular environment. We found that FAST-PETase purified from some transconjugant isolates could degrade about 40% of a 0.25 mm thick commercial PET film within 4 days at 50°C. We then demonstrated partial degradation of a post-consumer PET product over 5–7 days by exposure to conditioned media from isolates. These results have broad implications for addressing the global plastic pollution problem by enabling environmental bacteria to degrade PET.

INTRODUCTION

Plastics are versatile materials that are lightweight, strong and chemically resistant. These desirable properties have made them an integral part of daily activities, infrastructure and economies since their invention in the 20th century (Andrady & Neal, 2009). Although plastics have been an economic boon, particularly in packaging and construction applications, their use has produced unsustainable levels of waste. From 1950 to 2015, about 4900 megatons, accounting for 59% of all plastics ever produced, have been discarded into landfills and the environment (Geyer et al., 2017). Ninety percent of plastics produced—consisting of polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET), polyurethane (PU) and polystyrene (PS) (Geyer et al., 2017)—are projected to persist for hundreds of years; they are not biodegradable on timescales relative to their end of use (Chamas et al., 2020). Consequently, their bioaccumulation poses threats to ecosystems and, potentially, to human health (Wang et al., 2019; Xu et al., 2020; Yuan et al., 2022). Annual plastic waste generation is projected to almost triple by 2060 compared to 2019 levels if trends in current plastic usage continue (OECD, 2022). Even with immediate implementation of ambitious strategies for waste management, environmental recovery and reduction of plastic production, conservative estimates suggest that plastic waste entering terrestrial and aquatic ecosystems will exceed or remain close to 2016 levels between 2030 and 2040 (Borrelle et al., 2020; Lau et al., 2020).

Although PE, PP, PVC, PET, PU and PS are referred to as non-biodegradable, some microbes and insects have ways to metabolise these plastics into their constituent molecules, which can then be used

as carbon and energy sources (Brandon et al., 2018; Chattopadhyay, 2022; Jeon & Kim, 2016; Maheswaran et al., 2023; Yoshida et al., 2016). These natural processes are relatively slow, taking weeks to months for significant biodegradation to occur (Gao & Sun, 2021; Kumar et al., 2023; Maheswaran et al., 2023; Sekhar et al., 2016; Shah et al., 2008; Yoshida et al., 2016). Among the most produced plastics, PET is a hydrolysable polymer that is widely used in the packaging and textile industries. Compared to other common plastics, PET is more amenable to biodegradation due to the presence of hydrolysable ester bonds. Substantial progress has been made in enzymatic PET depolymerisation for resource recovery (Liu et al., 2023). For example, PET hydrolase (PETase) from *Ideonella sakaiensis* has been subject to numerous protein engineering efforts that have resulted in PETase variants with depolymerisation rates orders of magnitude higher than the wild-type enzyme, and with greater thermo- and chemostability (Bell et al., 2022; Cui et al., 2021; Lu et al., 2022; Son et al., 2019; Zurier & Goddard, 2023). Efforts have also been made to optimise expression and secretion of PET-degrading enzymes in some bacterial species other than *Escherichia coli* (Li et al., 2023; Wang et al., 2020; Yan et al., 2021), and developments are ongoing for employing naturally occurring and designer microbial communities to degrade PET (Gao & Sun, 2021; Maheswaran et al., 2023; Qi et al., 2021; Roberts et al., 2020).

Effluent and sludge from wastewater treatment plants are major pathways by which microplastics enter the environment (Liu et al., 2021; Sun et al., 2019; Zurier & Goddard, 2021). Notably, a recent study reported that 6% of plastic processed at a recycling facility was found as microplastics in the facility's wash water post-filtration (Brown et al., 2023). Secondary treatment processes and digestion of sludge offer opportunities for the removal of microplastics through bioaugmentation. As reviewed by Zurier and Goddard (2021), microbial communities could be supplemented with engineered bacteria that secrete plastic-degrading enzymes (i.e., cell bioaugmentation); this can be more cost-effective than continuously supplementing a cocktail of purified enzymes into a secondary treatment unit.

A major challenge with the cell bioaugmentation approach is persistence of the introduced microorganisms, which are often not adapted to the environment at hand and are therefore likely to be quickly eliminated (Albright et al., 2022; Ma et al., 2022). As an alternative strategy that would bypass these barriers to establishment, we propose genetically engineering microbes native to the environment to express metabolic pathways for biodegradation of plastic waste. This approach, known as genetic bioaugmentation, can be achieved through in situ delivery of broad-host-range conjugative plasmids. Genetic bioaugmentation has been shown to improve persistence of the introduced functionality when using donor strains native to their environment (Ronda et al., 2019). Genetic bioaugmentation has not yet been applied for bioremediation of plastic waste, but this technique has been used to introduce catabolic genes into microbial communities for bioremediation of non-plastic-based pollutants in laboratory and pilot scale settings (Bathe et al., 2004; Chettri et al., 2023; French et al., 2020; Garbisu et al., 2017; Ke et al., 2022; Ren et al., 2018; Top et al., 2002; Venkata Mohan et al., 2009).

In this proof-of-concept study, we lay a foundation for the use of genetic bioaugmentation for bioremediation of PET. We genetically engineered bacteria in a municipal wastewater sample to degrade PET plastics by delivering a broad-host-range, conjugating plasmid carrying the FAST-PETase gene (Lu et al., 2022), which codes for an engineered PET hydrolase that is more robust to pH and temperature ranges and is orders of magnitude more efficient than the wild-type enzyme (Lu et al., 2022). We assessed degradation of commercial PET film using purified FAST-PETase from several engineered isolates. We then measured degradation of post-consumer PET using conditioned media from some of the engineered isolates. This report provides a foundation to support the next steps in assessing the performance and efficacy of such a genetic bioaugmentation approach.

EXPERIMENTAL PROCEDURES

Bacterial strains

Escherichia coli DH5α (Matthew Scott Lab, University of Waterloo) and NEB10-β (New England Biolabs (NEB), Whitby, Ontario, Canada) were used for cloning steps. *E. coli* K-12 (Δ*ilvD*::*FRT*, Δ*galK*::*cfp-bla*, pSAS31, pTGD, pFAST-PETase-cis) was used as a conjugative donor strain for matings with wastewater samples. Engineered wastewater isolates (see "Experimental Procedures" section) were used for protein expression as indicated.

Plasmid construction

All polymerase chain reaction (PCR) amplification steps were performed using Phusion DNA polymerase (NEB, Whitby, Ontario, Canada). Primers used for PCR amplification were ordered from Integrated DNA Technologies (IDT, Coralville, Iowa, United States) and are listed in Data S3. PCR products were assembled using NEB Hifi DNA Assembly Master Mix (NEB) according to the manufacturer instructions. pNuc-trans-mCherry was constructed by replacing *mRFP* under the P_{bs} promoter on pNuc-trans-mRFP (Hamilton et al., 2019) (a gift from Thomas Hamilton

and David Edgell, Western University) with *mCherry*. The backbone of pNuc-trans-mRFP was amplified using primers AY-9 and AY-10, and *mCherry* from pBT1-proD-mCherry (Addgene plasmid #65823, gift from Xiaoxia Lin, University of Michigan) using primers AY-7 and AY-8, followed by heat shock transformation into *E. coli* DH5α. pFAST-PETase-trans was assembled using PCR-amplified fragments from pNuc-trans-mCherry (primers OM-5 and OM-6), pFA6a-link-yoEGFP-SpHis5 (primers OM-8 and OM-9) (Addgene plasmid #44836) and pBTK522::FAST-PETase (Lu et al., 2022) (gift from Hal Alper, University of Texas at Austin) (primers OM-1 and OM-2), and was subsequently transformed into *E. coli* DH5α by heat shock. pFAST-PETase-cis was constructed by amplifying pFAST-PETase-trans (primers DE-3124 and DE-3125) (Hamilton et al., 2019) with 60-bp homologous overlaps to the AvrII cut site in pTA-Mob (Strand et al., 2014). The pTA-Mob plasmid (gift from Thomas Hamilton and David Edgell, Western University) was linearised using AvrII (NEB) and combined with the amplified fragment from pFAST-PETase-cis using a modified NEB Hifi Assembly protocol with a 2 h assembly step at 50°C, followed by electroporation into electrocompetent *E. coli* NEB10-β.

Filter-mating conjugation and selection of engineered isolates

An auxotrophic donor strain was constructed by filter-mating *E. coli* NEB10-β (pFAST-PETase-cis) with *E. coli* K-12 (Δ*ilvD*::*FRT*, Δ*galK*::*cfp-bla*, pSAS31, pTGD) (gift from Xiaoxia Lin, University of Michigan) using a procedure similar to that described in Malwade et al. (2017). Transconjugants were selected on Luria-Bertani (LB) plates (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, 15 g/L agar) supplemented with ampicillin (100 μg/mL), gentamycin (50 μg/mL) and kanamycin (50 μg/mL) and were used as donors in filter-mating procedures with wastewater samples.

Untreated wastewater samples were collected from the City of Waterloo wastewater system access point (Wayne Parker lab, University of Waterloo, Ontario, Canada) and were processed the same day. About 50 mL of wastewater were filtered through a sterile 0.40 μm-pore polycarbonate filter (Sigma-Aldrich, Oakville, Ontario, Canada) under vacuum. The filter and its contents were then vortexed in 1 mL of 1× phosphate-buffered saline (PBS; 2.56 g/L $Na_2HPO_4{\cdot}7H_2O$, 8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH_2PO_4) for 1 min. Three washes, consisting of centrifuging for 5 min at 4000 × *g* followed by resuspension in 1× PBS, were then completed. The solution was resuspended to an OD_{600} of 0.5. The donor strain, which was grown overnight from a single colony in LB medium (10 g/L tryptone, 10 g/L NaCl and 5 g/L yeast extract) supplemented with appropriate antibiotics, was diluted 1:50 in LB with appropriate antibiotics and was grown at 37°C with shaking until exponential phase (OD_{600} of ~0.5). The donor culture was then pelleted by centrifugation and resuspended to an OD of 0.5 in 1× PBS. Filter-mating of the donor culture and collected solids from the wastewater sample was completed by adding 100 μL of each onto a sterile 0.4 μm-pore polycarbonate filter placed on R2A agar (0.5 g/L yeast extract, 0.5 g/L proteose peptone no. 3, 0.5 g/L casamino acids, 0.5 g/L glucose, 0.5 g/L soluble starch, 0.3 g/L sodium pyruvate, 2.2 mM KH_2PO_4, 0.2 mM $MgSO_4$ and 15 g/L agar). Mating was allowed to occur for ~24 h before the filters were vortexed in 1 mL of 1× PBS for 1 min, and serial dilutions were plated on M9 glucose agar (2 g/L glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 64 g/L $Na_2HPO_4{\cdot}7H_2O$, 15 g/L KH_2PO_4, 2.5 g/L NaCl, 5.0 g/L NH_4Cl and 15 g/L agar) supplemented with 50 μg/mL gentamycin and 100 μg/mL ampicillin. Plates were incubated at 30°C until transconjugants appeared, which were observed after 1–2 days as red/pink colonies. Colonies that were identified as expressing *mCherry* based on fluorescence at 670 nm (Cy5 channel) using a Chemidoc MP Imaging System (Bio-Rad, Mississauga, Ontario, Canada) were streaked onto M9 glucose plates supplemented with 50 μg/mL gentamycin and 100 μg/mL ampicillin until pure cultures were obtained.

Conjugation efficiency in wastewater conditions

Untreated wastewater samples were collected from the City of Waterloo wastewater system access point (Wayne Parker lab, University of Waterloo) and were processed the same day. The donor strain *E. coli* K-12 (Δ*ilvD*::*FRT*, Δ*galK*::*cfp-bla*, pSAS31, pTGD, pFAST-PETase-cis) was grown from a single colony overnight in LB supplemented with 0.2% glucose and appropriate antibiotics, then diluted 1:50 into fresh LB with glucose and antibiotics. Once the donor strain reached an OD_{600} = 0.4–0.6, 50 mL of donor culture was washed twice in sterile 1× PBS and resuspended in 50 mL of sterile 1× PBS. Then, 5 mL of donor culture were added to either 20 mL of wastewater or 20 mL of wastewater with 10× concentrated solids (prepared by centrifuging 200 mL of wastewater at 2550 × *g* for 15 min and decanting 180 mL of supernatant) in a 50 mL falcon tube, then incubated for 24 h at 30°C with 60 rpm shaking. After 24 h, 1 mL of culture was washed twice in sterile 1× PBS. Serial dilutions of this mixture were then plated on selective M9 glucose plates containing gentamycin and ampicillin and on non-selective M9 glucose plates. The plates were incubated at 30°C for 5 days. Transconjugants were enumerated by counting colonies identified as expressing *mCherry* by visual inspection and by imaging under the Cy5 channel on a Chemidoc MP Imaging System. Total recipients were

enumerated by counting colonies on non-selective plates. Conjugation efficiency was determined as the ratio of transconjugants to the total of recipients plus transconjugants.

Plasmid stability

A single colony was inoculated into 3 mL of LB media and grown at 30°C with shaking. The culture was diluted 1:1000 into 3 mL of fresh growth medium once per day. At selected time points (Days 1, 3, 7, 10 and 14), the culture was serially diluted in sterile 1× PBS and plated on selective (gentamycin at 50 μg/mL and ampicillin at 100 μg/mL) and non-selective LB plates. Plates were incubated at 30°C for 16–24 h, and colony forming units were counted manually on each plate to quantify the cell density.

Protein expression and purification

A single colony was inoculated into LB supplemented with ampicillin (100 μg/mL) and gentamycin (50 μg/mL) and grown overnight at 30°C with shaking. The overnight culture was then diluted 1:50 into 50 mL of LB supplemented with ampicillin and gentamycin. Protein expression was induced with 0.2% arabinose when the OD_{600} of the culture reached 0.6–0.8, after which the cells were cultured for ~20 h at 30°C with shaking. For purification of FAST-PETase from cell extracts, 50 mL of induced culture was centrifuged at 2550 × *g* for 15 min at 4°C and resuspended in 10 mL of cold lysis buffer (20 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 7.4). The cells were incubated on ice with 0.1 mg/mL lysozyme for 30 min. The cells were further lysed by sonication in three 1 min intervals each followed by 1 min cool-down (Qsonica XL-2000, 3.2 mm, 8 W). Cell debris was pelleted at 2550 × *g* for 30 min, and the 6× His-tagged FAST-PETase was purified from the supernatant by Ni-NTA affinity chromatography. To perform this purification, 1 mL of 50% Ni-NTA agarose (Qiagen, Toronto, Ontario, Canada) was equilibrated using 10 bed volumes of wash buffer (same formulation as lysis buffer). The cell lysate was passed through the column, followed by washing twice with 10 bed volumes of wash buffer twice. The bound proteins were eluted using an imidazole step gradient of 50, 75, 100 and 250 mM in five elution steps using two bed volumes of elution buffer (20 mM sodium phosphate, 300 mM NaCl, pH 7.4. Imidazole concentrations in each elution step were 50, 75, 100, 250 and 250 mM, respectively). For purification of FAST-PETase from culture supernatant, 50 mL of induced culture were centrifuged at 2550 × *g* and 4°C for 30 min, and FAST-PETase was purified from the supernatant using the same chromatography procedure described above.

Protein purity for each elution was checked by SDS-PAGE using stain-free imaging on a Chemidoc MP Imaging System. The purified protein from elutions containing the fewest contaminating proteins (typically elution steps 3–5) was concentrated in 1× PBS using a 5 kDa cut-off Vivaspin Centrifugal Concentrator (Sartorius, Oakville, Ontario, Canada). Protein concentrations were determined using the micro-BCA assay (Thermo Fisher Scientific, Mississauga, Ontario, Canada), where protein concentrations were correlated to bovine serum albumin standards at an absorbance of 562 nm in a plate reader.

PET sample preparation

Amorphous PET (aPET) film from GoodFellow (Pittsburgh, Pennsylvania, United States; product ES301445; specification: 0.25 mm thick, 1.300–1.400 g cm^{-3} density, 1.580–1.640 refractive index, 10 0×10^{-13} $cm^3 \cdot cm\, cm^{-2} s^{-1} Pa^{-1}$ permeability to water at 25°C) and post-consumer PET (pcPET) (coffee cup lid No. A626P, Amhil North America, Mississauga, Ontario, Canada) were prepared in circular form (aPET: 7 mm diameter, pcPET: 6 mm diameter) using office hole punchers. The PET discs were washed for 30 min with 1% SDS, 20% ethanol and distilled water, and dried overnight at 50°C. Prior to degradation experiments, individual PET discs were weighed on an analytical balance with an accuracy of ±0.1 mg.

PET degradation assay with purified FAST-PETase

A single, pre-weighed aPET disc was placed in a 2 mL polypropylene microcentrifuge tube with 600 μL of 0.1 M KH_2PO_4-NaOH buffer (pH 8.0) with 3.6 μg of purified protein concentrate (corresponding to 200 nM FAST-PETase). The tube was capped to prevent volatilisation. The sample was incubated at 50°C for 96 h, replenishing with fresh buffer and enzyme solution every 24 h to maximise the degradation rate. The PET samples were subsequently washed with 1% SDS, 20% ethanol and distilled water, dried for 24 h at 50°C and then weighed on an analytical balance to determine weight loss.

PET degradation with culture supernatant

A single colony was inoculated into 10 mL of M9 glycerol (4 g/L glycerol, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 64 g/L $Na_2HPO_4 \cdot 7H_2O$, 15 g/L KH_2PO_4, 2.5 g/L NaCl and 5.0 g/L NH_4Cl) supplemented with ampicillin (100 μg/mL) and gentamycin (100 μg/mL) and grown until saturation at 30°C with shaking. The saturated culture was then diluted 1:50 into 50 mL of M9 glycerol

supplemented with ampicillin and gentamycin. Protein expression was induced with 0.2% arabinose, not induced, or repressed with 0.2% glucose when the OD_{600} of the culture reached 0.3–0.5, after which the cells were cultured for 12–14 h at 30°C with shaking (220 rpm). The supernatant for each culture was collected by centrifuging 50 mL of culture at 2550 × *g* for 60 min at 4°C and stored for the duration of the experiment at 4°C. A single pcPET disc was placed in a 2 mL polypropylene microcentrifuge tube with 2 mL of supernatant. A replicate tube was prepared without PET for comparison to account for any change in absorbance from other compounds. All samples were incubated at 50°C for 120 h. After incubation, samples were centrifuged at 14,000 × *g* for 3 min. The supernatant was transferred to a quartz cuvette, and absorbance at 240 nm was measured using UV-spectrophotometry (Pirillo et al., 2021; Zhong-Johnson et al., 2021), where the spectrophotometer was blanked to 0.1 M KH_2PO_4-NaOH buffer (pH 8). The mean absorbance of samples without PET was subtracted from the absorbance reading of samples that contained PET.

For degradation of whole coffee cup lids, the lids were washed, dried and pre-weighed using the same procedure described in the "PET sample preparation" section. The lids were submerged in individual sealed containers with 150 mL of 0.1 M KH_2PO_4-NaOH buffer (pH 8) containing 1:80 culture supernatant from isolate WW2. The reaction was carried out for 168 h at 50°C. The final mass of the lids was measured after washing and drying using the same procedure described in the "PET sample preparation" section.

Scanning electron microscopy

PET samples were washed with 1% SDS, 20% ethanol and distilled water, then dried overnight at 50°C. The samples were attached to an aluminium stub using double-sided carbon tape, followed by sputter-coating (Polaron Instruments SEM Coating Unit E5100) with gold particles for 2 min at 20 mA in an argon atmosphere (layer thickness ~30 nm). The samples were then imaged by scanning electron microscopy (Tescan VEGA TS-5130) at 20 kV and 1000× magnification.

Differential scanning calorimetry

PET film crystallinity was determined using differential scanning calorimetry. PET film samples (9–11 mg) were loaded into aluminum Tzero pans (TA Instruments, Grimsby, Ontario, Canada; product 901683.901) with a Tzero lid (TA Instruments; product 901671.901) for solid samples. Samples were heated from 40 to 300°C at 10°C min^{-1}, held at 300°C for 1 min, cooled from 300 to 30°C at 10°C min^{-1} and held at 30°C for 1 min in a Q2000 calorimeter (TA Instruments) with an RCS90 refrigerated cooling system. Percentage crystallinity was calculated as:

$$\%\,\text{crystallinity} = \left[\frac{\Delta H_f - \Delta H_c}{\Delta H_f^o}\right] \times 100\,\%$$

where ΔH_f is the enthalpy of fusion at the melting point, ΔH_c is the enthalpy of crystallisation and ΔH_f^o is the enthalpy of fusion for a completely crystalline sample at its equilibrium melting temperature, which is 140 J g^{-1} (Mehta et al., 1978). TA Universal Analysis software was used to calculate ΔH_f and ΔH_c. ΔH_f and ΔH_c were measured by integrating from 110–120 to 150°C and 210 to 260°C, respectively, with a linear baseline. Crystallinity was measured in triplicate for each source of PET.

Statistical analysis

Experiments were performed in biological and technical replicates where mentioned in figure captions. All statistical analyses were performed in Python 3.9 using the SciPy (Virtanen et al., 2020) library (v1.7.3). Welch's one-sided *t*-test was used to compare the means of two groups. $p < 0.05$ was considered as statistically significant.

RESULTS

pFAST-PETase-cis conjugates to bacteria from wastewater

We constructed an IncP RK2-based (Strand et al., 2014) conjugative plasmid, pFAST-PETase-cis (Figure 1A; Figure S1; Data S4), which carries the FAST-PETase gene (Lu et al., 2022) under control of an arabinose-inducible promoter (P_{BAD}) (details in "Experimental Procedures" section). Our design of pFAST-PETase-cis retains the signal peptide SP_{pstu} and 6× His-tag from the backbone pBTK522::FAST-PETase (Lu et al., 2022; Figure 1B). These facilitate the secretion and purification of FAST-PETase. We chose to drive FAST-PETase expression from the P_{BAD} promoter to achieve tuneable gene expression for testing purposes. Moreover, this promoter has been shown to drive expression in a diverse range of gram-negative bacteria (Prior et al., 2010). The plasmid carries a fluorescent marker (*mCherry*) and two antibiotic resistance markers (Amp^R, Gm^R) to facilitate selection and plasmid maintenance.

We used *E. coli* K-12 (Δ*ilvD*::*FRT*, Δ*galK*::*cfp-bla*, pSAS31, pTGD, pFAST-PETase-cis) as conjugative donor. This strain is auxotrophic to isoleucine, leucine and valine, allowing for counter-selection on

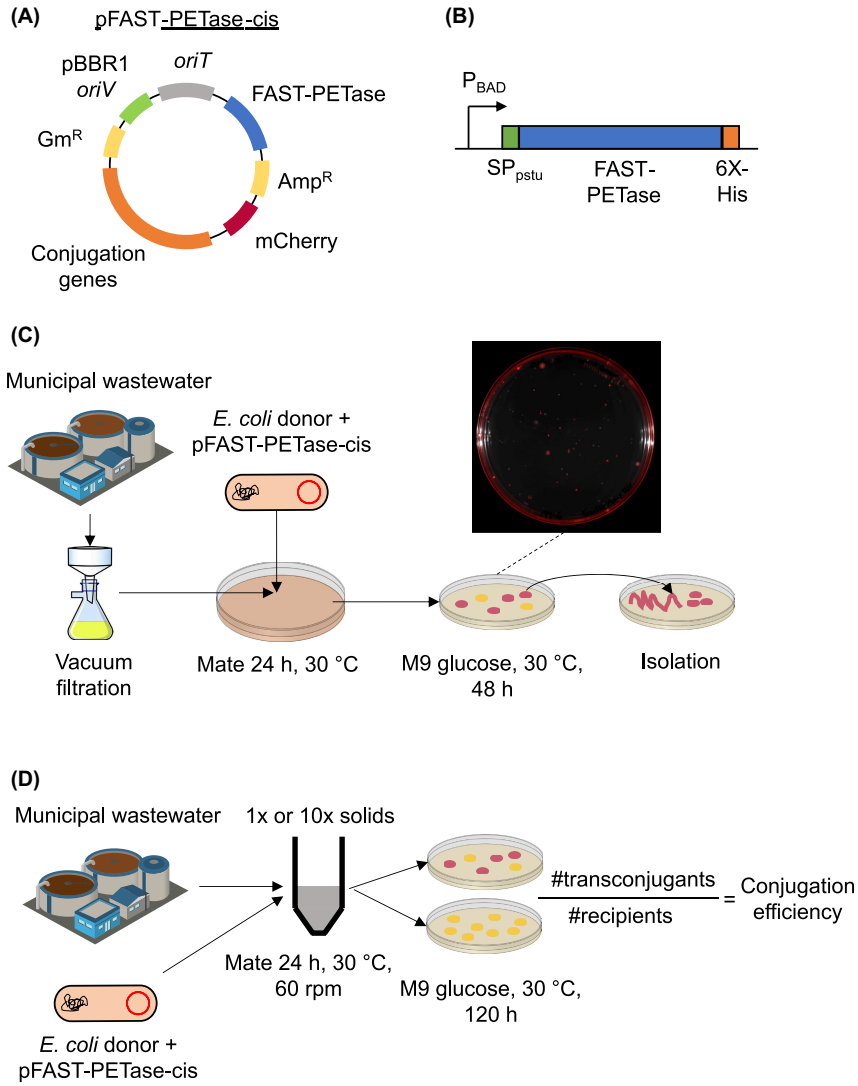

FIGURE 1 Conjugation of pFAST-PETase-cis into wastewater bacteria. (A) Schematic map of pFAST-PETase-cis (not to scale): *oriT*, RK2/RP4 conjugative origin of transfer; FAST-PETase, gene for FAST-PETase enzyme; Amp^R, ampicillin resistance gene (*bla-TEM*$_1$); *mCherry*, gene for fluorescent protein mCherry; conjugation genes, encoding the IncP RK2/RP4 conjugation system; Gm^R, gentamycin resistance gene; pBBR1 *oriV*, plasmid origin of replication. (B) FAST-PETase coding region (not to scale) highlighting the arabinose-inducible promoter (P_{BAD}), signal peptide (SP_{stu}) and 6× His-tag. (C) Experimental procedure for conjugation of pFAST-PETase-cis into bacteria from a wastewater sample. (D) Experimental procedure for measuring conjugation efficiency in wastewater suspension.

minimal media (to facilitate isolation of transconjugants). Untreated wastewater influent was sourced from the City of Waterloo, Ontario, Canada. This water source was chosen because wastewater influent is often a major source of microplastics that enter the environment through wastewater treatment plants (Liu et al., 2021; Sun et al., 2019). In addition, wastewater influent is representative of bacterial diversity inside wastewater treatment plants (Ye & Zhang, 2013). We filter-mated the donor strain with the wastewater sample and picked 32 transconjugant colonies expressing *mCherry* on M9 glucose agar plates supplemented with ampicillin and gentamycin (Figure 1C, details in "Experimental Procedures" section). No *mCherry* expression was detected in a wastewater sample incubated without donors (Figure S2). Based on bacterial internal transcribed spacer (bITS) sequencing (Milani et al., 2020) on all 32 colonies in a pooled sample (performed by Metagenom Bio Life Science, details in Methods S1), we observed that most isolates were *Gammaproteobacteria* from the *Enterobacteriaceae* family (Data S1), with one exception: *Pusillimonas* ssp., a gram-negative bacterium from the *Burkholderiaceae* family. From these picked colonies, we selected seven isolates with distinct colony morphologies to identify using bITS sequencing on each individual colony (Table 1; Data S2) and to investigate for PET degradation activity.

TABLE 1 Selected transconjugant isolates identified by bITS sequencing.

Isolate number	Species
WW2	*Pusillimonas* ssp.
WW5	*Enterobacter* ssp.
WW7	*Escherichia* ssp.
WW17	*Enterobacter* ssp.
WW21	*Enterobacter* ssp.
WW23	*Citrobacter* ssp.
WW24	*Citrobacter* ssp.

To confirm in situ delivery in wastewater conditions, we performed a liquid-mating assay where donors were mixed directly into municipal wastewater influent samples (1× or 10× solids) and allowed to mate for 24 h (Figure 1D). After washing the mating mixture twice in 1× PBS, the mixture was serially diluted and plated onto selective (ampicillin and gentamycin) and non-selective M9 glucose plates, which were incubated for 5 days at 30°C. After counting colonies showing *mCherry* activity, we found that the conjugation efficiency (defined as the ratio of transconjugants to transconjugants plus recipients) was on the order of 1% (1.2% with 10× solids, 1.9% with 1× solids). To confirm that observed colonies were not the donor strain, an exponentially growing donor culture was washed twice in 1× PBS and incubated at 30°C for up to 7 days on selective M9 glucose plates; no growth was observed (Figure S3).

pFAST-PETase-cis is lost in absence of selection

To assess the stability of pFAST-PETase-cis, we serially passaged four different transconjugant species once daily in LB media over the course of 14 days. The number of cells expressing ampicillin- and gentamycin-resistance (which are carried on pFAST-PETase-cis) decreased by 4–6 logs for WW2 (*Pusillimonas* ssp.) and WW7 (*Escherichia* ssp.), whereas it decreased by about one log for WW17 (*Enterobacter* ssp.) and WW23 (*Citrobacter* ssp.) (Figure 2). These results demonstrate that the plasmid is lost in the absence of selection, which highlights the need for additional measures to maintain the FAST-PETase gene in transconjugants.

Purified FAST-PETase expressed from transconjugant isolates degrades PET

Before beginning our activity assays, we used SDS-PAGE to characterise intracellular expression of FAST-PETase (Figure 3A). We then incubated commercial, amorphous PET (aPET) film (crystallinity 8.8 ± 1.0% determined by differential scanning calorimetry, "Experimental Procedures" section) with 3.6 μg of purified protein in 0.1 M KH_2PO_4-NaOH (corresponding to 200 nM FAST-PETase) from cell extracts. As a negative control, we incubated aPET film with the same buffer without FAST-PETase. Significant mass loss occurred for several isolates (Figure 3C) in comparison to the negative control. We then characterised the presence of FAST-PETase in the supernatant by SDS-PAGE (Figure 3D) and incubated an aPET film with 3.6 μg of purified protein from culture supernatant. Again, significant mass loss occurred (Figure 3D) compared to the negative control. Inspection of aPET film samples with scanning electron microscopy (SEM) showed that samples treated with FAST-PETase (Figure 3F) had a pitted surface, which is characteristic of enzymatic degradation (Lu et al., 2022; Yoshida et al., 2016). In contrast, an untreated aPET sample had a smooth surface (Figure 3E).

Culture supernatant containing FAST-PETase degrades post-consumer PET without enzymatic purification

After confirming the effectiveness of FAST-PETase isolated from transconjugants against commercial PET film, we next sought to confirm whether transconjugants could degrade PET without the need for enzymatic purification. FAST-PETase has been previously demonstrated to be effective against numerous post-consumer PET (pcPET) products (Lu et al., 2022), so we switched to using post-consumer PET to simulate a source of PET that is more representative of PET waste encountered in the field. To test whether FAST-PETase produced from the isolates would degrade pcPET without enzymatic purification, we incubated 6 mm hole punches of pcPET from a coffee cup lid (crystallinity 11.4 ± 0.2% determined from differential scanning

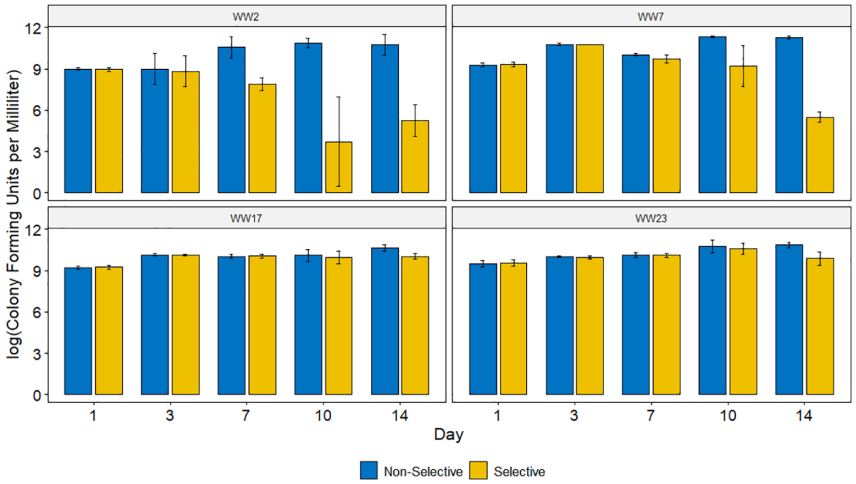

FIGURE 2 Cell density of wastewater transconjugants plated on selective (ampicillin and gentamycin) and non-selective plates over 14 serial passages in LB. Bars show the mean of three biological replicates. Error bars: ±SD.

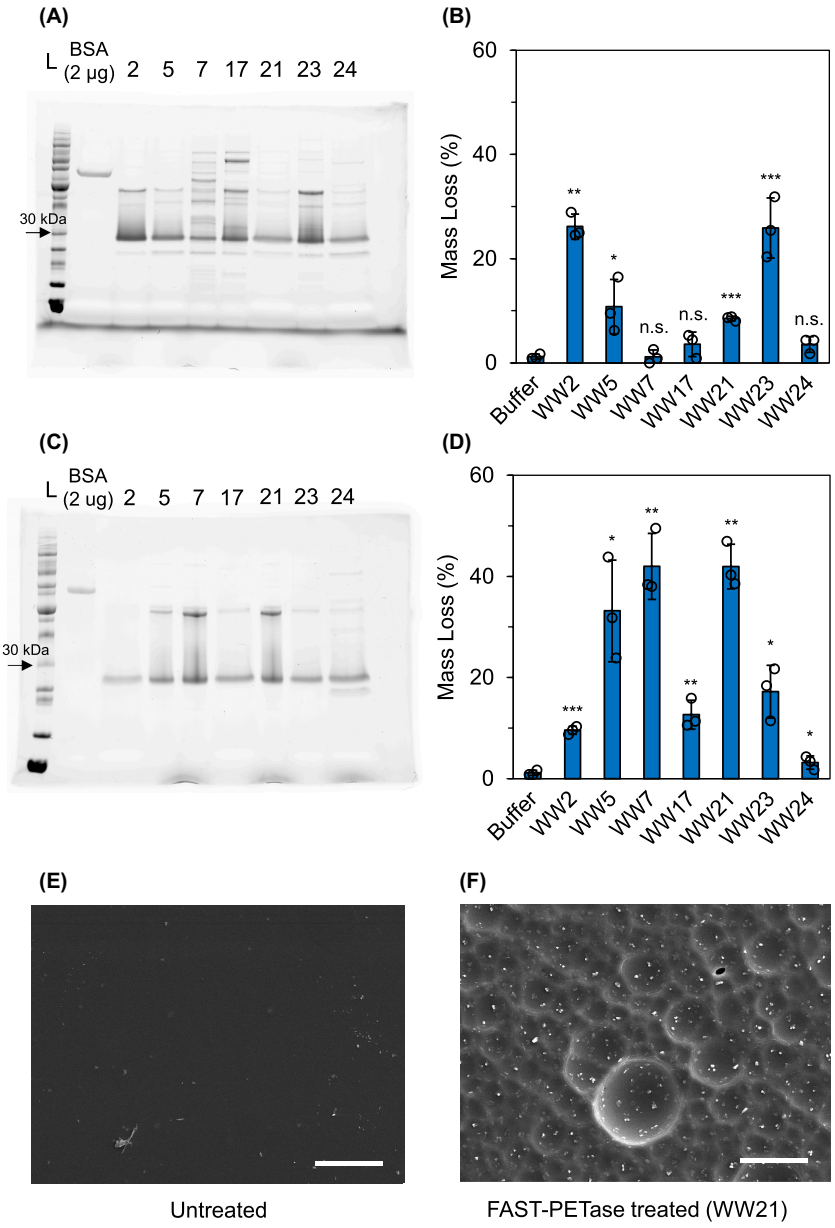

FIGURE 3 Enzymatic degradation of aPET film (7 mm diameter, 0.25 mm thick, ~11 mg) by purified FAST-PETase solution obtained from isolated transconjugants. SDS-PAGE gels of purified FAST-PETase are shown for (A) cell lysates and (C) culture supernatant. L: Protein ladder (PageRuler Unstained Protein Ladder, Thermo Fisher, no. 26614); BSA: Bovine serum albumin; numbers indicate isolate ID. Mass loss from exposure to FAST-PETase purified from (B) cell lysates and (D) supernatant was measured after 96 h incubation at 50°C. Bars show the mean of three technical replicates for each isolate and control. Error bars: ±SD. Statistical analyses were performed using Welch's one-sided *t*-test to compare mass loss of each sample to the negative control that contained no FAST-PETase (Buffer) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (E) Scanning electron microscopy (SEM) image of an untreated PET film. (F) SEM image of a PET film incubated at 50°C for 96 h with FAST-PETase purified from WW21 culture supernatant. Scale bars: 50 µm.

calorimetry, "Experimental Procedures" section) with culture supernatant (M9 glycerol medium) from WW2 (*Pusillimonas* ssp.), WW5 (*Enterobacter* ssp.) and WW7 (*Escherichia* ssp.) that was diluted in 0.1 M KH_2PO_4-NaOH buffer. We were unable to measure substantial mass loss within the accuracy of an analytical balance (accuracy of 0.1 mg; data not shown), so we measured degradation using a spectrophotometric technique ("Experimental Procedures" section) instead, which is more sensitive than gravimetric measurements. The PET degradation byproducts, terephthalic acid (TPA) and mono-(2-hydroxyethyl)terephthalate (MHET), have both been shown to have an absorbance peak around 240 nm (A_{240}) (Zhong-Johnson et al., 2021).

In comparison to samples where FAST-PETase expression was either repressed or not induced, a significant increase in A_{240} occurred in samples exposed to supernatant from cultures where FAST-PETase expression was induced (Figure 4A), indicating enzymatic degradation of the PET sample. Samples showing a significant increase in A_{240} also showed signs of degradation observable with the naked eye (Figure 4B). Further inspection by SEM showed roughening of the plastic surface of PET samples incubated with cultures induced to express FAST-PETase (Figure 4B), which is characteristic of enzymatic degradation of PET (Lu et al., 2022; Yoshida et al., 2016).

We hypothesised that higher enzyme concentration in undiluted culture supernatant would improve the PET degradation rate. We thus followed the same protocol with culture supernatant that had not been diluted in KH_2PO_4-NaOH buffer. Contrary to our hypothesis, we observed minimal activity: there was little to no

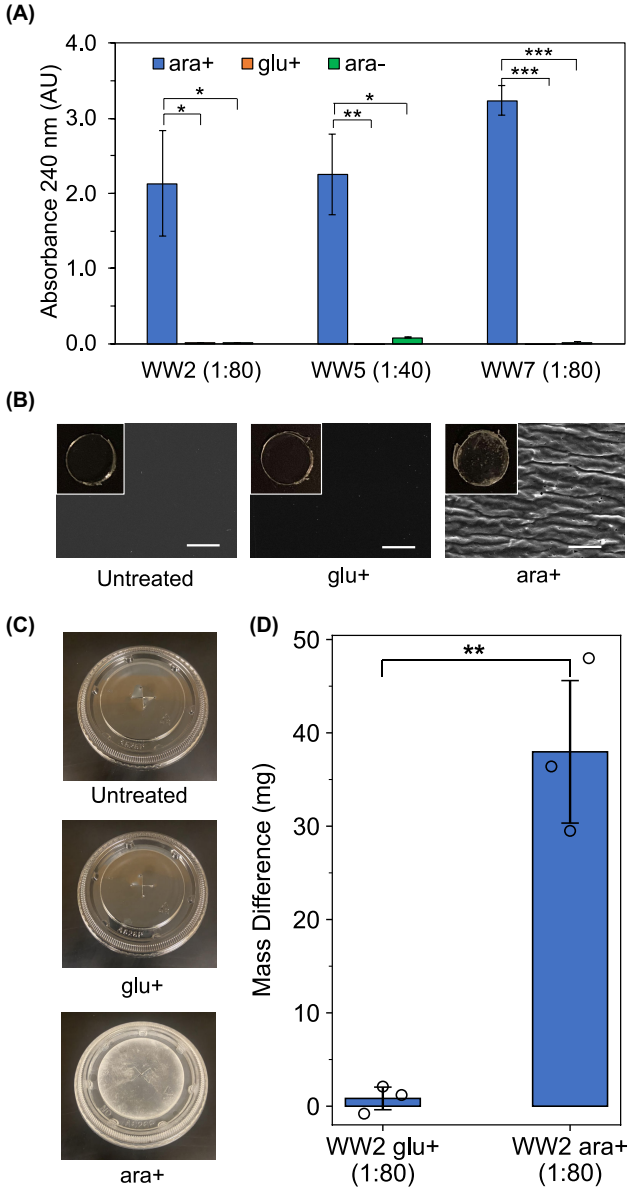

FIGURE 4 Degradation of a coffee cup lid using spent supernatant from engineered wastewater isolates diluted in 0.1 M KH_2PO_4-NaOH buffer (pH 8.0). (A) A_{240} measurements for diluted supernatant incubated with a 6 mm hole punch of pcPET for 120 h at 50°C. ara+: Protein expression induced with 0.2% arabinose; glu+: Protein expression suppressed with 0.2% glucose; ara-: No arabinose added. Bars show a mean of three technical replicates. Error bars: ±SD. (B) Photos (inset) and SEM images of PET samples prior to treatment (left) or incubated in culture supernatant from WW5 (1:40) for 120 h. Scale bars: 50 μm. (C) Coffee cup lid (bottom) shows macroscopic signs of degradation after 168 h incubation at 50°C with spent supernatant (ara+) from WW2 (1:80). An untreated lid (top) and lid incubated with spent supernatant (glu+) from WW2 (1:80) (middle) are shown for comparison. (D) Difference between initial and final mass of coffee cup lids incubated in supernatant from WW2 (1:80) after 168 h incubation at 50°C. Bars show the mean of three technical replicates. Error bars: ±SD. All statistical analyses were performed using Welch's one-sided *t*-test to compare ara+ samples to glu+ and ara- samples (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

increase in A_{240} relative to media where FAST-PETase expression was suppressed (Figure S4). We observed the same negative result when we repeated this experiment with an additional pH adjustment step (addition of 1 M NaOH) to modify the pH of the supernatant from 6.5 to 8.0, which is optimal for FAST-PETase activity (Lu et al., 2022) (Figure S4).

We tested scaling of pcPET degradation by increasing the total reaction volume, allowing an entire coffee cup lid (same pcPET product used in Figure 4A) to incubate in diluted supernatant from WW2 (*Pusillimonas* ssp.) for 168 h at 50°C. A lid that was incubated in supernatant where FAST-PETase expression was induced showed signs of degradation visible to the naked eye (Figure 4C) and significant mass loss (38 ± 9 mg, corresponding to 1.3 ± 0.3% of the initial mass of the lid; Figure 4D). In comparison, a lid incubated in supernatant where FAST-PETase expression was suppressed did not show any visible signs of degradation (Figure 4C) nor appreciable mass loss (Figure 4D).

DISCUSSION

Engineering microbiomes to enable in situ degradation of plastics in diverse ecological niches remains a key milestone for deploying biological solutions for plastic waste at scale (Engineering Biology Research Consortium, 2022). Biotechnological plastic degradation and upcycling can offer a means to achieve a circular life cycle for plastic waste in a sustainable and cost-effective manner, facilitating a broader move towards a circular economy. Developments in biotechnological approaches to plastic waste management will ideally complement related strategies for reduction of single-use plastics and innovations in biodegradable plastics.

In this work, we demonstrated a proof of concept for genetically engineering bacteria from municipal wastewater in situ to degrade PET. We achieved this by using a broad-host-range conjugative plasmid carrying the FAST-PETase gene. Compared to cell bioaugmentation efforts, genetic bioaugmentation bypasses the adaptation barrier of introducing exogenous microbes into a new environment and thus could be used to achieve long-term bioremediation solutions. In particular, an approach focused on degradation of specific plastic compounds could be useful for removing microplastics from the waste streams of plastic manufacturing and recycling facilities, which are a notable source of microplastic output into wastewater (Brown et al., 2023).

The approach demonstrated here may generalise to a variety of ecological niches: several studies have

utilised the RK2/RP4 conjugation machinery coupled with a broad-host-range origin of replication (or a library of such origins) to engineer microbes from diverse environments: soil (Klümper et al., 2015), mammalian guts (Ronda et al., 2019) and marine ecosystems (Goodman et al., 1993). A broad-host-range genetic engineering platform could facilitate the construction of microbial consortia for degradation of individual types of plastic polymers that have known biodegradation pathways (e.g. PET). Such libraries of consortia could eventually be further expanded for degradation of other common plastic polymers, potentially resulting in modular tools for degradation of mixed-waste plastics.

Our results demonstrate that active FAST-PETase released by the engineered isolates can degrade PET without the need for enzymatic purification (Figure 4). The assays using purified enzyme (Figure 3) illustrate the plastic-degrading potential of the engineered isolates, which can guide future optimisation efforts. However, we found that the solution conditions had a substantial effect on FAST-PETase activity. Our preliminary attempts to degrade commercial PET film in LB medium using a purified enzyme solution showed no activity (purified from WW5; data not shown). Furthermore, our attempts at degrading PET using untreated and pH-adjusted supernatant also proved unsuccessful (Figure S4), highlighting that further developments are necessary for effective in situ degradation. It was necessary to dilute the supernatant to achieve significant PET degradation, consistent with previous observations that FAST-PETase activity is inhibited at high enzyme concentrations (Avilan et al., 2023) and acidic conditions (Lu et al., 2022). We found that the secreted enzyme fraction performed better than the intracellular enzyme fraction at similar dosages (e.g. WW5 and WW7 in Figure 3B,D), with a lower abundance of contaminating proteins in the purified secreted fraction compared to the intracellular fraction (Figure 3A,C), suggesting that the presence of other proteins in the medium may hinder FAST-PETase activity. Improved activity could be achieved by a design in which expressed FAST-PETase is anchored to the cell surface; immobilising PETase enzymes has been shown to improve the stability and activity of the enzyme (Chen et al., 2020), including under simulated wastewater conditions (Zhu et al., 2022; Zurier & Goddard, 2022). Further optimisation of enzyme activity could be achieved by addressing environmental factors such as pH, temperature and biotic factors such as other secreted enzymes and metabolic waste products.

Alongside improving operating conditions, PET degradation efficiency could be enhanced by improving the genetic vector design. In pFAST-PETase-cis, FAST-PETase expression was regulated by the inducible P_{BAD} promoter for testing purposes. Regulating expression using a constitutive promoter could enable autonomous plastic degradation. Alternatively, expression induced by specific environmental conditions could be employed to minimise metabolic load and avoid unintended plastic degradation in, for example, products in use and infrastructure. Optimisation of ribosome-binding sites, transcriptional terminators and screening of signal peptides in members of the microbiome of interest could likewise prove beneficial (Low et al., 2013; Xu et al., 2019).

To achieve complete conversion of PET to its constituents, TPA and ethylene glycol (EG), the PETase activity investigated here could be complemented by a MHETase enzyme, which would enable conversion of the depolymerisation product, MHET, into TPA and EG; alternatively, MHETase might be present in the environment (produced by, e.g. *I. sakaiensis* (Yoshida et al., 2016)). The turnover rate of wild-type MHETase from *I. sakaiensis* (k_{cat} ~ 25 s^{-1} at 30°C) (Knott et al., 2020) is much higher than that of FAST-PETase (k_{cat} ~ 0.016 s^{-1} at 50°C),[1] so depolymerisation by PETase will likely be the rate-limiting step for the conversion of PET into TPA and EG. Moreover, MHETase has been observed to improve PETase activity by relieving product inhibition caused by MHET monomers (Erickson et al., 2022; Knott et al., 2020). Recently, a MHETase that is thermostable at the optimum operating temperature for FAST-PETase has been designed (Zhang et al., 2023), offering an attractive dual-enzyme system for applications in PET biodegradation and upcycling. If production of large quantities of TPA and EG poses ecological or environmental concerns, bioaugmentation of additional consortia could be considered, using, for example the consortium designed by Bao et al. (2023) for efficient conversion of TPA and EG into biomass.

Using *E. coli* as a donor strain, we found that pFAST-PETase-cis could conjugate on a filter-mating setup and in native wastewater conditions at efficiencies high enough to allow isolation of transconjugants by plating. Improvements in conjugation efficiency could be achieved by exploring alternative donor strains, conjugation systems and plasmid incompatibility classes; working towards applications, such efforts would need to account for environmental conditions (e.g. pH, temperature, seasonal variability). Moreover, the concentration of suspended solids (not measured) could affect conjugation efficiency by increasing the total surface area available for attachment, promoting spatial proximity between donor and recipient bacteria (Hamilton et al., 2019). We observed low taxonomic diversity among the transconjugant isolates, likely in part due to our reliance on *mCherry* expression, driven by the P_{bs} promoter

[1]Converted from the specific activity of 348 $\mu mol_{TPAeq}\, h^{-1}\, mg_{enzyme}^{-1}$ (Arnal et al., 2023).

whose activity has only been previously reported in *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* (Yang et al., 2018). Moreover, it is possible that conjugation efficiency from *E. coli* is higher towards closely related species (Stewart & Levin, 1977). Finally, the laboratory growth conditions likely had an impact on the results; reduced diversity of transconjugants has been reported in mating conditions that are less conducive to growth of diverse species (Ronda et al., 2019). Additional improvements for selection of the plasmid could be made by using a promoter library for selection markers and origins of replication (e.g. Ronda et al., 2019). Such efforts could target specific hosts that might be best suited to application (e.g. non-pathogenic strains known to be abundant in the conditions of interest).

We observed that pFAST-PETase-cis was lost in the absence of selection to varying extents based on the host species (Figure 2). This could be attributed to copy number variance arising from differences in host-encoded replication and regulatory proteins, plasmid oligomer formation in recombination-proficient hosts, and differences in metabolic load (Summers, 1991). Nonetheless, our observations highlight that long-term bioremediation applications will require efforts towards improving genetic stability. Improved maintenance of the function of interest could be achieved by incorporating plasmid maintenance systems such as toxin-antitoxin systems (Kroll et al., 2010; Lin et al., 2023), gene entanglement (Blazejewski et al., 2019; Chlebek et al., 2023) and plasmid partitioning systems (Danino et al., 2015) into the plasmid design, or by integrating the gene of interest into the chromosome of the host (Ronda et al., 2019). Chromosomal integration of the genetic cargo could be further constrained to non-pathogenic species by using species- and site-specific gene-editing tools (Rubin et al., 2022). Complementary selection efforts may be required to ensure long-term in situ maintenance of engineered strains (Raper et al., 2018).

Deployment of genetically engineered microorganisms for degrading plastics will require careful consideration of environmental and public safety and adhere to regional regulatory policies. To date, there have been few pilot-scale or field studies utilising genetic bioaugmentation for bioremediation purposes (Ripp et al., 2000; Sayler & Ripp, 2000; Venkata Mohan et al., 2009). One notable case was a field release in Estonia in 1989 of *Pseudomonas putida* cultures carrying a gene for metabolising phenol into a watershed contaminated from a phenol release caused by a subterranean oil shale mine fire (Peters et al., 1997). Six years after the release, the introduced operon was still found to be present in native microbiota (Peters et al., 1997). This study suggests that an introduced functionality could persist long-term in the environment if under selection for metabolism of an abundant pollutant. It will be prudent to test the use of engineered microbes for plastic degradation in contained systems while simultaneously assessing ecological risk, including: characterising the modified microorganisms and nature of the genetic modification, tracking the fate of these microorganisms and the introduced genes of interest, assessing the environmental impact of the release, and monitoring the effects of the release on non-target microorganisms (Gustafsson & Jansson, 1993). Genetic biocontainment of the genetic vector using conditional lethality, conditional genetic regulation, and conditional fitness control could help prevent unintended escape of the modified microorganisms (Lee et al., 2018). In wastewater treatment plants, disinfection through tertiary treatment operations could also offer an additional means of biocontainment. Moreover, the controlled environments in wastewater treatment units offer opportunities to assess ecological risks and explore the performance of in situ microplastic degradation at scale.

AUTHOR CONTRIBUTIONS

Aaron Yip: Conceptualization; investigation; writing – original draft; methodology; validation; visualization; writing – review and editing; project administration; funding acquisition. **Owen D. McArthur:** Methodology; investigation. **Kalista C. Ho:** Methodology. **Marc G. Aucoin:** Writing – review and editing; conceptualization; project administration; resources; supervision; funding acquisition. **Brian P. Ingalls:** Supervision; writing – review and editing; conceptualization; project administration; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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REFERENCES

Albright, M.B.N., Louca, S., Winkler, D.E., Feeser, K.L., Haig, S.J., Whiteson, K.L. et al. (2022) Solutions in microbiome engineering: prioritizing barriers to organism establishment. *The ISME Journal*, 16, 331–338.

Andrady, A.L. & Neal, M.A. (2009) Applications and societal benefits of plastics. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, 364, 1977–1984.

Arnal, G., Anglade, J., Gavalda, S., Tournier, V., Chabot, N., Bornscheuer, U.T. et al. (2023) Assessment of four engineered PET degrading enzymes considering large-scale industrial applications. *ACS Catalysis*, 13, 13156–13166.

Avilan, L., Lichtenstein, B.R., König, G., Zahn, M., Allen, M.D., Oliveira, L. et al. (2023) Concentration-dependent inhibition of mesophilic PETases on poly(ethylene terephthalate) can be eliminated by enzyme engineering. *ChemSusChem*, 16, e202202277.

Bao, T., Qian, Y., Xin, Y., Collins, J.J. & Lu, T. (2023) Engineering microbial division of labor for plastic upcycling. *Nature Communications*, 14, 5712.

Bathe, S., Mohan, T.V.K., Wuertz, S. & Hausner, M. (2004) Bioaugmentation of a sequencing batch biofilm reactor by horizontal gene transfer. *Water Science and Technology*, 49, 337–344.

Bell, E.L., Smithson, R., Kilbride, S., Foster, J., Hardy, F.J., Ramachandran, S. et al. (2022) Directed evolution of an efficient and thermostable PET depolymerase. *Nature Catalysis*, 5, 673–681.

Blazejewski, T., Ho, H.-I. & Wang, H.H. (2019) Synthetic sequence entanglement augments stability and containment of genetic information in cells. *Science*, 365, 595–598.

Borrelle, S.B., Ringma, J., Law, K.L., Monnahan, C.C., Lebreton, L., McGivern, A. et al. (2020) Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution. *Science*, 369, 1515–1518.

Brandon, A.M., Gao, S.H., Tian, R., Ning, D., Yang, S.S., Zhou, J. et al. (2018) Biodegradation of polyethylene and plastic mixtures in mealworms (larvae of *Tenebrio molitor*) and effects on the gut microbiome. *Environmental Science & Technology*, 52, 6526–6533.

Brown, E., MacDonald, A., Allen, S. & Allen, D. (2023) The potential for a plastic recycling facility to release microplastic pollution and possible filtration remediation effectiveness. *Journal of Hazardous Materials Advances*, 10, 100309.

Chamas, A., Moon, H., Zheng, J., Qiu, Y., Tabassum, T., Jang, J.H. et al. (2020) Degradation rates of plastics in the environment. *ACS Sustainable Chemistry & Engineering*, 8, 3494–3511.

Chattopadhyay, I. (2022) Role of microbiome and biofilm in environmental plastic degradation. *Biocatalysis and Agricultural Biotechnology*, 39, 102263.

Chen, Z., Wang, Y., Cheng, Y., Wang, X., Tong, S., Yang, H. et al. (2020) Efficient biodegradation of highly crystallized polyethylene terephthalate through cell surface display of bacterial PETase. *Science of the Total Environment*, 709, 136138.

Chettri, D., Verma, A.K. & Verma, A.K. (2023) Bioaugmentation: an approach to biological treatment of pollutants. *Biodegradation*, 35, 117–135.

Chlebek, J.L., Leonard, S.P., Kang-Yun, C., Yung, M.C., Ricci, D.P., Jiao, Y. et al. (2023) Prolonging genetic circuit stability through adaptive evolution of overlapping genes. *Nucleic Acids Research*, 51, 7094–7108.

Cui, Y., Chen, Y., Liu, X., Dong, S., Tian, Y., Qiao, Y. et al. (2021) Computational redesign of a PETase for plastic biodegradation under ambient condition by the GRAPE strategy. *ACS Catalysis*, 11, 1340–1350.

Danino, T., Prindle, A., Kwong, G.A., Skalak, M., Li, H., Allen, K. et al. (2015) Programmable probiotics for detection of cancer in urine. *Science Translational Medicine*, 7, 289ra84.

Engineering Biology Research Consortium. (2022) *Engineering Biology for Climate & Sustainability: A Research Roadmap for a Cleaner Future*. https://roadmap.ebrc.org/engineering-biology-for-climate-sustainability/

Erickson, E., Shakespeare, T.J., Bratti, F., Buss, B.L., Graham, R., Hawkins, M.K.A. et al. (2022) Comparative performance of PETase as a function of reaction conditions, substrate properties, and product accumulation. *ChemSusChem*, 15, e202101932.

French, K.E., Zhou, Z. & Terry, N. (2020) Horizontal 'gene drives' harness indigenous bacteria for bioremediation. *Scientific Reports*, 10, 15091.

Gao, R. & Sun, C. (2021) A marine bacterial community capable of degrading poly(ethylene terephthalate) and polyethylene. *Journal of Hazardous Materials*, 416, 125928.

Garbisu, C., Garaiyurrebaso, O., Epelde, L., Grohmann, E. & Alkorta, I. (2017) Plasmid-mediated bioaugmentation for the bioremediation of contaminated soils. *Frontiers in Microbiology*, 8, 1966.

Geyer, R., Jambeck, J.R. & Law, K.L. (2017) Production, use, and fate of all plastics ever made. *Science*, 3, e1700782.

Goodman, A.E., Hild, E., Marshall, K.C. & Hermansson, M. (1993) Conjugative plasmid transfer between bacteria under simulated marine oligotrophic conditions. *Applied and Environmental Microbiology*, 59, 1035–1040.

Gustafsson, K. & Jansson, J.K. (1993) Ecological risk assessment of the deliberate release of genetically modified microorganisms. *Ambio*, 22, 236–242.

Hamilton, T.A., Pellegrino, G.M., Therrien, J.A., Ham, D.T., Bartlett, P.C., Karas, B.J. et al. (2019) Efficient inter-species conjugative transfer of a CRISPR nuclease for targeted bacterial killing. *Nature Communications*, 10, 4544.

Jeon, H.J. & Kim, M.N. (2016) Isolation of mesophilic bacterium for biodegradation of polypropylene. *International Biodeterioration and Biodegradation*, 115, 244–249.

Ke, Z., Wang, S., Zhu, W., Zhang, F., Qiao, W., Jiang, J. et al. (2022) Genetic bioaugmentation with triclocarban-catabolic plasmid effectively removes triclocarban from wastewater. *Environmental Research*, 214, 113921.

Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J. et al. (2015) Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. *The ISME Journal*, 9, 934–945.

Knott, B.C., Erickson, E., Allen, M.D., Gado, J.E., Graham, R., Kearns, F.L. et al. (2020) Characterization and engineering of a two-enzyme system for plastics depolymerization. *Proceedings of the National Academy of Sciences*, 117, 25476–25485.

Kroll, J., Klinter, S., Schneider, C., Voß, I. & Steinbüchel, A. (2010) Plasmid addiction systems: perspectives and applications in biotechnology. *Microbial Biotechnology*, 3, 634–657.

Kumar, V., Sharma, N., Duhan, L., Pasrija, R., Thomas, J., Umesh, M. et al. (2023) Microbial engineering strategies for synthetic microplastics clean up: a review on recent approaches. *Environmental Toxicology and Pharmacology*, 98, 104045.

Lau, W.W.Y., Shiran, Y., Bailey, R.M., Cook, E., Stuchtey, M.R., Koskella, J. et al. (2020) Evaluating scenarios toward zero plastic pollution. *Science*, 369, 1455–1461.

Lee, J.W., Chan, C.T.Y., Slomovic, S. & Collins, J.J. (2018) Next-generation biocontainment systems for engineered organisms. *Nature Chemical Biology*, 14, 530–537.

Li, T., Menegatti, S. & Crook, N. (2023) Breakdown of polyethylene therepthalate microplastics under saltwater conditions using engineered vibrio natriegens. *AICHE Journal*, 69, e18228.

Lin, J., Guo, Y., Yao, J., Tang, K. & Wang, X. (2023) Applications of toxin-antitoxin systems in synthetic biology. *Engineering Microbiology*, 3, 100069.

Liu, F., Wang, T., Yang, W., Zhang, Y., Gong, Y., Fan, X. et al. (2023) Current advances in the structural biology and molecular engineering of PETase. *Frontiers in Bioengineering and Biotechnology*, 11, 1263996.

Liu, W., Zhang, J., Liu, H., Guo, X., Zhang, X., Yao, X. et al. (2021) A review of the removal of microplastics in global wastewater treatment plants: characteristics and mechanisms. *Environment International*, 146, 106277.

Low, K.O., Muhammad Mahadi, N. & Md. Illias, R. (2013) Optimisation of signal peptide for recombinant protein secretion in bacterial hosts. *Applied Microbiology and Biotechnology*, 97, 3811–3826.

Lu, H., Diaz, D.J., Czarnecki, N.J., Zhu, C., Kim, W., Shroff, R. et al. (2022) Machine learning-aided engineering of hydrolases for PET depolymerization. *Nature*, 604, 662–667.

Ma, H., Zhao, Y., Yang, K., Wang, Y., Zhang, C. & Ji, M. (2022) Application oriented bioaugmentation processes: mechanism, performance improvement and scale-up. *Bioresource Technology*, 344, 126192.

Maheswaran, B., al-Ansari, M., al-Humaid, L., Sebastin Raj, J., Kim, W., Karmegam, N. et al. (2023) In vivo degradation of polyethylene terephthalate using microbial isolates from plastic polluted environment. *Chemosphere*, 310, 136757.

Malwade, A., Nguyen, A., Sadat-Mousavi, P. & Ingalls, B.P. (2017) Predictive modeling of a batch filter mating process. *Frontiers in Microbiology*, 8, 461.

Mehta, A., Gaur, U. & Wunderlich, B. (1978) Equilibrium melting parameters of poly(ethylene terephthalate). *Journal of Polymer Science Polymer Physics Edition*, 16, 289–296.

Milani, C., Alessandri, G., Mangifesta, M., Mancabelli, L., Lugli, G.A., Fontana, F. et al. (2020) Untangling species-level composition of complex bacterial communities through a novel metagenomic approach. *mSystems*, 5, e00404-20.

OECD. (2022) *Global plastics outlook: policy scenarios to 2060*. Paris: Organisation for Economic Co-operation and Development.

Peters, M., Heinaru, E., Talpsep, E., Wand, H., Stottmeister, U., Heinaru, A. et al. (1997) Acquisition of a deliberately introduced phenol degradation operon, pheBA, by different indigenous pseudomonas species. *Applied and Environmental Microbiology*, 63, 4899–4906.

Pirillo, V., Pollegioni, L. & Molla, G. (2021) Analytical methods for the investigation of enzyme-catalyzed degradation of polyethylene terephthalate. *The FEBS Journal*, 288, 4730–4745.

Prior, J.E., Lynch, M.D. & Gill, R.T. (2010) Broad-host-range vectors for protein expression across gram negative hosts. *Biotechnology and Bioengineering*, 106, 326–332.

Qi, X., Ma, Y., Chang, H., Li, B., Ding, M. & Yuan, Y. (2021) Evaluation of PET degradation using artificial microbial consortia. *Frontiers in Microbiology*, 12, 778828.

Raper, E., Stephenson, T., Anderson, D.R., Fisher, R. & Soares, A. (2018) Industrial wastewater treatment through bioaugmentation. *Process Safety and Environment Protection*, 118, 178–187.

Ren, C., Wang, Y., Tian, L., Chen, M., Sun, J. & Li, L. (2018) Genetic bioaugmentation of activated sludge with dioxin-catabolic plasmids harbored by *Rhodococcus* sp. strain p52. *Environmental Science & Technology*, 52, 5339–5348.

Ripp, S., Nivens, D.E., Ahn, Y., Werner, C., Jarrell, J., Easter, J.P. et al. (2000) Controlled field release of a bioluminescent genetically engineered microorganism for bioremediation process monitoring and control. *Environmental Science & Technology*, 34, 846–853.

Roberts, C., Edwards, S., Vague, M., León-Zayas, R., Scheffer, H., Chan, G. et al. (2020) Environmental consortium containing pseudomonas and bacillus species synergistically degrades polyethylene terephthalate plastic. *mSphere*, 5, e01151-20. Available from: https://doi.org/10.1128/msphere.01151-20

Ronda, C., Chen, S.P., Cabral, V., Yaung, S.J. & Wang, H.H. (2019) Metagenomic engineering of the mammalian gut microbiome in situ. *Nature Methods*, 16, 167–170.

Rubin, B.E., Diamond, S., Cress, B.F., Crits-Christoph, A., Lou, Y.C., Borges, A.L. et al. (2022) Species- and site-specific genome editing in complex bacterial communities. *Nature Microbiology*, 7, 34–47.

Sayler, G.S. & Ripp, S. (2000) Field applications of genetically engineered microorganisms for bioremediation processes. *Current Opinion in Biotechnology*, 11, 286–289.

Sekhar, V.C., Nampoothiri, K.M., Mohan, A.J., Nair, N.R., Bhaskar, T. & Pandey, A. (2016) Microbial degradation of high impact polystyrene (HIPS), an e-plastic with decabromodiphenyl oxide and antimony trioxide. *Journal of Hazardous Materials*, 318, 347–354.

Shah, A.A., Hasan, F., Akhter, J.I., Hameed, A. & Ahmed, S. (2008) Degradation of polyurethane by novel bacterial consortium isolated from soil. *Annales de Microbiologie*, 58, 381–386.

Son, H.F., Cho, I.J., Joo, S., Seo, H., Sagong, H.Y., Choi, S.Y. et al. (2019) Rational protein engineering of thermo-stable PETase from *Ideonella sakaiensis* for highly efficient PET degradation. *ACS Catalysis*, 9, 3519–3526.

Stewart, F.M. & Levin, B.R. (1977) The population biology of bacterial plasmids: a priori conditions for the existence of conjugationally transmitted factors. *Genetics*, 87, 209–228.

Strand, T.A., Lale, R., Degnes, K.F., Lando, M. & Valla, S. (2014) A new and improved host-independent plasmid system for RK2-based conjugal transfer. *PLoS One*, 9, e90372.

Summers, D.K. (1991) The kinetics of plasmid loss. *Trends in Biotechnology*, 9, 273–278.

Sun, J., Dai, X., Wang, Q., van Loosdrecht, M.C.M. & Ni, B.-J. (2019) Microplastics in wastewater treatment plants: detection, occurrence and removal. *Water Research*, 152, 21–37.

Top, E.M., Springael, D. & Boon, N. (2002) Catabolic mobile genetic elements and their potential use in bioaugmentation of polluted soils and waters. *FEMS Microbiology Ecology*, 42, 199–208.

Venkata Mohan, S., Falkentoft, C., Venkata Nancharaiah, Y., Sturm, B.S.M.S., Wattiau, P., Wilderer, P.A. et al. (2009) Bioaugmentation of microbial communities in laboratory and pilot scale sequencing batch biofilm reactors using the TOL plasmid. *Bioresource Technology*, 100, 1746–1753.

Virtanen, P., Gommers, R., Oliphant, T.E., Haberland, M., Reddy, T., Cournapeau, D. et al. (2020) SciPy 1.0: fundamental algorithms for scientific computing in python. *Nature Methods*, 17, 261–272.

Wang, N., Guan, F., Lv, X., Han, D., Zhang, Y., Wu, N. et al. (2020) Enhancing secretion of polyethylene terephthalate hydrolase PETase in *Bacillus subtilis* WB600 mediated by the SPamy signal peptide. *Letters in Applied Microbiology*, 71, 235–241.

Wang, W., Gao, H., Jin, S., Li, R. & Na, G. (2019) The ecotoxicological effects of microplastics on aquatic food web, from primary

producer to human: a review. *Ecotoxicology and Environmental Safety*, 173, 110–117.
Xu, N., Wei, L. & Liu, J. (2019) Recent advances in the applications of promoter engineering for the optimization of metabolite biosynthesis. *World Journal of Microbiology and Biotechnology*, 35, 33.
Xu, S., Ma, J., Ji, R., Pan, K. & Miao, A.-J. (2020) Microplastics in aquatic environments: occurrence, accumulation, and biological effects. *Science of the Total Environment*, 703, 134699.
Yan, F., Wei, R., Cui, Q., Bornscheuer, U.T. & Liu, Y.-J. (2021) Thermophilic whole-cell degradation of polyethylene terephthalate using engineered clostridium thermocellum. *Microbial Biotechnology*, 14, 374–385.
Yang, S., Liu, Q., Zhang, Y., du, G., Chen, J. & Kang, Z. (2018) Construction and characterization of broad-spectrum promoters for synthetic biology. *ACS Synthetic Biology*, 7, 287–291.
Ye, L. & Zhang, T. (2013) Bacterial communities in different sections of a municipal wastewater treatment plant revealed by 16S rDNA 454 pyrosequencing. *Applied Microbiology and Biotechnology*, 97, 2681–2690.
Yoshida, S., Hiraga, K., Takehana, T., Taniguchi, I., Yamaji, H., Maeda, Y. et al. (2016) A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science*, 351, 1196–1199.
Yuan, Z., Nag, R. & Cummins, E. (2022) Human health concerns regarding microplastics in the aquatic environment—from marine to food systems. *Science of the Total Environment*, 823, 153730.
Zhang, J., Wang, H., Luo, Z., Yang, Z., Zhang, Z., Wang, P. et al. (2023) Computational design of highly efficient thermostable MHET hydrolases and dual enzyme system for PET recycling. *Communications Biology*, 6, 1–18.
Zhong-Johnson, E.Z.L., Voigt, C.A. & Sinskey, A.J. (2021) An absorbance method for analysis of enzymatic degradation kinetics of poly(ethylene terephthalate) films. *Scientific Reports*, 11, 928.
Zhu, B., Ye, Q., Seo, Y. & Wei, N. (2022) Enzymatic degradation of polyethylene terephthalate plastics by bacterial curli display PETase. *Environmental Science & Technology Letters*, 9, 650–657.
Zurier, H.S. & Goddard, J.M. (2021) Biodegradation of microplastics in food and agriculture. *Current Opinion in Food Science*, 37, 37–44.
Zurier, H.S. & Goddard, J.M. (2022) Directed immobilization of PETase on mesoporous silica enables sustained depolymerase activity in synthetic wastewater conditions. *ACS Applied Bio Materials*, 5, 4981–4992.
Zurier, H.S. & Goddard, J.M. (2023) PETase engineering for enhanced degradation of microplastic fibers in simulated wastewater sludge processing conditions. *ACS ES&T Water*, 3, 2210–2218.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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