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Biotransformation of 2,4-dinitrotoluene in a phototrophic co-culture of engineered Synechococcus elongatus and Pseudomonas putida

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Summary

In contrast to the current paradigm of using microbial mono-cultures in most biotechnological applications, increasing efforts are being directed towards engineering mixed-species consortia to perform functions that are difficult to programme into individual strains. In this work, we developed a synthetic microbial consortium composed of two genetically engineered microbes, a cyanobacterium (Synechococcus elongatus PCC 7942) and a heterotrophic bacterium (Pseudomonas putida EM173). These microbial species specialize in the co-culture: cyanobacteria fix CO2 through photosynthetic metabolism and secrete sufficient carbohydrates to support the growth and active metabolism of P. putida, which has been engineered to consume sucrose and to degrade the environmental pollutant 2,4-dinitrotoluene (2,4-DNT). By encapsulating S. elongatus within a barium–alginate hydrogel, cyanobacterial cells were protected from the toxic effects of 2,4-DNT, enhancing the performance of the co-culture. The synthetic consortium was able to convert 2,4-DNT with light and CO2 as key inputs, and its catalytic performance was stable over time. Furthermore, cycling this synthetic consortium through low nitrogen medium promoted the sucrose-dependent accumulation of polyhydroxyalkanoate, an added-value biopolymer, in the engineered P. putida strain. Altogether, the synthetic consortium displayed the capacity to remediate the industrial pollutant 2,4-DNT while simultaneously synthesizing biopolymers using light and CO2 as the primary inputs.

Introduction

In nature, bacteria typically coexist in communities with hundreds to thousands of species, creating a complex web of interspecies metabolic reactions (Little et al., 2008; Saxena, 2015; Dolinsek et al., 2016; Goldford et al., 2018). Most consortia exhibit a high degree of division of labour, where individual species display specialized metabolism and exchange small molecules and signals with neighbours (Tan et al., 2015). Interactions range from the cooperative degradation of toluene and other aromatic compounds (Nikel et al., 2014; Tecon and Or, 2017) to the consumption of metabolic waste products (Wilkinson et al., 1974). Compartmentalization of biochemical transformations across distinct species can confer metabolic capabilities on a consortium that may be difficult to engineer within any one individual species (Roell et al., 2019). Natural microbial consortia also exhibit other desirable features, such as robustness in the face of fluctuating environmental conditions and invasive microbes (Blasche et al., 2017). Against this background, there has been increasing interest in rationally engineering microbial consortia for desired outputs.
to capitalize upon some of these potential benefits (Ortiz-Marquez et al., 2013; McCarty and Ledesma-Amaro, 2019; Tsoi et al., 2019).

Recently, cyanobacteria have been engineered to efficiently produce and secrete simple sugars, making them attractive as alternative feedstock crops to produce carbohydrates for consumption by other industrially relevant microbes (Carrieri et al., 2012; Xu et al., 2013; Hays and Ducat, 2015; Song et al., 2016; Hays et al., 2017; Santos-Merino et al., 2019; Vijay et al., 2019). In one such example, the heterologous expression of a sucrose permease gene (scsB) in Synechococcus elongatus PCC 7942 (hereafter, S. elongatus CscB) enabled light-driven secretion of sucrose at rates estimated to exceed the productivities of traditional plant feedstock crops (Ducat et al., 2012). One approach to utilize the sugars secreted by such cyanobacteria is to cultivate them with a heterotrophic partner that can convert the carbohydrate into value-added products. Recent reports have shown that S. elongatus CscB can be paired with a variety of heterotrophic microbes, including Escherichia coli and Bacillus subtilis (Hays et al., 2017), Azotobacter vinelandii (Smith and Francis, 2016, 2017), Halomonas boliviensis (Weiss et al., 2017), Pseudomonas putida (Löwe et al., 2017a), Saccharomyces cerevisiae (Ducat et al., 2012) and Cryptococcus curvatus and Rhodotorula glutinis (Li et al., 2017). Such co-cultures are typically stable over long time periods (weeks to months) and the heterotrophic partner strain can be programmed to output value-added biological products, such as \( \alpha \)-amylase (Hays et al., 2017), fatty acids (Li et al., 2017) and poly-hydroxyalkanoates (PHAs) [e.g. poly(3-hydroxybutyrate) (Smith and Francis, 2016; Smith and Francis, 2017; Weiss et al., 2017; Löwe et al., 2017a,b)].

The limited availability of potable water influences the feasibility of algal and cyanobacterial applications; cultivation using marginal water supplies may ultimately be more sustainable (Barry et al., 2016). Industrial wastewater streams are often unsuitable for plant-based agriculture because of the toxic contaminants they contain. 2,4-Dinitrotoluene (2,4-DNT) is a nitroaromatic xenobiotic that is produced as a by-product during the synthesis of polyurethane, pesticides and explosives (Spain, 1995; Ju and Parales, 2010). A single 2,4,6-trinitrotoluene (TNT)-manufacturing plant can contaminate up to 500 000 gallons of water with nitroaromatics in a single day (Yinon, 1990) – and 2,4-DNT is a significant toxic contaminant found at dangerous levels in water and soils (Hooker and Skeen, 1999). 2,4-DNT is highly stable within the environment and remediation costs via incineration have been estimated at 400 USD per cubic yard of soil (Griest et al., 1995, 1998).

An alternative strategy for remediation of sites contaminated with 2,4-DNT is the use of bacterial strains capable of mineralizing this toxic nitroaromatic (Symons and Bruce, 2006; Aburto-Medina et al., 2017). Generally, two biological pathways that transform 2,4-DNT are found in nature. The non-specific reductive pathway is thought to involve reduction of 2,4-DNT by common electron carriers such as flavin- or iron center-containing electron donors (e.g. nitroreductases; French et al., 2001; Shemer et al., 2018). Reduction of the nitro group of 2,4-DNT creates highly reactive nitroso-intermediates and hydroxylamino-derivatives, which are also toxic due to their capacity to bind a range of biomolecules, frequently causing cellular damage by forming DNA and protein adducts (Spain, 1995; Achtnich et al., 1999; Padda et al., 2003). The toxic derivatives formed by the reductive pathway are not fully mineralized by most bacteria and also have long persistence times in natural environments (Achtnich et al., 1999; Shemer et al., 2018). A separate pathway that initiates with 2,4-DNT oxidation has been identified in some prokaryotes, including Burkholderia sp. R34 (McCormick et al., 1978; Spanggord et al., 1991; Suen et al., 1996), where it is encoded in a single gene cluster (Suen and Spain, 1993; Haigler et al., 1994; Parales et al., 1996; Johnson et al., 2000; Nishino et al., 2000). Enzymes encoded by the dnt gene cluster catalyse the oxidation of 2,4-DNT through a series of steps ultimately resulting in the release of nitrite, pyruvate and propionyl-coenzyme A (CoA) (Johnson et al., 2002). Because the products of the oxidative pathway are non-toxic metabolites common to most microorganisms, the oxidative pathway is preferable from the perspective of bioremediation.

In this study, we expanded upon prior efforts of engineering synthetic cyanobacteria/heterotroph consortia (Hays and Ducat, 2015; Smith and Francis, 2016, 2017; Hays et al., 2017; Weiss et al., 2017; Li et al., 2017; Löwe et al., 2017a) with the goal of generating co-cultures that can utilize contaminated water supplies and perform a bioremediation function while also producing a valuable bioproduct from light and CO\(_2\) (Fig. 1). We desired a heterotrophic partner that was genetically tractable and which could tolerate the toxic effects of 2,4-DNT – therefore, we turned our attention to the soil bacterium P. putida, an emerging microbial chassis (Calero and Nikel, 2019) recognized for its high tolerance to aromatic organic compounds, versatile central metabolism and genetic tractability (Park et al., 2006; Nikel et al., 2016; Nikel and de Lorenzo, 2018; Wirth et al., 2020). We explored the capacity of S. elongatus CscB to support engineered strains of P. putida in direct co-culture without supplementation of other organic carbon sources. Furthermore, P. putida was engineered to express the oxidative 2,4-DNT degradation pathway and the capacity of these synthetic co-cultures to remove this common toxin from the stream was evaluated both in batch and repeated-batch cultures. Finally, we explored
PHAs) as an accessory function. 

Results

Alginate-encapsulated S. elongatus CscB cells are protected from the toxic effects of 2,4-DNT

2,4-DNT is broadly toxic to many prokaryotes and eukaryotes (Yoon et al., 2006; Kuperman et al., 2009; Rocheleau et al., 2010); therefore, we first characterized the susceptibility of S. elongatus to this nitroaromatic compound at concentrations relevant for subsequent coculture applications. We observed that low concentrations of 2,4-DNT (8 μM) started to cause a substantial (ca. 60%) growth impairment in planktonic cultures of S. elongatus, while growth was arrested at concentrations ≥ 15 μM (Fig. 2A). Likewise, the content of chlorophyll a [Chl(a)], the loss of which is an indicator of physiological stress and cell death (Sauer et al., 2001; Latifi et al., 2009; Korosh et al., 2018), declined rapidly in S. elongatus cells exposed to 2,4-DNT concentrations above 30 μM (Fig. 2B and Fig. S1). In particular, we could detect a decrease of ca. 40% in the relative Chl(a) content over the first 6 h of incubation – and visible bleaching of cultures occurred by 48 h at 2,4-DNT concentrations ≥ 30 μM. By comparison, soils and water from contaminated sites are routinely reported to have 2,4-DNT levels near or above 100 μM (Griest et al., 1995). These preliminary experiments indicated that low 2,4-DNT levels could significantly affect the growth and physiological status of planktonic S. elongatus cells, a complication that could preclude their use to support bioremediation of this compound in co-cultures.

Previously, we have shown that S. elongatus cells encapsulated within an alginate hydrogel remain metabolically active for months under prolonged nitrogen-depletion stress (Weiss et al., 2017). Similarly, immobilization of a variety of cell types in hydrogels has been reported to increase stress tolerance and cell longevity (Bailliez et al., 1985; Gilleta et al., 2000; Leino et al., 2012; Therien et al., 2014; Ruiz-Guereca and Sánchez-Saavedra, 2016). We therefore examined the viability of alginate-encapsulated S. elongatus CscB cells and found that they maintained relative Chl(a) contents at a level similar to that of planktonic cells, even when exposed to 2,4-DNT concentrations near the solubility limits (250 μM) (Fig. 2C and Fig. S2 and S3). As indicated in Fig. S3B, the Chl(a) content per cell in alginate-encapsulated S. elongatus CscB was ca. 1 × 10^{-8} μg cell^{-1}, irrespective of the addition of 250 μM 2,4-DNT and 1 mM IPTG (the inducer needed to stimulate sucrose excretion by means of the CscB exporter). Furthermore, these encapsulated S. elongatus CscB cells also retained metabolic activity for at least 5 days when exposed to 250 μM 2,4-DNT – as assayed by the sucrose secreted to the supernatants of IPTG-induced cultures (Fig. 2D). In particular, the sucrose concentration in supernatants of cultures added with 250 μM 2,4-DNT reached ca. 1.2 g l^{-1} by the end of the experiment. Therefore, these experiments indicate that alginate encapsulation of S. elongatus CscB sufficiently protects cells from the acute toxicity brought about by 2,4-DNT addition without affecting the capacity of the cells to produce and secrete sugars, which led us to explore co-cultivation strategies as indicated below.

Co-cultivation of engineered derivatives of P. putida EM173 with alginate-encapsulated S. elongatus CscB

We next constructed a strain of P. putida that is capable of (i) utilizing sucrose as a carbon source and (ii) transforming 2,4-DNT through the oxidative pathway from Burkholderia sp. R34. P. putida does not naturally utilize sucrose as a carbon source, but has been previously engineered to grow on this substrate (Löwe et al., 2017a,b). We first transformed the genetically tractable, prophage-less strain EM173 of P. putida (Martínez-García et al., 2015) with the low-copy-number plasmid pSEVA221-cscRABY (Löwe et al., 2019) that bears the sucrose-utilization genes from P. protegens Pf-5 (Fig. 3A). The resulting strain was named P. putida EM-S. Plasmid pSEVA221-cscRABY, a derivative of vector pSEVA221 (Silva-Rocha et al., 2013), constitutively expresses the genes encoding a sucrose hydrolase (CscA, PFL_3237) and a sucrose permease (CscB, PFL_3238). These functions enable the uptake and catabolism of the disaccharide, together with a cognate transcriptional regulator (CscR, PFL_3236). Separately, a...
synthetic mini-Tn7 transposon, carrying the functions required for 2,4-DNT biotransformation from *Burkholderia* sp. R34 (Fig. 3A), was constructed as indicated in Experimental Procedures. The *dnt* gene cluster in this transposon was delivered into the unique *att/C1 Tn7* site within the chromosome of *P. putida* EM/C1S, resulting in a stable, engineered strain designed for both oxidative 2,4-DNT transformation and sucrose consumption (hereafter referred to as *P. putida* EM/C1DNT/C1S).

We then examined the sucrose catabolism capacity of these modified *P. putida* strains under a range of physiologically relevant growth conditions. We observed that the pSEVA221-cscRABY plasmid was sufficient to confer sucrose utilization in both *P. putida* EM-S and *P. putida* EM-DNT-S when grown in M9 minimal medium supplemented with 20 g l\(^{-1}\) sucrose (Fig. 3B). Both *P. putida* EM-S and *P. putida* EM-DNT-S grew exponentially over the course of the first 9 h post-inoculation, and by 24 h had reached final cell densities of OD\(_{600}\) (optical density measured at 600 nm) of 4.3 and 6 respectively. The specific growth rates of the two engineered strains were very similar, and the pattern of sucrose consumption was almost identical in either case. Indeed, during this incubation period, sucrose concentration declined in a nearly linear fashion from 20 to <3 g l\(^{-1}\) at 24 h (Fig. 3B). We similarly evaluated the growth profile of engineered *P. putida* at lower sucrose concentrations within M3 minimal medium, designed for cyanobacterial growth (derived from BG-11 minimal medium; see Table S1). To this end, *P. putida* EM-DNT-S was first inoculated in M3 minimal medium without supplementing a carbon source and incubated overnight in order to deplete internal carbon stores, then washed and re-inoculated into fresh M3 minimal medium supplemented with a range of sucrose concentrations (from 0 to 10 g l\(^{-1}\)). Bacterial growth was evident at sucrose concentrations ranging from 1.25 to 10 g l\(^{-1}\), after a lag phase longer than that observed in M9 minimal medium (Fig. 3C). In particular, we observed that at sugar concentrations >5 g l\(^{-1}\) the bacterial cultures attained OD\(_{600}\) values well over 1 unit after 48 h of incubation. Taken together, these results indicate that this cyanobacterial culture medium supports growth of engineered *P. putida* strains when sucrose is present as the sole carbon source – thereby enabling its use for designing co-cultures.

Next, we implemented and evaluated co-cultures by inoculating *P. putida* EM/S and *P. putida* EM-DNT/S into flasks containing either empty alginate beads or alginate-encapsulated *S. elongatus* CscB (Fig. 3D). Within the first 48 h, *P. putida* cells grew in all flasks – even in those
containing empty alginate beads. Residual carbon stores within *P. putida* and/or trace amounts of sugars present in the alginate beads likely drove the residual bacterial growth observed in flasks containing empty beads, and the growth of these cultures was minor compared to that of cells co-cultivated with alginate-encapsulated (and sucrose-producing) *S. elongatus* CscB (Fig. 3D). Furthermore, when the alginate beads were re-suspended in fresh M3 minimal medium at 96 h post-inoculation, growth of engineered *P. putida* strains was only observed in flasks with alginate-encapsulated *S. elongatus* CscB cells, where they grew exponentially. Additionally, we did not observe degradation of the alginate beads in these (nor in subsequent) experiments, indicating that *P. putida* cannot utilize the hydrogel itself as a carbon source, in agreement with previous work (Löwe et al., 2017a). With these results at hand, we next evaluated the use of this co-culture setup for 2,4-DNT transformation.

**Biotransformation of 2,4-DNT by *P. putida* EM-DNT-S**

As indicated previously, the *dnt* gene cluster of *Burkholderia* sp. R34 encodes the enzymes needed for oxidative breakdown of 2,4-DNT through oxygenation reactions (Spain, 1995; Symons and Bruce, 2006). The enzymes within the 2,4-DNT degradation pathway catalyse two successive oxygenation reactions mediated by DntA and DntB (Fig. 4A). 2,4-DNT is firstly dioxygenated by DntA to form 4-methyl-5-nitrocatechol (4M5NC), a compound with a strong absorption peak at 420 nm (de las Heras et al., 2011). 4M5NC is the substrate of DntB, which yields 2-hydroxy-5-methylquinone (2H5MQ), an intermediate with an absorption peak at 485 nm (de las Heras et al., 2011). In the native context of this catabolic route, 2H5MQ is then processed by the products of a number of additional genes encoded in the pathway to finally yield pyruvate and propionyl-CoA (Fig. 4A).

We next examined the functional capacity of the *dnt* gene cluster introduced into *P. putida* EM-DNT-S by following the kinetics of 2,4-DNT transformation in axenic cultures. M3 minimal medium, supplemented with 250 μM 2,4-DNT and 2 g l⁻¹ sucrose, was inoculated with *P. putida* EM-S or *P. putida* EM-DNT-S at an initial OD₆₀₀ = 0.1. Consistent with the sequential formation of two expected oxidative intermediates, the supernatant of the *P. putida* EM-DNT-S cultures visibly changed.
pigmentation over the course of the growth experiment (Fig. 4B), whereas cultures of *P. putida* EM-S did not show any visible changes in coloration over the entire incubation period. Within 2–4 h following inoculation of *P. putida* EM-DNT-S, the supernatant turned yellow, consistent with the accumulation of the first intermediate, 4M5NC. Subsequently, the supernatant transitioned from yellow to orange (around 4 h) and then to red (22 h after inoculation), which reveals the presence of the second intermediate, 2H5MQ, and the breakdown of 4M5NC. These results are indicative of the biotransformation of 2,4-DNT and, as reported previously, *P. putida* strains carrying the dnt gene cluster were not able to grow on 2,4-DNT as the only carbon source (Akkaya et al., 2018). A separate quantitative analysis of the absorbance spectra of *P. putida* EM-DNT-S supernatants focused upon the first 8 h following 2,4-DNT addition, revealing a characteristic absorbance rise at 420 nm (between 1-4 h post-

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Fig. 4. 2,4-DNT biotransformation in mono-cultures of engineered *P. putida*.

A. Pathway for the oxidative degradation of 2,4-DNT in *Burkholderia* sp. R34. The first step, executed by DntA, is a di-oxygenation reaction that leads to the formation of 4-methyl-5-nitrocatechol (4M5NC). This intermediate is the substrate for the DntB mono-oxygenase, yielding 2-hydroxy-5-methylquinone (2H5MQ). In the native context, the pathway leads to the conversion of 2,4-DNT into pyruvate and propionyl-coenzyme A (CoA).

B. Representative images of axenic cultures of *P. putida* EM-DNT-S and *P. putida* EM-S grown in the presence of 250 μM 2,4-DNT over time (left column); the zoom-in of supernatants from these cultures shows the characteristic changes in pigmentation (right column).

C. Time-course of averaged spectral signatures (*n* = 3) in supernatants from *P. putida* EM-DNT-S cultures grown in the presence of 250 μM 2,4-DNT, as measured via scanning spectrophotometry. The signature absorbance peaks of 4M5NC and 2H5MQ are indicated. A.U., arbitrary units.

D. LC-MS analysis of culture supernatants of *P. putida* EM-DNT-S (top panel) and *P. putida* EM-S (bottom panel) after 4 h of incubation with 250 μM 2,4-DNT. The peak corresponding to the monoisotopic mass of 4M5NC (mass 169.037 Da) is indicated by a yellow line.

E. LC-MS quantification of the 4M5NC concentration (conc.) in supernatants of *P. putida* EM-S and *P. putida* EM-DNT-S cultures after 4 h incubation with 250 μM 2,4-DNT. All cultures were grown in M3 minimal medium with sucrose at either 20 g l⁻¹ (B) or 2 g l⁻¹ (C–E). N.D., not detected.
inoculation). A second absorption peak arose at 485 nm (4–8 h), during the time period where the characteristic peak of 4M5NC gradually decreased (Fig. 4C). These spectroscopic changes were neither observed in control experiments with the non-degrading P. putida EM-S strain (Fig. S4) nor in the absence of 2,4-DNT. We directly confirmed the accumulation of 4M5NC by liquid chromatography coupled to mass spectrometry (LC-MS). P. putida EM-DNT-S supernatants exhibited a peak matching the 4M5NC standard (Fig. 4D), whereas no 4M5NC was detected in control (P. putida EM-S) cultures under any condition. Direct quantification of 4M5NC levels by LC-MS indicated a peak consistent with 4M5NC accumulation (ca. 8 μM) at 4 h post-inoculation (Fig. 4E). Again, we could not detect any 4M5NC formation in control cultures of the non-degrading strain P. putida EM-S. As an additional control of the activity of the first enzymes within the pathway, we carried experiments in which the release of nitrite from the substrate was measured by a colorimetric method. Nitrite could be detected in culture supernatants when P. putida EM-DNT-S was incubated in the presence of 250 μM 2,4-DNT and using sucrose at 2 g l⁻¹ as the sole carbon source. After 4 h, the nitrite concentration reached 7.3 ± 0.2 μM, and it peaked at 59.8 ± 1.3 μM by 24 h, suggesting that the oxidative pathway for 2,4-DNT degradation plays a significant role in the removal of the nitroaromatic. The decrease in the 4M5NC together with the release of nitrite are indicative of 2,4-DNT biotransformation. As previously mentioned, no bacterial growth was detected when P. putida EM-DNT-S cells were incubated in the mineral medium containing 2,4-DNT but no added carbon source – which indicates that this engineered strain can transform the substrate without net assimilation of carbon (or at least not to the extent that would support growth on 2,4-DNT).

The disappearance of 2,4-DNT from the supernatant of P. putida cultures was directly assessed by gas chromatography coupled to mass spectrometry (GC-MS) in all the experiments. We observed that the soluble fraction of 2,4-DNT declined significantly within the first 4 h, and dropped to undetectable levels by 22 h. This kinetic pattern of 2,4-DNT transformation is similar to what has been reported for Burkholderia sp. R34 (Pérez-Pantoja et al., 2013) and other engineered P. putida strains (Akkaya et al., 2018, 2019). Soluble levels of 2,4-DNT declined in cultures of P. putida EM-S as well (Fig. S5A), whereas the concentration of the nitroaromatic remained constant in control flasks that were not inoculated with P. putida cells (Fig. S5B). These observations can be accounted for by the fact that a small fraction of 2,4-DNT became most likely adsorbed (i.e. physically associated) to cell surfaces, a known property of nitroaromatic compounds (Achtinich et al., 1999). Additionally, the reductive pathway (catalysed by non-specific nitroreductases, which have been reported to be present in many bacterial species) could result in the conversion of 2,4-DNT into other intermediates (Fig. S5C). To investigate this possibility, we assessed culture supernatants for the presence of 2-amino-4-nitrotoluene/4-amino-2-nitrotoluene (2A4NT/4A2NT), two key isomer intermediates of the reductive pathway, by LC-MS. These two compounds were detected at low levels (<2.5 μM) in the supernatant of cultures of both P. putida EM-DNT-S and P. putida EM-S, but the formation of 2A4NT/4A2NT was significantly lower in cultures of P. putida EM-DNT-S (Fig. S5D). This result suggests that the oxidative route competes with the non-specific reductive pathway in removing 2,4-DNT in engineered P. putida strains, with a predominant role of the former over the later in P. putida EM-DNT-S. With this information at hand, we set to establish co-cultures of engineered P. putida and S. elongatus as indicated in the next section.

**Biotransformation of 2,4-DNT by a synthetic consortium of engineered P. putida and S. elongatus CscB**

We next introduced 2,4-DNT into co-cultures of P. putida with alginate-encapsulated S. elongatus CscB cells. Both P. putida EM-S and P. putida EM-DNT-S were able to grow with the beaded S. elongatus CscB cells in the presence of 250 μM 2,4-DNT (Fig. 5A). Cultures containing only alginate-encapsulated S. elongatus CscB or empty alginate beads served as controls for both optical density measurements as well as any potential 2,4-DNT adsorption to abiotic surfaces. As previously observed, no noticeable bacterial growth was detected in the absence of S. elongatus CscB in these experiments. GC-MS analysis of supernatants demonstrated that cultures containing engineered P. putida or S. elongatus cells removed 2,4-DNT from the culture, while the 2,4-DNT concentration did not change in empty-bead control experiments (Fig. 5B). Similarly, the supernatant of co-cultures containing P. putida EM-DNT-S exhibited visual changes in colour similar to those reported in Fig. 4, indicating activity of the oxidative dnt pathway. Accordingly, LC-MS analysis validated that the oxidative pathway intermediate 4M5NC transiently accumulated in the supernatants of P. putida EM-DNT-S-containing co-cultures (Fig. 5C), reaching a concentration of ca. 3 μM within the first hour of incubation and completely disappearing after 24 h. Conversely, 4M5NC was not detected in P. putida EM-S-containing co-cultures. These results indicate that the synthetic consortium can efficiently remove 2,4-DNT in a single batch culture.

Based on these results, we next evaluated the capacity of the co-culture to remediate multiple (i.e. repeated) batches of 2,4-DNT-containing streams by extending the co-culture experiment to 15 days and exchanging the
culture supernatant every 4–5 days with fresh medium containing 250 μM 2,4-DNT (Fig. 5D). In each case, \textit{P. putida} was able to quickly re-grow from residual planktonic cells after removal and replacement of the culture supernatant—a phenomenon likewise observed for \textit{P. putida} EM/C1S and \textit{P. putida} EM/C1DNT/C1S. Again, colorimetric changes in the supernatant were evident only in the \textit{P. putida} EM/DNT/C1S–containing co-cultures after each back-dilution, with added 2,4-DNT. Direct measurement of the residual nitroaromatic concentration indicates that the total mass of 2,4-DNT cleared from solution by the \textit{P. putida} EM/DNT/C1S strain over this period in repeated-batch co-cultures amounts to 136 mg l\(^{-1}\).

**Simultaneous removal of 2,4-DNT and accumulation of PHA by engineered strains of \textit{P. putida} in a synthetic consortium**

Wild-type \textit{P. putida} strains are known to accumulate PHA as a carbon storage (López \textit{et al.}, 2015; Prieto \textit{et al.}, 2016), particularly under nitrogen-depleted conditions in the presence of an abundant carbon source (Hoffmann and Rehm, 2004), which prompted us to investigate the opportunity to both remove 2,4-DNT and simultaneously produce a valuable by-product. In these experiments, engineered \textit{P. putida} cells were inoculated into the alginate-encapsulated and IPTG-induced \textit{S. elongatus} CscB culture flasks at an OD\(_{600}\) ~ 0.5 in the presence of 250 μM 2,4-DNT (Fig. 6A). These flasks contained either M3 or M3-N minimal medium, the latter of which has a reduced nitrogen content to trigger polymer accumulation (2 mM NaNO\(_3\); Table S1). The pattern of cell growth was very similar for the two strains under evaluation, and bacterial growth was not significantly affected by the reduced nitrogen conditions in M3-N minimal medium. Twenty-four hours after cycling co-cultures into either nitrogen-rich or nitrogen-depleted media (i.e. M3 and M3-N minimal medium, respectively), planktonic \textit{P. putida} cells were harvested and the intracellular PHA content was evaluated by means of LC-MS.
measurements of crotonic acid as indicated in Experimental Procedures. Both engineered P. putida strains were able to accumulate the polymer under nitrogen-limited conditions – but not in co-cultures run in nitrogen-rich M3 minimal medium (Fig. 6B). In particular, P. putida EM-S and P. putida EM-DNT-S co-cultures grown under nitrogen-depleted conditions in M3-N minimal medium accumulated the polymer at a specific volumetric productivity of 4.9 and 5.1 mg PHA l⁻¹ day⁻¹ of culture, with a polymer content in biomass of 22 and 23.4 mg of PHA g cell dry weight⁻¹, respectively. These results indicate that the accumulation of PHA from sucrose as the only carbon source by engineered P. putida is not affected by the presence of 2,4-DNT (or its transformation products) and that the polymer can be obtained in co-cultures designed for 2,4-DNT removal as an added-value by-product.

Discussion

Rational engineering of microbial communities is an emerging area of study that has implications for both applied and fundamental microbiology. Most natural microbial biochemistry occurs in the context of complex microbial consortia that are unlike the mono-cultures routinely cultivated under controlled laboratory conditions (Jessup et al., 2004). Desirable system-level features (e.g. robustness) are thought to emerge from simple interactions between individual species [e.g. by the division of metabolic labour (McCann, 2000; Ives and Carpenter, 2007; Voike and Nikel, 2018)], although these phenomena remain poorly understood. Gaining a deeper understanding of the design principles underlying natural microbial ecologies will likely require the application of top-down, systems-level analyses (e.g. metagenomic approaches) and the development of flexible bottom-up platforms that permit the assembly of simple, tractable microbial mixed cultures (Jessup et al., 2005; Momeni et al., 2011; Goers et al., 2014).

In this work, we constructed microbial co-cultures capable of transforming the industrial toxin 2,4-DNT by utilizing light and CO₂ as the primary inputs. Our present contribution builds upon a flexible co-cultivation system by demonstrating that (engineered) heterotrophs can be incorporated into designer communities in order to programme them for removal of environmental contaminants – thus extending the utility of synthetic consortia beyond the mere coexistence of microbial strains into performing complex biodegradation tasks. We chose 2,4-DNT as a model toxicant, since this nitroaromatic compound is a chemical contaminant that poses a significant bioremediation challenge due to toxicity and persistence in natural environments. This synthetic consortium described herein consists of engineered strains of S. elongatus and P. putida designed to divide metabolic labour between the partner species – with S. elongatus CscB specializing in photosynthetic processes, generating and secreting soluble sugars sufficient to promote the growth and distinct metabolic activities of co-cultivated engineered P. putida. We demonstrated that co-cultures were reproducibly stable over a series of back-dilutions (repeated-batch cultivation), permitting the removal of soluble 2,4-DNT in successive batch cultures for an extended period of time.

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The selection of the partner heterotroph in our system is not arbitrary. *P. putida* has gained significant attention as a chassis for industrially relevant synthetic biology due to its metabolic diversity, high tolerance of oxidative stress, and capacity to conduct complex biochemical reactions involving aromatic substrates (Poblete-Castro et al., 2017). In this context, the still-evolving 2,4-DNT degradation pathway from *Burkholderia* sp. R34 has been reported to lead to accumulation of oxidative stress by an increased rate of mutation in its native context, whereas *P. putida* heterologously expressing the *dnt* gene cluster (*P. putida* EM-DNT and derivatives thereof) does not exhibit the same rate of DNA damage and mutation (Akkaya et al., 2019). *P. putida* EM-DNT-S cocultures removed 2,4-DNT from the soluble phase partly via this oxidative degradation pathway. However, under the conditions of our cocultures, a portion of 2,4-DNT is also transformed via reductive processes (Fig. S5A) and/or gets adsorbed to surfaces. Because multiple products and nitroamino-adducts are formed downstream of 2,4-DNT reduction, we could not precisely quantitate the flux through the oxidative pathway with our data, but we do see a ca. 25% reduction in the accumulation of the reductive products 2A4NT and 4A2NT. Fitting our kinetic data of 2,4-DNT transformation (Fig. 4 and Fig. S5A) to a simple mass-action model of a branched metabolic pathway suggests that up to 40% of 2,4-DNT may be fully mineralized via the oxidative pathway (see Experimental Procedures). Further engineering efforts may be required to increase the fraction of 2,4-DNT that is completely catabolized and/or incorporated into biomass, e.g. by improving the affinity of enzymes in the oxidative pathway for 2,4-DNT (Monti et al., 2005; Ju and Parales, 2010) or by coupling the growth of engineered strains to fully oxidative catabolism of the aromatic substrate (Durante-Rodriguez et al., 2018; Fernández-Cabezón et al., 2019).

In tandem with the transformation of 2,4-DNT, co-cultures were programmed to synthesize a valuable by-product in the form of PHA. Co-cultures of engineered *P. putida* and *S. elongatus* accumulated the polymer with specific volumetric productivities of ca. 5 mg PHA l\(^{-1}\) day\(^{-1}\) using gaseous CO\(_2\) as the primary carbon source—which is lower than the productivity reported in recent publications describing bacterial cocultures or mono-cultures of cyanobacteria directly engineered to express PHA-producing pathways (Löwe et al., 2017a,b). Nevertheless, we have demonstrated that polymer accumulation can occur in *P. putida* the presence of 2,4-DNT and that PHA synthesis could be coupled to the removal of the nitroaromatic. Therefore, PHA produced from these co-cultures represents a value-added by-product—and a first-case example of polymer accumulation coupled to bioremediation of an environmental pollutant. The economic prospects of the process can be improved by optimizing PHA yields (e.g. by modifying the regulation of the PHA synthesis to render it insensitive to nitrogen availability) or by incorporating pathways leading to higher-value bioproducts in the system. Further modifications could include the addition of other microbial partners in the consortium, thereby multiplying the range of products that can be obtained in these co-culture setups. One way or the other, the rational pairing of cyanobacteria and *P. putida*, together with the plethora of synthetic biology approaches created to engineer novel properties in whole-cell biocatalysts, allows for the design of systems with the potential to transform current production platforms.

**Experimental procedures**

**Bacterial strains and culture conditions**

Planktonic *S. elongatus* strains were grown in a Multitron Pro (Infors HT, Annapolis Junction, MD, USA) photobiological shaker at 32°C, 2% (v/v) CO\(_2\), and a constant illumination of ~70 μmol m\(^{-2}\) s\(^{-1}\) (15 W Grow-Lux; OSRAM Sylvania Inc., Wilmington, MA, USA) as previously described (Weiss et al., 2017). Selection for the genomically integrated *cscB* module was achieved by addition of 12.5 μg ml\(^{-1}\) chloramphenicol to the culture medium. Engineered *P. putida* strains were constructed essentially as reported previously (Martínez-García et al., 2014; Löwe et al., 2017a,b, 2019; Akkaya et al., 2018). During routine cultivation, *P. putida* strains carrying an integrated *dnt* gene cluster were maintained in the presence of 25 μg ml\(^{-1}\) gentamicin, while strains transformed with plasmid pSEVA221-cscRABY were maintained with 50 μg ml\(^{-1}\) kanamycin. Lysogeny broth (LB) medium cultures were at 32°C in a Multitron (Infors HT) incubator with rotary agitation at 150 rpm. For coculture experiments, *P. putida* cultures grown in LB medium were re-suspended in M3 minimal medium (Table S1) with either 20 g l\(^{-1}\) or 2 g l\(^{-1}\) sucrose, as indicated in the main text. These cultures were then used as inoculum for subsequent experiments after pelleting cells, rinsing the pellet, and re-suspending cells in fresh media to prevent the carryover of carbohydrates and other soluble metabolites. Antibiotic selection was omitted for all co-culture experiments.

**Encapsulation of *S. elongatus* CscB cells in alginate beads**

Alginate encapsulation was performed as previously described (Weiss et al., 2017) with minor adjustments. Briefly, planktonic *S. elongatus* CscB cells grown in minimal BG-11 medium containing 1 g l\(^{-1}\) HEPES (pH = 8.3; Sigma-Aldrich Co., St. Louis, MO, USA) were
harvested at an optical density at 750 nm (OD750) of 2.0 via centrifugation at 3500 g for 30 min and concentrated in 3 ml of sulfur-free BG-11 minimal medium. Concentrated cells were mixed into a sterile and degassed volume of 3% (w/v) sodium alginate at a final OD750 = 5.0, thereby resulting in a 2.75% (w/v) sodium alginate-S. elongatus CscB suspension. In a sterile biosafety cabinet, a vertically oriented syringe pump (KD Scientific, Holliston, MA, USA) was used to dispense the solution dropwise through a 30 G needle into a ≥20-fold larger volume of 20 mM BaCl2. The drops travelled ~35 cm from needle to the slowly stirred BaCl2 solution and were allowed to equilibrate in the solution for at least 20 min. Solidified beads were rinsed once with BG-11 medium and incubated overnight in M3 minimal medium (without NaCl); the mean diameter of cured alginate beads used in these experiments was ~1.8 mm. To acclimate cells within the alginate hydrogel and precipitate residual Ba2+, beads were transferred through a series of media washes in the following days. The first day post-encapsulation, beads were rinsed and re-suspended in fresh M3 minimal medium (without NaCl), transferred to 250-ml baffled Erlenmeyer flasks, and placed into a Multitron Pro incubator (Infors HT) with rotary shaking (125 rpm) for 24 h. Subsequently, the M3 minimal medium was exchanged, and the alginate bead suspension was portioned into ~10-ml aliquots into 125-ml baffled Erlenmeyer flasks. To acclimate cells to the final working salt concentrations, the spent medium was replaced with M3 minimal medium with only 25 mM NaCl for 24 h, then M3 minimal medium with 50 mM NaCl for 24 h, and finally to complete M3 minimal medium (containing 100 mM NaCl, Table S1). The culture medium was subsequently refreshed daily for 3–5 days prior to initiation of experiments. In the cases where the beads were utilized in co-culture, cscB expression was induced with the addition of IPTG at 1 mM 24 h prior to the addition of P. putida cells. The culture medium was exchanged a final time coincident with addition of P. putida cells.

Analytical methods

Culture optical densities were measured with a Genesys 20 (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer. The density of planktonic S. elongatus CscB was measured at OD750, and both co-culture experiments and P. putida mono-cultures were measured at OD600. This spectrophotometer was also used to measure Chl(a) concentrations of planktonic S. elongatus cultures (Zavrel et al., 2015). For Chl(a) measurements in alginate-encapsulated S. elongatus, 1 ml of pre-chilled methanol was added to four alginate beads in three technical replicates. Beads were vortexed and incubated at 4°C for 30 min in the dark to extract Chl(a). The absorbance of the supernatant was measured in cuvettes at 720 nm and 665 nm to calculate the final Chl(a) concentration as previously described (Zavrel et al., 2015).

Sucrose concentration of beaded cultures was measured as indicated previously (Weiss et al., 2017). Briefly, 1 ml of culture supernatant was withdrawn at the indicated time points, cells were pelleted at 17 000 g for 10 min, and sucrose was quantified via a sucrose/o-glucose assay kit (Megazyme, Bray, Ireland) with three technical replicates for each sample. Culture supernatant spectra were scanned using a DU800 Spectrophotometer (Beckman Coulter Inc., Brea, CA, USA) to assess the presence of coloured metabolites within the 2,4-DNT degradation pathway. Cell-free culture supernatant was obtained by centrifugation for 10 min at 17 000 g. The supernatant was then transferred to a cuvette for spectral analysis.

Direct 2,4-DNT measurements were made using an Agilent 5975 GC/single quadrupole MS (Agilent Technologies Inc., Santa Clara, CA, USA). Culture samples were centrifuged at 17 000 g for 10 min and 10 µl of cell-free supernatant were removed to a new tube. A 50-µl aliquot of ethyl acetate was added to the supernatant and incubated at room temperature for 30 min. The ethyl acetate phase was then transferred to a GC vial and injected (the injection volume was 1 µl). Samples were separated with a 5% (w/w) phenyl-methyl capillary column (Agilent Technologies Inc.) and measured by mass selective detector (MSD). A temperature of 275°C was set for the split/splitless injector (ratio of 10:1). Helium gas was used as the carrier gas at a flow rate of 1 ml min−1. The release of inorganic nitrite (NO2−) from nitroaromatic compounds was assessed in culture supernatants by a colorimetric method essentially as described by Zhang et al. (2001).

LC-MS measurements of metabolites within the 2,4-DNT degradation pathway were made using a Waters Xevo G2-XS UPLC-MS/MS (Waters Corp., Milford, MA, USA). Culture samples were harvested and pelleted via centrifugation (17 000 g for 10 min), and a 100-µl aliquot of the supernatant was transferred to a clean tube, lyophilized, and re-suspended in 1 ml of deionized water for measurements. HPLC measurements of crotonic acid (as a proxy of PHA accumulation) were made with a Waters 2695 HPLC. Samples were processed and analysed as previously described (Weiss et al., 2017). Briefly, cells were centrifuged at 17 000 g for 10 min, the supernatant was decanted, and the pellet was lyophilized. The cell biomass was suspended in 1 ml of concentrated H2SO4 and heated to 90°C for 1 h to promote dehydration of 3-hydroxybutyrate into crotonic acid. After cooling the reaction mixture to room temperature, particulate material was removed by filtration and the mixture was diluted 100-fold with deionized water for measurements.
water. A 20-μl aliquot of the resulting solution was injected onto an Aminex 300-mm HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA), and 0.028 N \( \text{H}_2\text{SO}_4 \) was used as the mobile phase at a flow of 1 ml min \(^{-1}\). The column temperature was maintained at 60° C and the UV absorption of crotonic acid was monitored at 210 nm. Two standards of commercial PHA (Sociedad de Laboratorios) were similarly treated and used for quantification purposes.

A simple mass-action model was used to explore the parameter space that could sufficiently fit our kinetic data sets of 2,4-DNT transformation (Fig. 4 and Fig. SS; Voit, 2000, 2017). Briefly, metabolite flux through both reductive and oxidative branches was modelled for the initial steps of 2,4-DNT transformation as a first-order kinetic dependent upon the concentration of each substrate \( \frac{d[P]}{dt} = k_1[S] - k_2[P] \); where \( P \) = product, \( S \) = substrate, and \( k_1 \) and \( k_2 \) are coefficients related to each branch point within the metabolic network. Reactions were modelled to be irreversible based on the unidirectionality of the early steps of 2,4-DNT transformation. Models were capable of producing simulated metabolite profiles that fit our kinetic profiles (within an allowed 25% error of our experimentally measured values), including the loss of 2,4-DNT from solution, and gain of the colorimetric products 4M5NC and 2H5MQ across a range of parameter space without making further assumptions of more complex pathway regulation (e.g. feedback or feed-forward regulatory steps), including oxidative branch flux between 15 and 40% of the competing reductive pathway.

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Conflict of interest

None declared.

Author contributions

D.T.F., P.I.N. and D.C.D. designed and directed the project and experiments. P.C. designed and engineered the \( P. \ putida \) strains utilized in this work. D.T.F. and P.S. performed the experiments and recorded the results. D.T.F., P.S., P.C., P.I.N. and D.C.D. analysed and interpreted the data. D.T.F. drafted the manuscript and figures. D.T.F., P.S., P.C., P.I.N. and D.C.D. provided commentary and edits to the manuscript and figures and prepared the final version of the article.

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Supporting information

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

**Table S1.** Chemical composition of BG-11, M3, and M3-N minimal media used in this study.

**Fig. S1.** Influence of 2,4-DNT on growth of *S. elongatus* PCC 7942.

**Fig. S2.** Effect of 2,4-DNT on alginate-encapsulated *S. elongatus* CscB.

**Fig. S3.** Chlorophyll *a* content per cell comparison in planktonic and alginate-encapsulated *S. elongatus* CscB.

**Fig. S4.** Spectral analysis of supernatants of *P. putida* EM S cultures.

**Fig. S5.** Kinetics of 2,4-DNT transformation by engineered *P. putida* through the oxidative and reductive pathway.