Conjugation-dependent 'gene drives' harness indigenous bacteria for bioremediation

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Abstract

Engineering bacteria to clean-up oil spills is rapidly advancing but faces regulatory hurdles and environmental concerns. Here, we develop a new technology to harness indigenous soil microbial communities for bioremediation by flooding local populations with catabolic genes for petroleum hydrocarbon degradation. Overexpressing three enzymes (almA, xylE, p450cam) in *E.coli* led to degradation rates of 60-99% of target hydrocarbon substrates. Mating experiments, fluorescence microscopy and TEM revealed indigenous bacteria could obtain these vectors from *E.coli* through conjugation. Inoculating petroleum-polluted sediments from a former refinery with engineered *E.coli* showed that the *E.coli* die after five days but a variety of bacteria received and carried the vector for over 120 days after inoculation. This approach could prime indigenous bacteria for degrading pollutants while providing minimal ecosystem disturbance.

Keywords: bioremediation, synthetic biology, petroleum hydrocarbons, oil spill, horizontal gene transfer

Introduction

Oil spills in recent decades have left a long-term mark on the environment, ecosystem functioning, and human health (*1–3*). In the Niger Delta alone, the roughly 12,000 spills since the 1970s have left wells contaminated with benzene levels 1000x greater than the safe limit established by the World Health organization and have irreparably damaged native mangrove ecosystems (*4,5*). Continued economic reliance on crude oil and legislation supporting the oil industry mean that the threat of spills is unlikely to go away in the near future (*6*).

At present, there are few solutions to cleaning up oil spills but engineering bacteria to hyper-degrade petroleum could present a viable solution in the near future. Previous studies have identified which bacterial enzymes are involved in petroleum hydrocarbon degradation (reviewed in references *7,8*) and have engineered bacterial enzymes like p450cam for optimal *in vivo* and *in vitro* degradation of single-substrate alkanes under lab conditions (*9,10*). However, there are several critical gaps in or knowledge of engineering bacteria for oil-spill bioremediation. First, we know little about how the performance of these enzymes compare and which enzyme would present an ideal target for over-expression in engineered organisms. Second, it is unclear how well engineered organisms can degrade petroleum hydrocarbons compared to native wild-type bacteria which naturally degrade alkanes, such as *Pseudomonas putida*. Third, the environmental effects of engineered bacteria on native soil populations are unclear. For example, do these bacteria persist over time in contaminated soils? Although the use of genetically modified bacteria in bioremediation is attractive, this solution faces significant regulatory hurdles which prohibit the release of genetically modified organisms in the environment (*11*).

Here, we propose a new bioremediation strategy which combines synthetic biology and microbial ecology and harnesses natural processes of horizontal gene transfer in soil ecosystems. We screened five enzymes involved in petroleum degradation in *E. coli* (alkB, almA, xylE, ndo and p450cam) in *E. coli* to identify 1) where these enzymes localize and their effect on crude oil using advanced microscopy and 2) to asses each enzyme's ability to degrade three petroleum hydrocarbon substrates (crude oil, dodecane, and benzo(a)pyrene) compared to two wild type bacteria (*Pseudomonas putida* and *Cupriavidus* sp. OPK) using bioassays and SPME GC-MS. Based on these results, we selected one vector (pSF-OXB15-p450camfusion) to determine whether these vectors could be transferred to indigenous bacteria found in petroleum-polluted sediments.

Results

Our research suggests that there is a strong connection between enzyme expression pattern and bacterial behavioral responses to crude oil. We inserted five enzymes (alkB, almA, xylE, ndo, and p450cam) and any required electron donors into the vector backbone pSF-OXB15 using Gibson Assembly (*12*) (**SI Fig. 1**). To identify where each enzyme localized within *E. coli*, we tagged each enzyme with a fluorophore (gfp or mcherry). Fluorescence microscopy revealed that alkB was localized to bacterial cell membranes and almA was found throughout the cytoplasm. The camphor-5-monooxygenase camC from the p450cam operon was expressed throughout the cell cytoplasm while another enzyme in the operon, the 5-exo-hydroxycamphor dehydrogenase camD, was expressed within a small compartment at one end of the cell (**Fig. 1A**). The dioxygenase ndoC from the ndo operon was also localized to a small compartment at one end of the cell. The dioxygenase xylE was found in small amounts in the bacterial cell membrane and larger amounts in a microcompartment at one end of the cell. In all cases, these compartments were 115-130 nm wide and could be seen in young, mature and dividing cells. The presence of microcompartments

in *E. coli* expressing p450cam, ndo, and xylE could reflect the known use of protein-based microcompartments by bacteria to concentrate highly reactive metabolic processes (*13*).

Over-expression of all five enzymes imbued *E. coli* with metabolism-dependent chemotactic behavior, where cell movement is driven towards substrates affecting cellular energy levels (*14*). For example, *E. coli* expressing alkB were seen 'clinging' to oil droplets in several cases (**Fig. 1B**) and those expressing xylE seemed to use the compartment-bound enzyme as a 'guide' towards crude oil (**SI Fig. 2**). Both behaviors mimic the interactions of wild-type oil-degrading bacteria (*15*).

Fluorescence microscopy also revealed for the first time the key role of extracellular enzymes in degradation of petroleum hydrocarbons. Three enzymes, alkB, almA, and p450cam were found in extracellular vesicles ranging in size from 0.68 µm to 1.67 µm (**SI Fig. 3**). These vesicles were only seen when *E. coli* was exposed to petroleum hydrocarbons. They are larger than minicells (which range from 200-400 nm in diameter) (*16*) and seem to serve some other function. Confocal images suggest that these vesicles may come into contact with oil droplets, potentially attaching to (or merging with) their surface (**SI Fig 4**).

We also found three enzymes, alkB, xylE, and p450cam, within the *E. coli* exopolysaccharide (EPS) matrix. AlkB and xylE were concentrated around the 500 nm pores within the EPS and found dispersed in smaller amounts throughout the exopolysaccharide. In contrast, p450cam was distributed in high amounts throughout the EPS (**Fig. 1C**). Cryotome sectioning of the EPS from bacteria expressing p450cam indicates that the monooxygenase camA from the p450cam operon co-localized with a second enzyme involved in hydrocarbon degradation, the dehydrogenase camD (**SI Fig. 5**). Protein levels of EPS from bacteria expressing alkB (0.84 ± 0.04 mg/mL), xylE (0.85 ± 0.13 mg/mL), and p450cam (0.97 ± 0.06 mg/mL) were also significantly higher than *E. coli* expressing the empty vector pSF-OXB15 (0.44 ± 0.02 mg/mL) (**SI Fig 6**) and were comparable to the protein level of *Cupriavidus* sp. OPK (1.2 ± 0.15 mg/mL), a bacteria known to use biofilms to degrade crude oil (*15*). Although previous studies suggest that EPS may be involved in the extra-cellular metabolism of environmental pollutants (*17*), this is the first study to identify several enzymes which may play a role in this process.

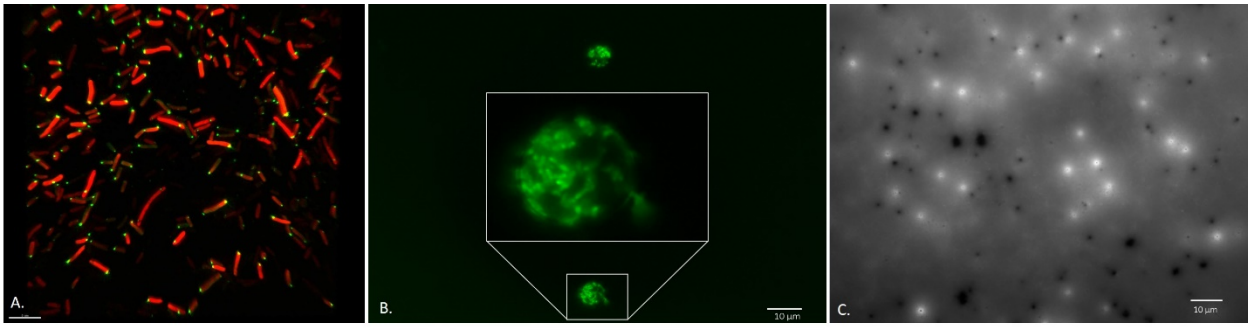

Fig. 1 The role of extracellular enzymes in crude oil degradation. A. Structured Illumination Microscopy (SIM) image of *E.coli* expressing proteins involved in petroleum degradation (cam A,B, C and D) from the CAM plasmid in *E.coli*. camC (fused to mcherry) was found throughout the cell while camD (fused to gfp) localized to a microcompartment at one end of the cell. The scale bar is 5 µm. B. *E. coli* expressing alkB fuse to gfp were found clinging to spheres containing crude oil, mimicking a behavior seen in wild-type oil-degrading bacteria. C. EPS from *E. coli* expressing

xylE. Gfp-tagged xylE were found around small pores (ca. 500 nm) within the EPS matrix. Figures 1B and 1C were taken using the GFP filter on a Zeiss AxioImager M1.

Finally, extracellular enzyme expression also influenced the size of the oil droplets within the cell culture media. For example, *E. coli* expressing xylE produced very small oil droplets (primarily >1µm in diameter) of crude oil while those expressing alkB and almA produced droplets ranging in size from 1 µm-120 µm in diameter (**SI Fig. 7**). Although xylE was not seen in vesicles, confocal suggests that crude oil droplets can flow through pores in biofilms and may become coated in EPS in the process. Potentially, attachment of enzymes to oil droplets (through fusion with vesicles or contact with EPS) may influence how fast a droplet is degraded over time.

To determine which enzymes were most useful for degrading long-chain hydrocarbons, PAHs, and crude oil, we conducted 96-well plate assays exposing wild-type and genetically engineered bacteria to 1% of dodecane, benzo(a)pyrene or crude oil. We found that bacteria engineered to over-express specific enzymes in petroleum degradation were able to degrade single-carbon substrates better than the wild-type bacteria *P. putida* and *Cupriavidus* sp. OPK. ANOVA of the assay data showed that there was significant variation in bacterial growth when exposed to dodecane ($F_{8,31}$ = 33.4, p = <0.001), benzo(a) pyrene ($F_{8,31}$ = 73.03, p = <0.001) and crude oil ($F_{8,31}$ = 240.6, p = <0.001). When exposed to dodecane, *E. coli* expressing p450cam increased in biomass the most (139.4%), followed by *E. coli* expressing xylE (136.3%), alkB (120.8%), and almA (97.6%) (**Fig. 2A**). Expressing p450cam and xylE led to significantly greater conversion of dodecane to biomass compared to *P. putida* (t = 4.71, df = 3.17, p < 0.01 and t = 4.41, df = 3.17, p < 0.01 respectively). SPME GC/MS analysis of these cultures revealed that all three bacteria degraded 99% of dodecane in 10 days. When exposed to benzo(a)pyrene, *P. putida* had the greatest increase in biomass (119.2%) followed by *E. coli* expressing almA (117.1%), xylE (94.8%), and p450cam (90.8%) (**Fig. 2B**). T-tests showed there was no significant difference in the biomass of *P. putida* and these three strains (p > 0.10). SPME GC-MS showed that *E.coli* expressing P450cam, almA and xylE degraded 90%, 97% and 98% of the benzo(a)pyrene respectively while *P. putida* degraded 86%.

In contrast, when engineered and wild-type bacteria were exposed to crude oil, *P. putida* converted the oil to biomass more efficiently, increasing in biomass by 110.9% (**Fig. 2C**). Only two genetically engineered bacteria, *E. coli* expressing p450cam and almA, had comparable increases in biomass to *Cupriavidus* sp. OPK (61.93%, 52%, and 48.7% respectively). The assay was repeated with crude oil stained with Nile Red and rates of degradation were determined according to French and Terry (*15*). *P. putida* degraded 79% of crude oil while *E. coli* expressing p450cam and almA degraded 64% and 60% respectively. *E. coli* expressing alkB, xylE, and ndo only grew ~25% and degraded 35-40% of crude oil. The high performance of p450cam when exposed to crude oil likely reflects the enzyme's known substrate promiscuity (*18,19*) which makes it a better catalyst for degrading crude oil, a complex substrate made of over 1,000 compounds (*20*).

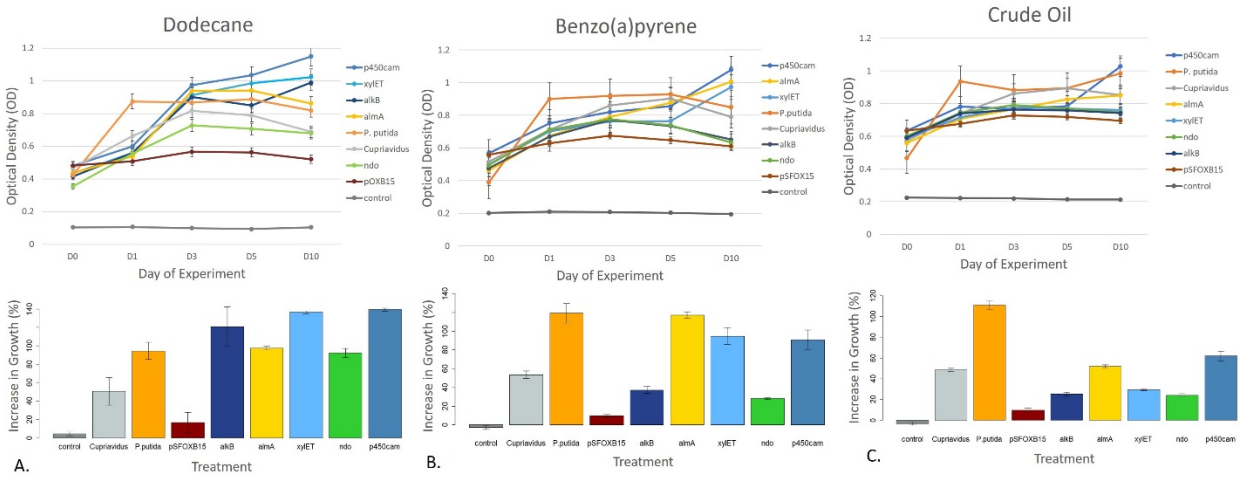

Figure 2 Growth of wild-type and engineered bacteria on dodecane (A), benzo(a)pyrene (B), and crude oil (C). Wild type strains are denoted as '*Cupriavidus*' and '*P. putida*.' Synthetic strains are denoted according to what enzyme they are engineered to express (e.g. alkB, almA). The two negative controls are a control with the carbon substrate but no cells and *E. coli* transformed with the vector backbone used in the experiment (pSFOXB15) but without genes inserted for hydrocarbon degradation.

To determine whether our engineered bacteria could transfer vectors containing petroleum-degrading genes to indigenous soil and marine bacteria, we conducted a series of mating experiments. We found that wild-type bacteria readily received the vector pSF-OXB15-p450camfusion through horizontal gene transfer (HGT) (**SI Fig. 8 and 9**). Rates for transformation ranged from 19 to 84% in 48 hours depending on the recipient species (**SI Table 1**) and were >90% after seven days of incubation for all tested species. Plasmid expression was stable for over three months in the absence of antibiotic pressure. HGT can occur through transformation, transduction, conjugation, transposable elements, and the fusing of outer membrane vesicles (OMVs) from one species to another (*21,22*). HGT is thought to play a role in the degradation of environmental toxins (*23*) but this is the first study to provide evidence for gene transfer between genetically engineered organisms and wild soil microbiota.

Advanced microscopy suggests conjugation is the primary mechanism of vector-transfer between transgenic *E. coli* and wild-type bacteria (*24*). Using fluorescence microscopy, we observed our engineered *E. coli* exhibiting possible mating behavior wild-type bacteria. We initially attempted to use PCR to amplify genes related to conjugation from *E. coli* expressing pSF-OXB15-p450camfusion before and after exposure to wild-type cells. However, PCR products were often 20-100 bp off from expected product size and/or the sequenced products did not match the expected sequence. Conjugative plasmids have high rates of homologous recombination (*25*), which may explain these results. Consequently, we used transmission electron microscopy (TEM) to verify the presence of conjugative pilii. TEM showed *E. coli* expressing p450cam tethered to wild-type cells by conjugative pili over long distances (**Fig 3A**), the formation of mating pair bridges between wild-type cells and *E.coli* (**Fig 3B**), and *E.coli* with conjugative pili (**Fig. 3C**).

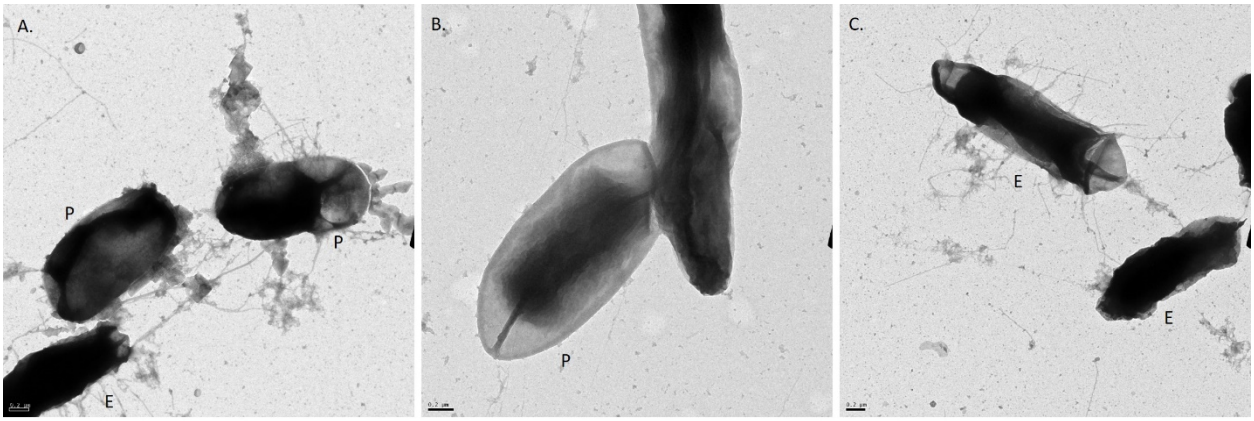

Figure 3 TEM confirmation of conjugation among *E. coli* expressing pSF-OXB15-p450camfusion and *P. putida*. A. *E. coli* (E) connected to *P. putida* (P) via conjugative pili. B. Mating-pair bridge between *P. putida* and *E. coli*. C. *E. coli* with conjugative pili.

To further confirm that conjugation is likely the main mechanism for plasmid exchange, we tested whether wild-type bacteria could take up naked plasmids from cell culture by adding 1 µl of purified plasmid (at a concentration of 1 ng/µl and 10 ng/µl) to LB cultures containing wild-type bacteria. We saw no transformed cells. In addition, neither fluorescence microscopy or TEM showed OMV production or release by the *E.coli* strains created in this study. OMVs are 50-250 nm in diameter (*26*), much smaller than any of the vesicles produced by our strains.

We conducted an additional experiment to determine the survival rate of engineered bacteria in petroleum polluted sediment from a former Shell Pond refinery in Bay Point, CA and whether these genes could be transferred to native, complex soil microbial communities. This sediment is contaminated with high levels of petroleum hydrocarbons, arsenic, heavy metals, and carbon black. At D_0, *E.coli* containing the plasmid pSF-OXB15-p450camfusion were seen in aliquots of contaminated sediment and there were no autofluorescent bacteria visible in the media. After Day_5, the population of *E.coli* had declined. Instead, a number of diverse native soil bacteria now contained the plasmid, a trend which continued over the course of the experiment. Based on morphology, these bacteria belonged to the *Pseudomonas*, *Flavobacteria*, and *Actinomycete* genera (among others). Plating out aliquots of soil at regular time points confirmed the data gathered by microscopy: the number and diversity of bacteria expressing the plasmid increased 40-fold over the 30-day experiment (**Fig. 4A-C**). The spider-silk-like biofilms formed by native soil microbiota present in the soil were also fluorescent (**Fig. 4D**), suggesting the p450cam enzymes also play a role in extracellular degradation of petroleum hydrocarbons under real-world conditions. We left the experiment running and after 90 days bacteria carrying the vector were still prolific (**SI Fig. 10**). This suggests native soil microbial communities will continue to carry vectors with useful catabolic genes, likely until the metabolic cost of replicating the vector no longer presents an advantage.

Our results suggest transgenic bacteria can easily transfer genes to wild-type soil bacteria. Potentially, engineered bacteria could be used in soil-based 'gene drives.' Previous studies have proposed this form of ecological engineering (*27,28*), but the best of our knowledge no studies have shown that such drives would be successful. *E.coli* engineered to carry plasmids containing genes involved in degradation of environmental toxins could be used to augment the capacity of native soil microbial communities to

degrade pollutants of interest. Replacing antibiotic selection markers with chromoprotein ones (*29*) would eliminate the release of antibiotic resistance genes into the environment.

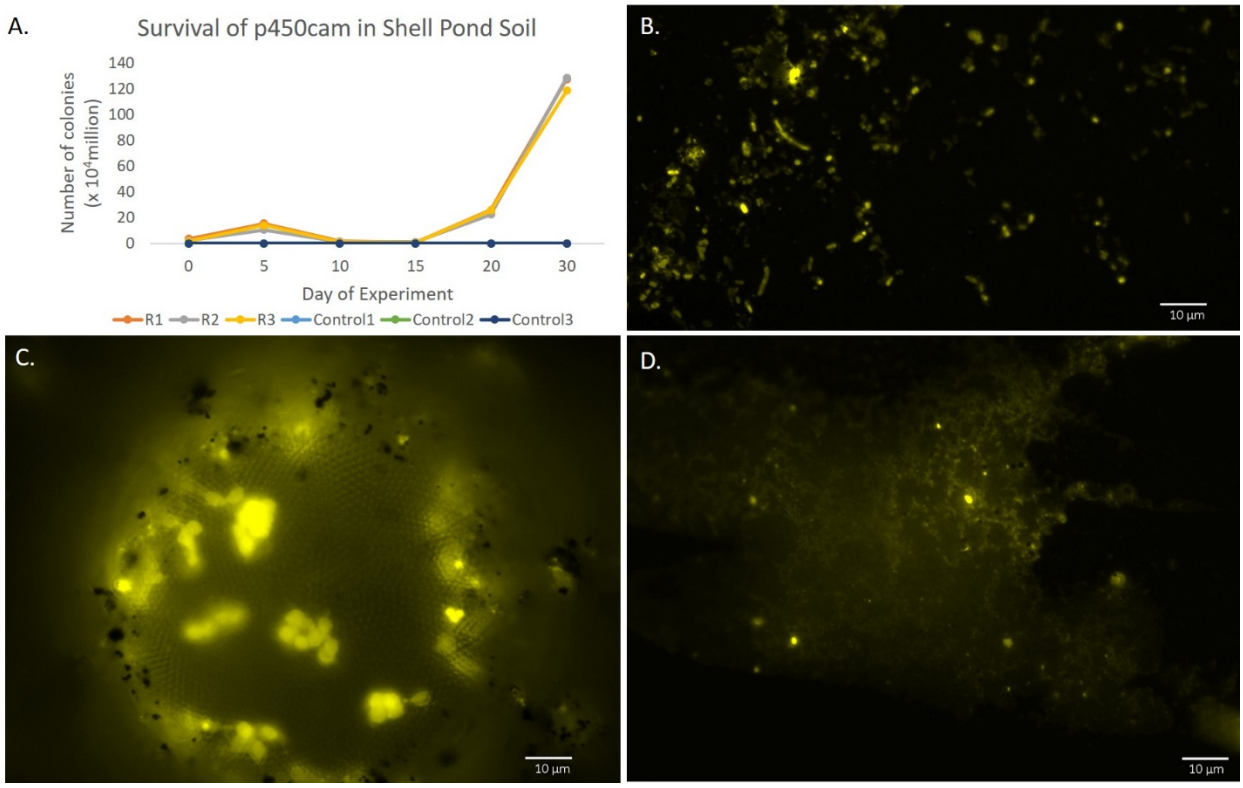

Figure 4 Expression of vector pSF-OXB15-p450cammcherry in soil microbial communities found in sediment from a former Shell Oil refinery (Shell Pond, Bay Point, CA). A. Number of colonies on kanamycin plates from soil aliquots (8 µg) diluted from Shell Pond soil inoculated with *E. coli* DH5α harboring the plasmid pSF-OXB15-p450camfusion (R1-3) or no inoculation (control 1-3). The number of colonies declined from D10-D15 then continued to increase exponentially. B. Aliquot of Shell Pond soil showing diverse bacteria expressing the vector on D_{21}. C. Example of native soil bacteria expressing the vector. These bacteria construct spheres made of polymers in a honey-comb pattern. D. Expression of p450cam tagged with mcherry in biofilm matrices within sediment from Shell Pond. The biofilms form thin nets over large soil particles; small soil particles can be seen as black 'dots' sticking to the biofilm surface. In all microscopy images, mcherry is false-colored yellow. All images were taken using the Texas Red filter on a Zeiss AxioImager M1 (excitation/emission 561/615).

Conclusion

Cleaning up environmental contamination from human activities is one of the greatest un-met challenges of the 21st century. Our research has shown that transferring catabolic genes involved in petroleum degradation from *E. coli* to indigenous bacteria may be a viable solution. This system could be adapted to exploit genes from local microbial populations which are already primed for degradation. This could be achieved by isolation and identification of native strains which degrade petroleum and proteomic identification and screening of candidate enzymes for over-expression. Future research is needed to determine 1) how long these plasmids are maintained under field conditions, 2) whether genetic

mutations accumulate over time that might impact enzyme functioning, and 3) how vector-based gene drives harnessing natural processes of conjugation may affect local microbial community composition and soil metabolic functions. Concentrated efforts among microbiologists, ecologists, synthetic biologists and policy makers in this new area of research may usher in a new era of how we respond to environmental disasters and toxic waste management in the Anthropocene.

Author contributions

KF created the vectors used in this study, performed the activity assays and microscopy, conducted the mating experiments, performed the statistical analysis of the data, and wrote the paper. ZZ conducted SPME GC/MS of cultures to detect dodecane and benzo(a)pyrene degradation. NT provided funding for this research.

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