BIOSYSMO

Deliverable D3.8

Intermediate report on genetically enhanced and adapted microorganisms able to improve biodegradation of pollutants

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Executive Summary

The work in D3.8 seeks to produce engineered microbial strains for the degradation of problematic pollutants persisting in soil and ground water. The work focuses on engineering the bacterium *Pseudomonas putida* KT2440 as it is an industrially relevant microbial chassis with an extensive molecular toolkit available for its genetic manipulation. Key pollutants to target have been selected based on analysis of sites identified in WP1, combined with a search of the relevant literature to identify known metabolic pathways for degradation. The target compounds include the pesticide lindane and the herbicide atrazine. We report the construction and validation of genetic modules encoding degradation pathways for these compounds encoded in mobilizable plasmids suitable for genetic augmentation of natural microbial communities. Engineered microbial strains have first been tested *in vitro* to validate the functionality of the recombinant degradation systems. Our results show preliminary evidence of growth in lindane when using a nanoparticle assay developed by JSI but not in suspension. Moreover, the strains engineered for atrazine mineralisation show enzymatic activities for the biodegradation of the pesticide as well as the ability to use cyanuric acid resulting from atrazine degradation as the sole nitrogen source. These strains will be evaluated by UBU in soil microcosm assays to assess their degradation performance in a more natural matrix. Data generated in the characterisation of engineered strains will be used in Task 2.2 to refine and improve Genome-Scale Metabolic Models (GEMs) initially constructed based on information available in public databases.

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Table of Abbreviations

Abbreviation	Definition
°C	Degrees Celsius
µg	Microgram
µL	Microlitre
ABC	ATP-Binding Cassette
bp	Base Pairs
CDS	Coding Sequence
CNB-CSIC	Centro Nacional De Biotecnología-Consejo Superior De Investigaciones Científicas
g	Gram
GEMs	Genome-Scale Metabolic Models
h	Hour
IDT	Integrated Dna Technologies
kan	Kanamycin
LB	Lysogeny Broth
MCS	Multiple Cloning Site
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
msfGFP	Mono-Function Superfolder Green Fluorescent Protein
NCBI	National Centre For Biotechnology Information
OD	Optical Density
ORF	Open Reading Frame
oriT	Origin Of Transfer
PAH	Polycyclic Aromatic Hydrocarbons
PIA	Pseudomonas Isolation Agar
RBS	Ribosome Binding Site
rpm	Revolutions Per Minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
WHO	World Health Organisation

1 Introduction

1.1 Overall strategy

One of the target pollutants is the γ-hexachlorocyclohexane isoform of lindane which has been used historically in agriculture as a broad-spectrum insecticide to target invertebrate pests. The World Health Organisation (WHO) classify lindane as 'moderately hazardous' due to its persistence in the soil following use and its neurotoxic effects [1], lindane has been observed to affect the nervous system and impact liver and kidney function, as well as exhibiting carcinogenic effects. Although its use is now banned in most countries, its persistence in soils and groundwater mean that it still poses significant risk, particularly in areas of former production sites, and in certain locations where spillage and dumping has historically occurred. Within the BIOSYSMO project, soil and water samples obtained from Site 13 carry heavy lindane contamination, resulting from a lindane factory previously located on that site.

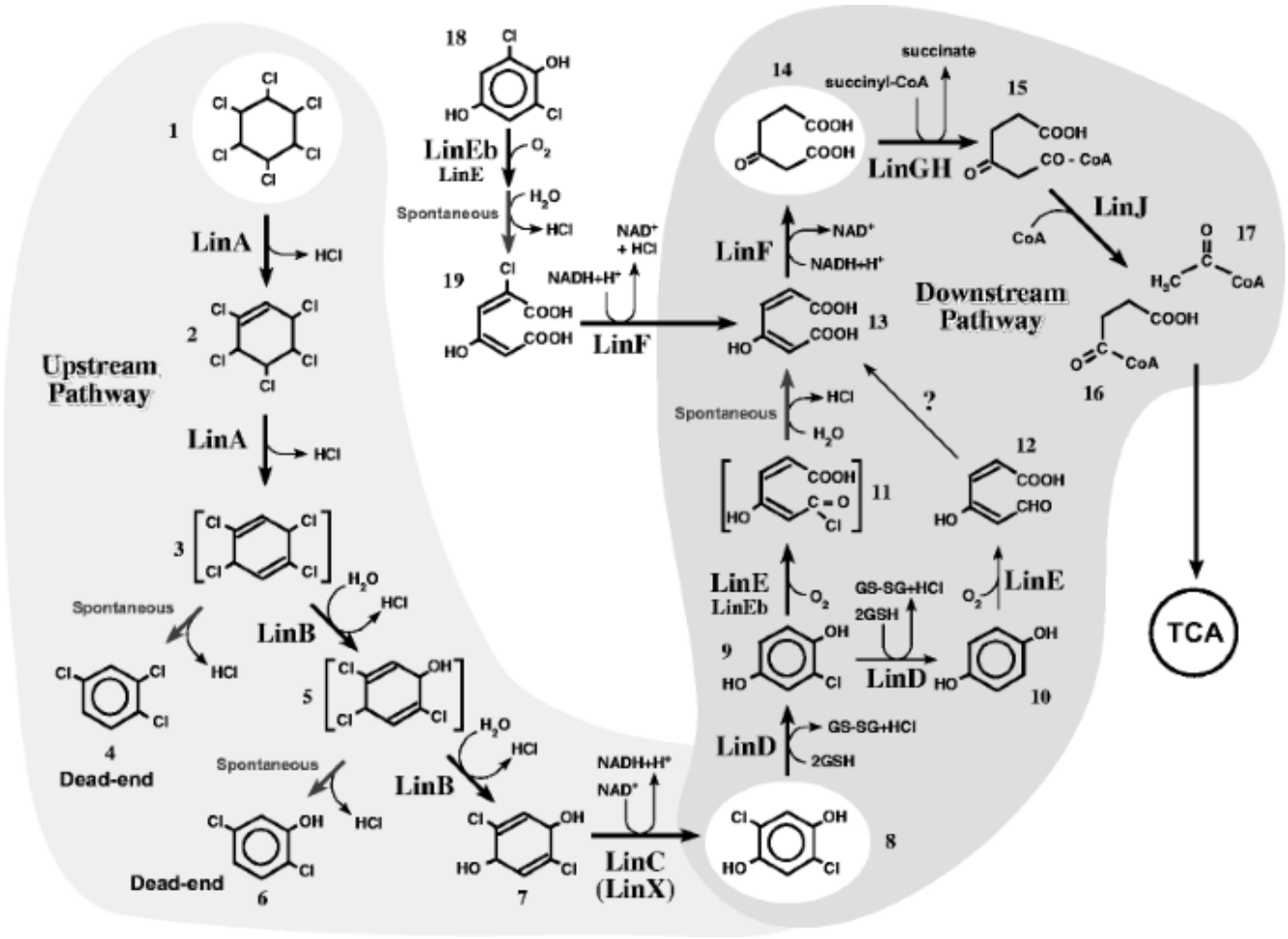

Figure 1. Lindane (γ-hexachlorocyclohexane) degradation pathway from *S. japonicum* UT26 [3].

Current methods for the treatment and management of lindane-polluted soil and water involve excavation or pumping of contaminated sites and subsequent containment or disposal in dedicated landfill sites, however, this does not destroy or remove the contamination from the soil or water matrix itself. Combustion of contaminated waste is an alternative treatment method; however, it offers poor cost effectivity, and the combustion process also has the potential to release harmful toxins such as dioxins and furans. An alternative to these methods is to take a bioremediation approach which is the goal of BIOSYSMO. Biological treatment methods offer the greatest promise as a means to degrade

lindane to lower toxicity degradation products, and also have the potential to accumulate compounds in biomass which can then be removed from affected sites.

There are a limited number of microbial species which exhibit lindane degradation abilities, many of which belong to the *Sphingomonas* and *Sphingobium* genera. In *Sphingobium japonicum* UT26, 15 genes have been identified dispersed within the genome which together permit *S. japonicum* to utilise lindane as a sole source of carbon and energy [2]. This includes 11 genes involved in the degradation of lindane through to acetyl-CoA (**Fig. 1**), and an additional four genes (*linKLMN*) comprising an ABC transporter system [3] to help in the transport of lindane to the bacterial cytoplasm.

Other problematic pollutants identified in the consortium are also being addressed, and degradation pathways for specific compounds which have been previously reported in the literature are constructed following the same format as the lindane degradation pathway. A second target compound is the herbicide atrazine which has been identified as a pollutant present at Site 1 (WP1). Atrazine can be degraded enzymatically *via* a six-step pathway (*atzABCDEF*) yielding cyanuric acid which can subsequently provide an accessible nitrogen source for many bacteria (**Fig. 2**) [4].

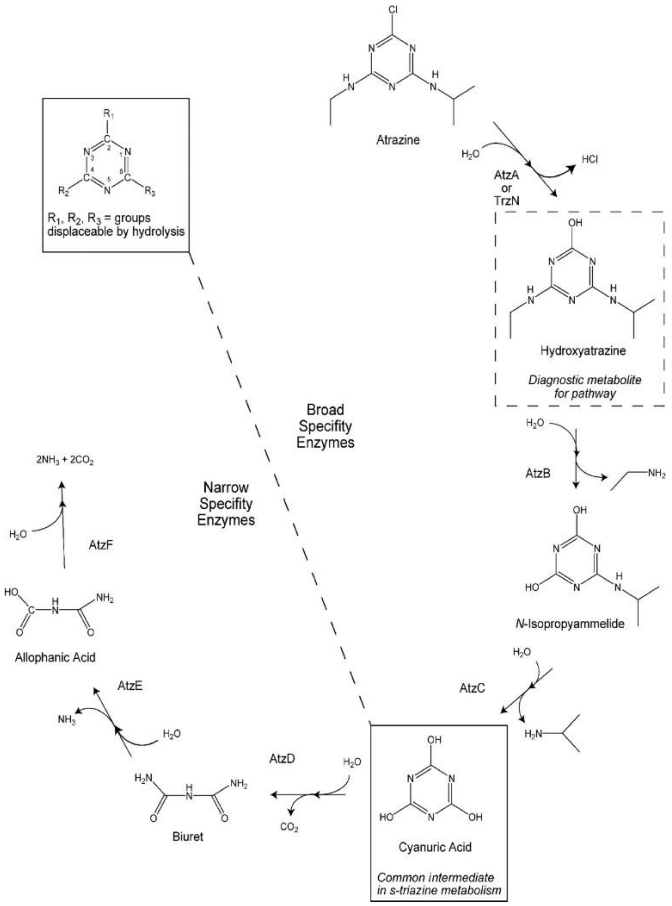

Figure 2. Microbial metabolism of atrazine [4].

One of the goals of T3.4 is to engineer the model organism *Pseudomonas putida* KT2440 for the biodegradation of the pollutant lindane and atrazine. There is a well-established toolkit for the genetic engineering of *Pseudomonas* spp. and moreover, *P. putida* is a popular chassis for industrial biotechnology with a high tolerance to physicochemical stresses [5].

2 Materials and Methods

2.1 Synthetic construct design

2.1.1 *In silico* design strategy for lindane and atrazine biodegradative pathways

The nucleotide sequences of the genes encoding the lindane degradation pathway and ABC transporter system in *S. japonicum* UT26, as well as the genes involved in the atrazine degradation pathway in *Pseudomonas* sp. ADP, were obtained from the National Center for Biotechnology Information (NCBI) database. The Integrated DNA Technologies (IDT) Codon Optimization Tool (https://eu.idtdna.com/pages/tools/codon-optimization-tool) was used to generate coding sequences (CDSs) optimised for expression in *P. putida*. A 15 bp region upstream of each coding sequence (CDS) was extracted from the *S. japonicum* genome to include the native ribosome binding site (RBS), while a 20 bp upstream region was used for the genes in the atrazine degradation pathway. All genes and RBSs were arranged successively orientated in the forward direction. The constitutive pEM7 promoter was included upstream of the RBS region preceding *linA* or *atzA*. The complete transcription units for lindane and atrazine degradation, comprising 15 and six genes, respectively, along with their associated RBS regions, were inserted into the multiple cloning site (MCS) of the plasmid pSEVA221. This plasmid also carries a kanamycin resistance gene for selection of positive transformants and an RK2 origin of replication, enabling replication across a broad range of Gram-negative hosts [6]. This resulted in the 16.94 kb plasmid pSEVA221-lindane and the 12.38 kb plasmid pSEVA221-atrazine (**Fig. 3**).

Figure 3. pSEVA221-lindane and pSEVA221-atrazine plasmid map.

2.1.2 Construct synthesis and assembly

The lindane and atrazine transcription unit was synthesised by GenScript (genscript.com) using the GenBrick synthesis service with subcloning into the pSEVA221 vector. The plasmid vector was prepared using the QIAGEN Plasmid Midi Kit following the Quick-Start Protocol; the DNA quality and yield analysed by BioDrop spectrophotometer (biochrom.co.uk); and the plasmid supplied directly to GenScript for subcloning.

2.1.3 Introduction of additional genes into the constructs

An additional copy of the lindane-degrading dioxygenase LinE and missing *atzGH* genes involved in the last steps of atrazine metabolism were added to the constructs. To introduce *linEb* and *atzGH* into the pSEVA221-lindane and pSEVA221-atrazine plasmids, all genes were codon-optimised using the same IDT Codon Optimization Tool. Exceptions were made when the 15 or 20 bp upstream region overlapped with a coding sequence; in these cases, the original sequence was retained to preserve native regulatory elements.

All required gene fragments were synthesised by Twist Bioscience, with 20 bp overlaps included to facilitate DNA assembly. The constructs were assembled using the NEBuilder® HiFi DNA Assembly Kit (New England Biolabs) according to the manufacturer's instructions. The assembled plasmids were transformed into *E. coli* 5-α competent cells (New England Biolabs), following the recommended protocol. Colonies were screened by colony PCR, and positive clones were verified by full plasmid sequencing (Fullcircle). Confirmed colonies were inoculated into culture and archived as 15% glycerol stocks for long-term storage.

2.2 Microbial engineering

2.2.1 Transformation of *Escherichia coli* chemically competent cells

The synthetic DNA construct supplied by GenScript (pSEVA221-lindane and pSEVA-atrazine) was delivered to One Shot TOP10 Chemically Competent *E. coli* cells (ThermoFisher Scientific) using the Chemical Transformation Procedure. Positive transformants were selected for by plating transformation mixture onto LB agar (LB Broth with agar (Miller) 40 g/L) supplemented with 50 µg/mL kanamycin and incubating overnight at 37°C. Long-term stocks of the strain were prepared by mixing equal quantities of overnight liquid culture (inoculated with a single colony) and 30 % glycerol in a cryovial for storage at -80°C.

2.2.2 Transformation of *Pseudomonas putida* by tri-parental mating

The pSEVA221-lindane and pSEVA221-atrazine plasmids were introduced to *P. putida* KT2440 *via* tri-parental mating which is preferred for the transfer of large genetic constructs [7]. Briefly, overnight 5 mL cultures of the donor (the *E. coli* strain used for cloning of the constructs), recipient (*P. putida* KT2440 used for expression), and helper (carrying the pRK600 plasmid with the *oriT* required for mobilisation of the target plasmid [8,9]) strains were grown in LB broth (LB Broth (Miller) 25 g/L) with the appropriate antibiotic selection and growth conditions (**Table 1**). Following incubation, 300 µL of each strain were combined in a 1.5 mL microcentrifuge tube and the cells pelleted for 1 min *via* centrifugation. The cells were washed twice in 10 mM $MgSO_4$, and the resultant cell pellet resuspended in 20 µL 10 mM $MgSO_4$ and spotted onto an LB agar plate void of antibiotics. The plate was air dried then incubated overnight at 30°C to allow conjugation to occur. The following day, the cells were scraped from the plate using an inoculation loop and resuspended in 1 mL 10 mM $MgSO_4$; the suspension was then streaked onto selective medium (M9 agar + 10 mM citrate + 50 µg/mL kanamycin) and incubated at 30°C until transconjugant colonies appeared (1-2 days). M9 agar was prepared by combining 80 mL 5X M9 salts (M9 Minimal salts 56.4 g/L) with 320 mL ddH_2O autoclaved with 6 g agar giving a final concentration of 1.5% agar; additionally, the 400 mL 1X M9 agar was supplemented with 800 µL 1M $MgSO_4$, 40 µL 1M $CaCl_2$, 40 µL 10,000X vitamin solution (nicotinic acid 0.01 g/mL, thiamine 0.005 g/mL, pABA (aminobenzoic acid) 0.001 g/mL, biotin 0.1 mg/mL), 400 µL 1,000X trace elements ($ZnSO_4.7H_2O$ 0.1 g/L, $MnCl_2.4H_2O$ 0.03 g/L, H_3BO_3 0.3 g/L, $CoCl_2.6H_2O$ 0.2 g/L, $CuCl_2.2H_2O$ 0.01 g/L, $NiCl_2.6H_2O$ 0.02 g/L, $Na_2MoO_4.2H_2O$ 0.03 g/L).

Table 1. Strains and growth conditions for tri-parental mating.

Strain	Role	Antibiotic	Temperature
*E. coli*_pSEVA221-lindane or *E. coli*_pSEVA221-atrazine	Donor	kanamycin 50 µg/mL	37°C
P. putida KT2440	Recipient	None	30°C
E. coli HB101	Helper	chloramphenicol 34 µg/mL	37°C

2.2.3 Microbial engineering using the pMATING plasmid series

To propagate beneficial traits within a microbial community, plasmids conferring the ability to transfer between individual cells without the need for a helper strain can be implemented. To initially test the trait-propagation capability of pMATINGα-msfGFP, conjugations were performed using the *E. coli* strain carrying pMATINGα-msfGFP and *P. putida* KT2440. A collection of *E. coli* strains carrying plasmids from the pMATING series were obtained from Victor de Lorenzo's Molecular Environmental Microbiology Laboratory (CNB-CSIC, Madrid)[9]. Briefly, overnight cultures grown in 5 mL LB broth at 37°C and 30°C respectively were adjusted to an OD_{600} = 0.1. 1 mL of each diluted culture was spun to pellet the cells, and the pellet resuspended in 1 mL 10 mM $MgSO_4$. 100 µL aliquots of each strain were combined, 100 µL10 mM $MgSO_4$ added, the cells pelleted again, and the supernatant removed. The resulting pellet was resuspended in 20 µL 10 mM $MgSO_4$ and plated as a single drop onto an LB agar plate void of antibiotics, then incubated overnight at 30°C. Following incubation, cells were collected using an inoculation loop and resuspended in 1 mL 10 mM $MgSO_4$. 10-, 100-, 1,000-, and 10,000-fold dilutions were made and 50 µL of each was spread onto *Pseudomonas* isolation agar (PIA) (*Pseudomonas* isolation agar 45 g/L, glycerol 20 mL/L) supplemented with 50 µg/mL kanamycin then incubated overnight at 30°C.

2.3 Growth assays

2.3.1 Lindane toxicity assay

A range of media were prepared by supplementing 1 x M9 minimal medium (prepared as described in 2.2.2 but without agar) with different concentrations of lindane ranging from 0-5 mM; 0.4% glucose was also included to support growth of untransformed *P. putida* KT2440 lacking the pSEVA221-lindane plasmid. The conditions containing 0 mM lindane represent standard growth conditions used as a control. Growth assays were prepared as 1 mL cultures in a 24-well microtitre plate inoculated with 2 µL of an overnight *P. putida* KT2440 culture grown in LB broth at 30°C with shaking at 180 rpm. Growth in the 24-well plate was monitored at 15 min intervals for 24 h using a CLARIOstar® microplate reader to record absorbance at OD_{600} with incubation at 30°C and shaking at 200 rpm.

2.3.2 Lindane degradation assay

A range of media were prepared by supplementing 1 x M9 minimal medium (prepared as described in 2.2.2 but without agar) with different concentrations of lindane ranging from 0-5 mM to create a minimal growth medium with lindane as the sole carbon source; 50 µg/mL kanamycin was also included to select for *P. putida* KT2440_pSEVA-lindane. Conditions containing 0 mM lindane served as a negative control, in which no growth was expected due to the lack of an accessible carbon source. Growth assays were prepared as 1 mL cultures in a 24-well microtitre plate inoculated with 2 µL of an overnight *P. putida* KT2440_pSEVA-lindane culture grown in LB+kan at 30°C with shaking at 180 rpm. Growth in the 24-well plate was monitored at 15 min intervals for 24 h using a CLARIOstar® microplate reader to record absorbance at OD_{600} with incubation at 30°C and shaking at 200 rpm.

2.3.3 Degradation assay in the presence of different surfactants

Degradation assays were performed as described in Section 2.3.2, with the addition of surfactants. For lindane degradation, 5 mM or 10 mM lindane was used; for atrazine, a 5 mM concentration was applied. Surfactants tested included rhamnolipid, Triton X-100, and Tween 80, added to the cultures at final concentrations ranging from 0.1% to 1%. Growth and degradation were monitored in 24-well plates using a CLARIOstar® microplate reader, recording OD600 at 15-minute intervals over 24 hours. Cultures were incubated at 30°C with shaking at 200 rpm.

2.3.4 Degradation assay in the glass vials

Degradation assays were also performed in 2 mL screw-top vials with caps (Agilent). Growth setups were prepared similarly to those described in Section 2.3.1. *P. putida* KT2440 strains carrying the plasmids were cultured in 1× M9 minimal medium supplemented with 0.33 mM lindane as the sole carbon source or 0.28 mM atrazine as the sole nitrogen source depending on whether they carried, respectively, the plasmids for lindane or atrazine mineralisation. Cultures were incubated at 30°C with shaking at 200 rpm, and growth was monitored over seven days.

2.3.5 Degradation assay in valerite nanoparticles

Vaterite particles were prepared by controlled precipitation of calcium chloride and sodium carbonate, loaded with hydrophobic compounds (atrazine or lindane) via freeze-thaw cycles, and dried overnight at 60°C (see detailed protocol in D4.2 Annex III).

P. putida KT2440 strains (wild-type, empty vector control, and engineered strains with atrazine/lindane degradation plasmids) were maintained at -80°C and cultured on LB medium with kanamycin (50 µg/mL) as needed. Pre-cultures were grown in LB broth (3-4 days, 30°C, 250 rpm) then transferred to M9 medium with 0.02% glucose. Cells were harvested, washed with 0.9% NaCl, and stained with SYTO 11 (5 µM, 40 min, dark). Vaterite particles (10 mg) were resuspended in 2 mL M9 medium by vortexing and sweeping (7 min, repeated once).

Assays were performed in 2 mL tubes with bacterial inoculum at OD_{600} 0.5-0.6, incubated at 30°C with shaking. Growth was monitored by flow cytometry measuring FL1-A fluorescence intensity. Cell growth was determined by SYTO fluorescence decay due to dye dilution during cell division. Relative fluorescence decay was calculated as: Relative decay (%) = (MFI_0 - MFI_x / MFI_0) × 100%

2.4 Activity assays

2.4.1 Halo formation assay

P. putida KT2440 strains carrying pSEVA221-lindane or pSEVA221-atrazine were grown overnight from glycerol stocks in LB broth. 10 µL of each overnight culture were spotted onto small Petri dishes containing LB agar supplemented with 10 mM lindane or atrazine. Each strain was tested on both substrates, alongside one another, serving as a negative control on the non-cognate compound. Plates were incubated at 30°C, and halo formation was monitored over seven days.

2.4.2 Colourimetric assay for LinE activity

The enzyme LinE, involved in the lindane degradation pathway, catalyses the conversion of hydroquinone into γ-hydroxymuconic semialdehyde (γ-HMSA), which produces a characteristic yellow coloration. KT2440 strains carrying pSEVA221-lindane and a negative control strain were cultivated overnight on LB agar plates. The colonies were then sprayed with 0.5 M hydroquinone solution, and colour changes were observed over a 2-hour period at room temperature.

2.4.3 Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS - PAGE)

To test the expression of different degradation relevant proteins in the engineered strains, SDS-PAGE was performed to assess the expression of degradation pathway proteins in the engineered strains, analysing both intracellular and extracellular fractions.

Overnight cultures (20 mL) were centrifuged at 13,000 rpm for 30 minutes. For extracellular proteins, 1 mL of supernatant was concentrated twice using Pierce™ PES Protein Concentrators (3000 Da cut-off, Thermo Fisher) at 13,000 rpm for 1 hour. Intracellular proteins were prepared by washing cell pellets with PBS, followed by sonication (Sonics VCX-130, 50% amplitude, six cycles of 10 s on/10 s off). Lysates were centrifuged to separate soluble proteins from debris, with the pellet resuspended in 4 M urea. Protein concentrations were measured using a NanoDrop spectrophotometer. Samples containing 15 µg of protein in 15 µL of water were denatured at 80°C for 10 minutes, then mixed with Tris-SDS sample buffer (TSS) containing 0.1% SDS. Protein samples (30 µL) from three biological replicates were loaded onto Invitrogen™ Novex™ 10% Tricine Mini Protein Gels and run at 160 V for 1 hour. Gels were stained with Coomassie Brilliant Blue and destained with water.

3 Results

3.1 Engineering microbial strains for biodegradation of pollutants

3.1.1 Synthesis of pSEVA221-lindane and pSEVA221-atrazine

pSEVA221-lindane and pSEVA221-atrazine were successfully synthesised by GenScript and delivered to *E. coli* for preservation and subsequent delivery to *P. putida.* Colonies containing the plasmid were obtained through growth on selective media (LB agar+kan). Overnight cultures from single colonies were grown in LB broth+kan, combined in equal volumes with 30% glycerol, and stored at -80°C in cryovials for preservation.

3.1.2 Engineering microbial strains for pollutant degradation

pSEVA221-lindane and pSEVA221-atrazine were successfully delivered to *P. putida* KT2440 *via* tri-parental conjugation, and transconjugants were obtained through growth on selective medium (M9 agar+citrate+kan) to counter select the donor and helper strains. Overnight cultures from single colonies were grown in LB broth+kan, combined in equal volumes with 30% glycerol, and stored at -80°C in cryovials for preservation.

3.1.3 Lindane toxicity and degradation assays

Preliminary assessment of the lindane degradation system was initially performed *via* growth assays to test for both lindane toxicity and the ability to use lindane as a sole carbon source. Untransformed *P. putida* KT2440 was grown in M9+glucose supplemented with a range of lindane concentrations. Engineered *P. putida* KT2440_pSEVA-lindane was grown in M9 minimal medium supplemented with a range of lindane concentrations as the sole carbon source (**Fig. 4**). Lindane exhibited poor solubility in M9 minimal medium, and as a result, undissolved solids in the medium contributed to a degree of noise in the plate reader measurements. Despite this, in the toxicity assay, the growth of *P. putida* KT2440 in M9+glucose without lindane was comparable to growth where lindane was also included. This suggests that the inclusion of lindane in growth assays at concentrations of 5 mM and below does not have a negative impact in growth.

3.1.4 Improved degradation assays

To reduce background noise caused by lindane precipitation, we tested the growth of P. putida KT2440 carrying pSEVA221-lindane in the presence of 10 mM lindane, compared to 10 mM glucose, using filtered media to remove undissolved particles. No significant growth was observed in the lindane condition (**Fig. 5**).

At this stage we incorporated atrazine as a growth substrate for the strain containing the pSEVA-atrazine plasmid. It is worth noting that atrazine is typically used as a nitrogen source, hence we tested growth in the presence of atrazine, or atrazine with glucose and no additional ammonia in the culture. Given the low aqueous solubility of both lindane and atrazine, we next explored strategies to increase substrate availability. One approach was the addition of surfactants to the medium. Initially, 0.1% rhamnolipid was added to lindane cultures, which resulted in a noticeable increase in growth (**Fig. 6**).

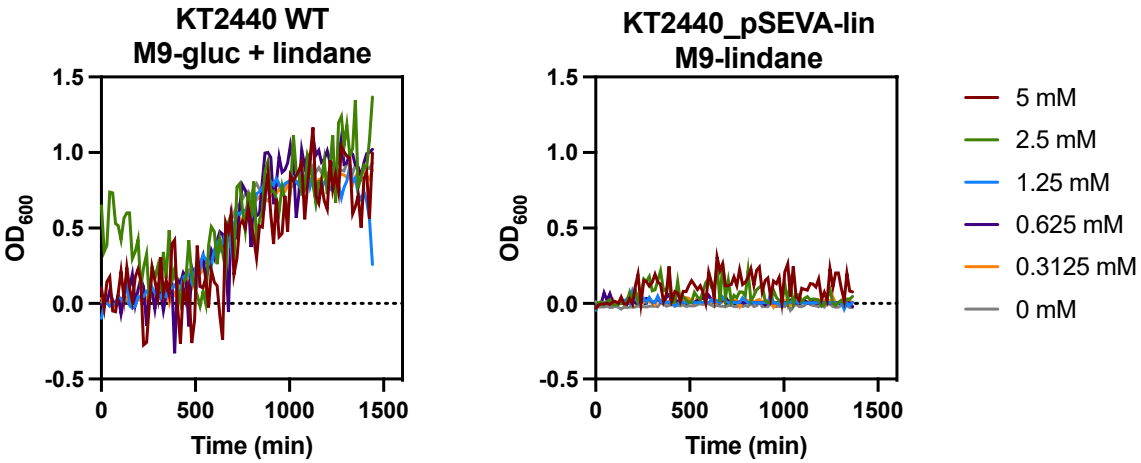

Figure 4. Growth assays to assess lindane toxicity in *P. putida* KT2440 (left), and the ability of engineered *P. putida* KT2440_pSEVA221-lindane to use lindane as a sole carbon source (right).

We then expanded the tests to include Triton X-100 and Tween 80, increasing concentrations up to 1%, and applied the same approach to cultures of the atrazine-degrading strain. The addition of rhamnolipid and Tween 80 dramatically enhanced the growth of the lindane-degrading strains. However, for atrazine cultures, interactions between the surfactants and atrazine led to culture turbidity, complicating growth measurements (**Fig. 7**).

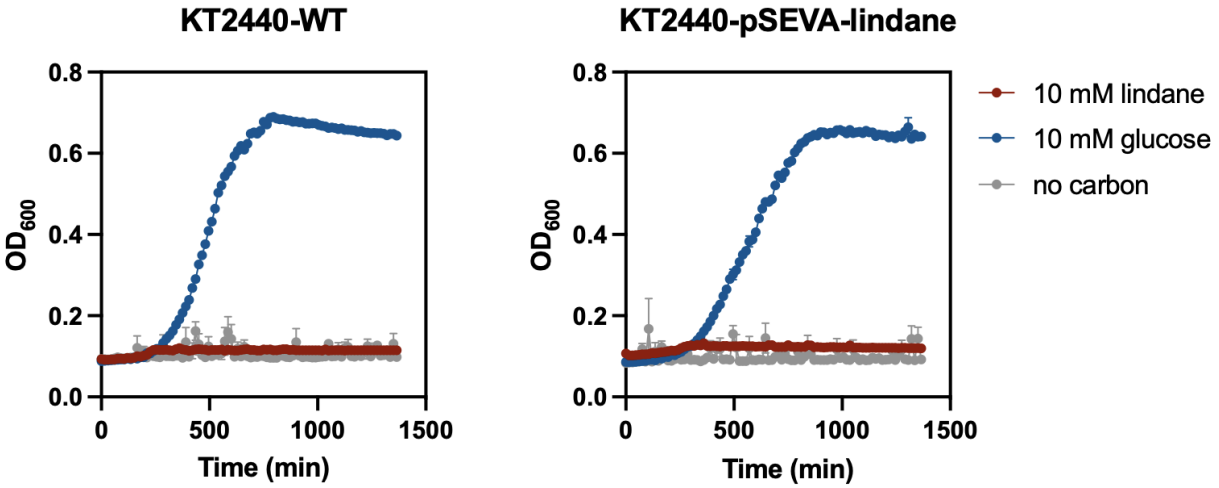

Figure 5. Growth assays to assess lindane degradation in *P. putida* KT2440 (left) and the engineered *P. putida* KT2440_pSEVA221-lindane (right) after filtration.

To determine whether the surfactants themselves could support bacterial growth, we subsequently tested *P. putida* strains in media containing 1% rhamnolipid, Tween 80 or Triton X-100 as the sole carbon source. Both rhamnolipid and Tween 80 supported significant growth, indicating that the observed increases in biomass in the degradation assays were largely attributable to the additional carbon provided by the surfactants, rather than enhanced lindane degradation alone (**Fig. 8**).

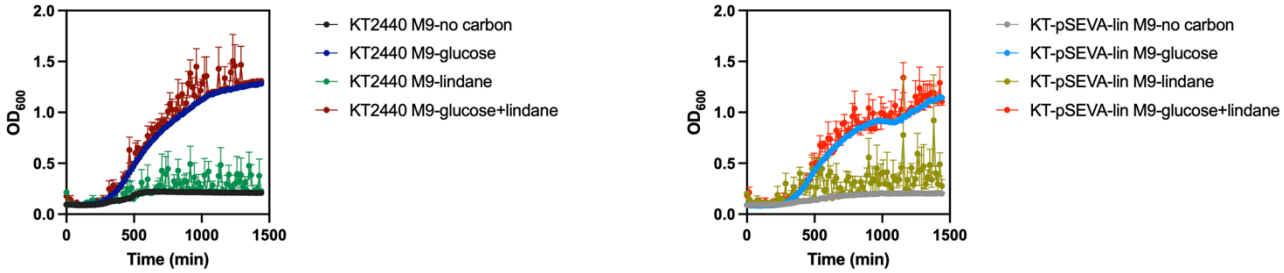

Figure 6. Preliminary growth assay comparing cultures without (left) and with (right) the addition of 0.1% rhamnolipids in M9 minimal medium supplemented with 10 mM of different carbon sources.

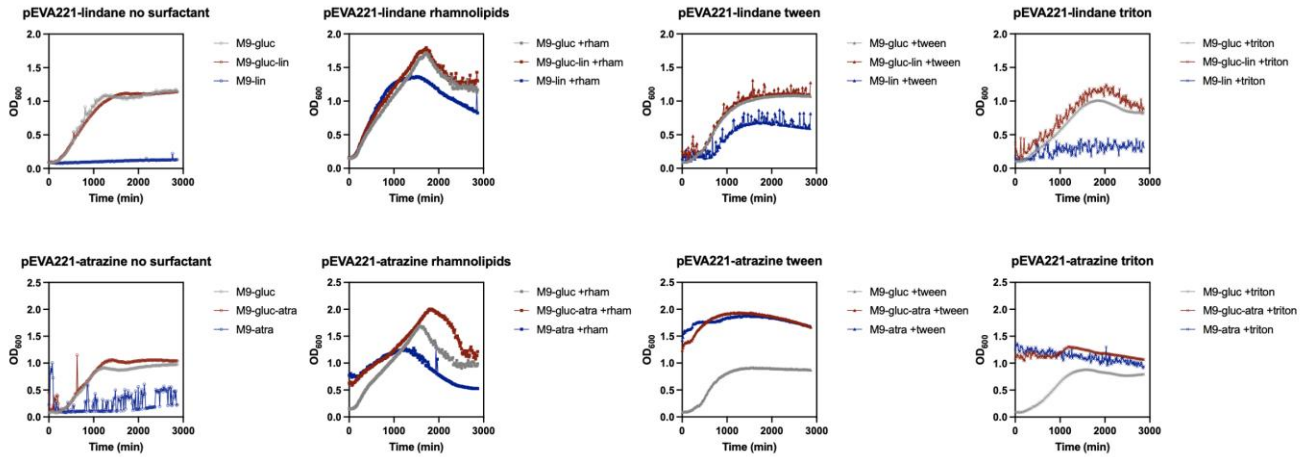

Figure 7. Growth of P. putida KT2440 carrying pSEVA221-lindane and pSEVA221-atrazine on different media conditions: M9-glucose, M9-glucose supplemented with pollutants, and M9 with pollutants alone. Cultures were tested with a 1% supplementation of various surfactants.

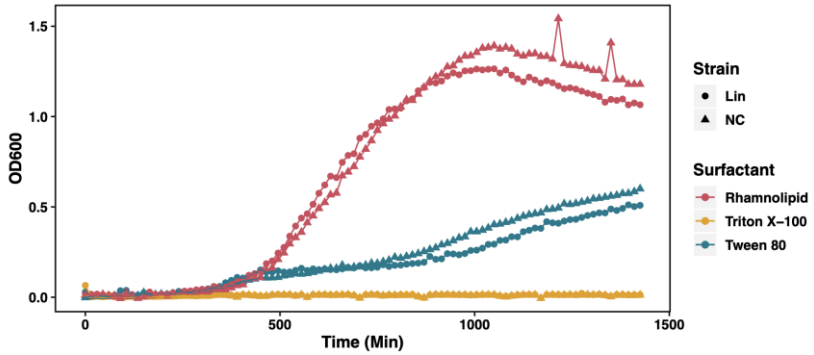

Figure 8. Growth of *P. putida* KT2440 carrying pSEVA221-lindane and empty pSEVA221 vector in M9 minimal medium supplemented with 1% of various surfactants. Lin stands for lindane while NC is the negative control containing the empty plasmid.

In a further effort to reduce experimental artifacts such as pollutant adhesion to plastic surfaces, we also cultured *P. putida* KT2440 carrying pSEVA221-lindane, pSEVA221-atrazine, and the empty vector control in glass vials. No growth was observed over one week of incubation.

Despite our efforts, we could not achieve a detectable solubility of lindane in aqueous culture media and this was confirmed by HPLC analyses. However, considering the excellent results of JSI isolating microorganisms on valerite nanoparticles coated with hydrophobic substrates described in previous tasks, we decided to test this system to monitor the growth of the engineered strains (**Fig. 9**). We observed an interesting phenotype with control strains not being able to grow on the nanoparticles, despite the use of glucose as a positive control. However, the *lin* containing strains did exhibit limited growth, albeit insignificant. These results, show promise and the method will continue to be implemented and validated in the coming months.

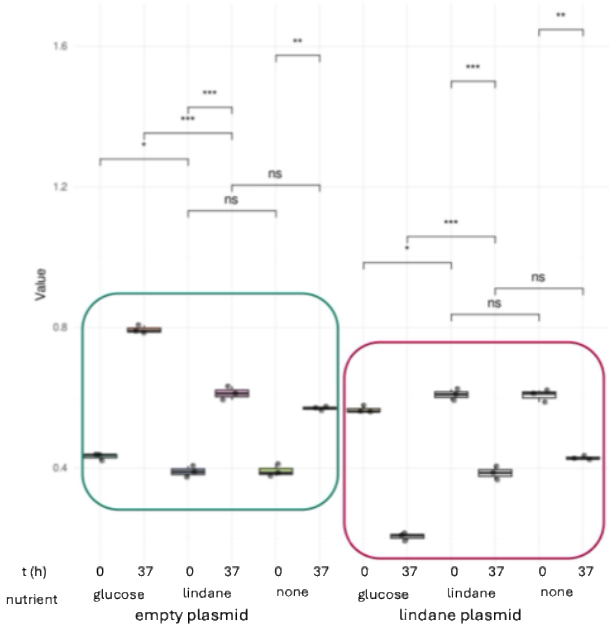

Figure 9. Changes in fluorescence intensity due to growth on lindane coated nanoparticles. Microbial growth in this system developed by JSI is observed through the dilution (decrease in fluorescence) of a fluorophore used

to stain the seeding population of cells after 37 h of incubation. The green box refers to control *P. putida* KT2440 containing the empty pSEVA221 plasmid and the red box shows the strains contain the lindane degradation pathway. This strain shows a decrease in fluorescence (mild growth) in all carbon sources tested including in the presence and absence of lindane

3.1.5 Testing the activity of key proteins in the pathway

Our results show that both pesticides can not be used as carbon sources by the engineered strains in the conditions tested. However, this does not mean that they could not be at least partially degraded by the enzymes recombinantly expressed. We evaluated this possibility by determining the presence of activities of enzymes present in both pathways. To this end we cultured cells carrying the recombinant genes in plates containing rich medium supplemented with either lindane or atrazine.

No halo was observed for *P. putida* KT2440 carrying pSEVA221-lindane. Further colorimetric assays also showed no detectable LinE activity, indicating that the *linE*-encoded step was non-functional under the current design. To address this, we introduced an additional copy of *linEb* into the pathway as *S. japonicum* contains two homologues of these genes (**Fig. 1**) to investigate whether this modification could improve the degradation capacity in the future.

In contrast, we observed the presence of a clear halo *P. putida* KT2440 carrying pSEVA221-atrazine when grown on LB agar containing atrazine (**Fig. 10**). This suggests that the initial enzymes of the *atz* pathway are functional.

While this result is important and points to atrazine degradation, it does not explain why the molecule can not be used for growth. Upon detailed analysis of the genome cluster described in the literature, we identified two open reading frames (ORFs) neglected in previous publications and likely coding for functional subunits of the biodegradative enzymes. We decided to extend the construct by including these two additional genes, *atzG* and *atzH* (**Fig. 11**), to test whether this would enable complete atrazine degradation and support growth using atrazine as the sole nitrogen source[10].

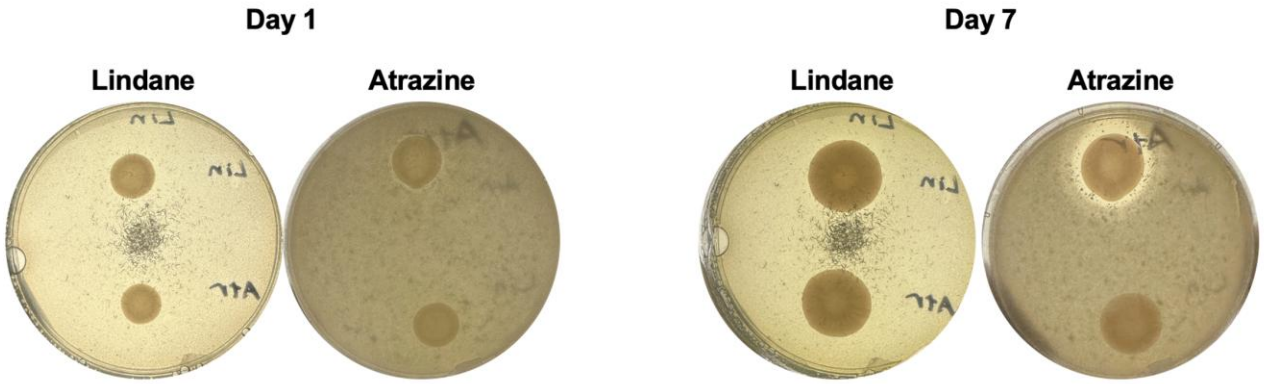

Figure 10. Halo formation assay of KT2440 carrying pSEVA221-lindane or pSEVA221-atrazine. Images were taken after one day and seven days of incubation.

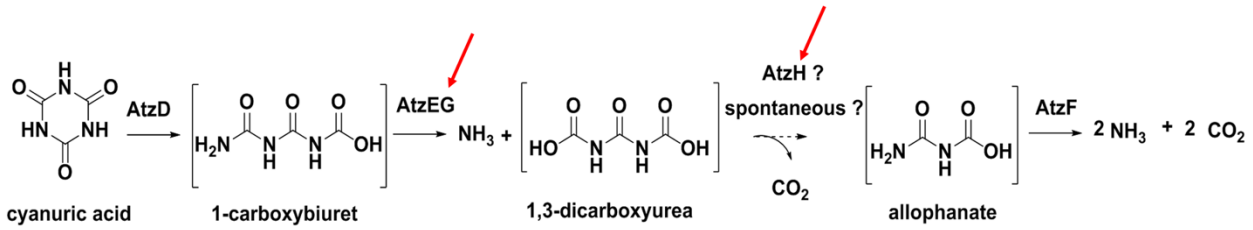

Figure 11. The additional two genes in the atrazine degradation pathway from *Pseudomonas* sp. ADP.

3.1.6 Growth assay of atrazine degradation strain with the additional genes

Following the introduction of *atzG* and *atzH* into the atrazine degradation pathway, we performed a growth assay using cyanuric acid, a more soluble intermediate of atrazine degradation, as the sole nitrogen source.

The new construct demonstrated significantly faster growth compared to the strain carrying the empty vector, indicating that the additional genes improved the strain's ability to metabolize cyanuric acid (**Fig. 12**). These results suggest that extending the *atz* pathway enhances atrazine degradation and may enable complete nitrogen assimilation from the compound.

3.1.7 Protein expression in the degradation strains

We also investigated the expression of the genes present in the recombinant plasmids for both linane and atrazine by extracting total proteins from the engineered strains and compared them to a strain carrying the empty vector.

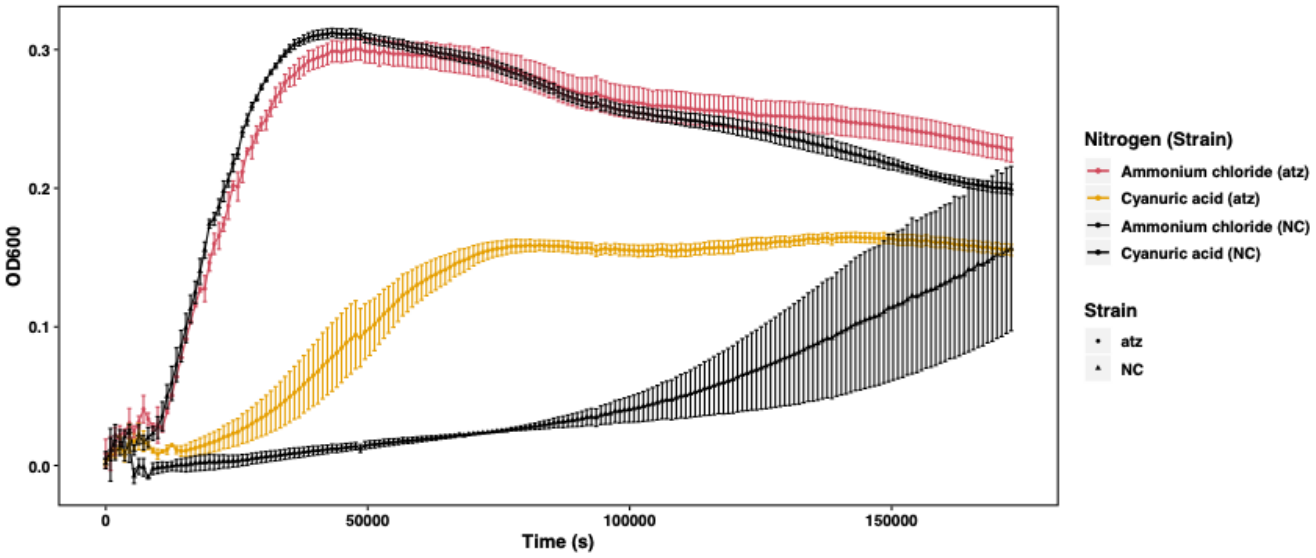

Figure 12. Growth of *P. putida* KT2440 carrying the pSEVA221-atrazine (harbouring *atzG* and *atzH*) and empty pSEVA221 vector in M9 minimal medium supplemented with either ammonium chloride (positive nitrogen source control) or cyanuric acid (test nitrogen source). Atz stands for the strain carrying the atrazine degradation genes while NC is the negative control containing the empty plasmid.

No distinct overexpressed proteins were detected in the intracellular soluble or insoluble fractions, nor in the extracellular protein samples (**Fig. 13**). One possible explanation is that pSEVA221 carries a low-copy RK2 origin of replication, which may limit expression levels. Moreover, the sensitivity of PAGE-

SDS is low and samples will be analysed using proteomics (mass spectrometry) in the near future. Further work will include testing the constructs in a host strain optimised for protein expression that has been developed by our group in a different project and successfully validated for the recombinant expression of biosynthetic pathways for several molecules including natural dyes (violacein), surfactants (rhamnolipids) and siderophores (ferrioxamines).

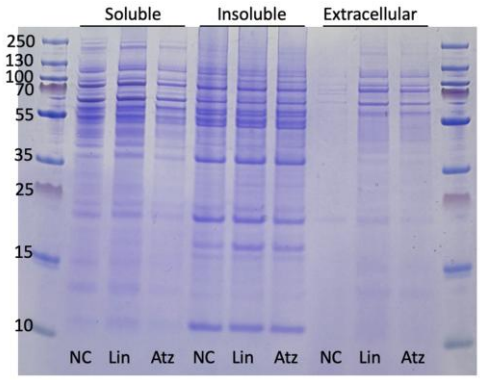

Figure 13. SDS-PAGE analysis of soluble, insoluble, and extracellular protein fractions extracted from Pseudomonas putida KT2440 carrying the empty pSEVA221 vector, pSEVA221-lindane, or pSEVA221-atrazine.

3.2 Engineering microbial strains for trait propagation

3.2.1 Validation of self-transferring plasmids

The pMATING series of plasmids have been constructed to contain the promiscuous conjugation machinery found in the RP4 plasmid. Microbial strains carrying variants of the pMATING plasmid series are able to transfer these plasmids to other individuals without the need for a helper strain. The long-term application of the pMATING plasmids is to engineer plasmid variants to carry degradation pathways for pollutants of interest, such that desirable traits are able to propagate within microbial communities.

Delivery of pMATINGα-msfGFP to P. putida KT2440

To confirm the activity of the RP4 machinery, the pMATINGα-msfGFP plasmid was used in initial tests to confirm that the plasmid is able to move between microbial strains without the need for a helper strain. The conjugation assay (2.2.3) was performed using *P. putida* KT2440 both with and without the addition of the *E. coli* strain carrying the pMATINGα-msfGFP plasmid. Dilution series of the conjugation mix were plated side-by-side onto M9+citrate and M9+citrate supplemented with kanamycin to select for the engineered individuals (M9-citrate provides an accessible carbon source to *P. putida* but not *E. coli*, so therefore counter selects for the recipient strain). Fluorescent colonies expressing the msfGFP protein were visible only where pMATINGα-msfGFP had been included in the transformation (**Fig. 14**). Comparisons of the plated transformation provide a qualitative indication of the efficiency of the conjugation.

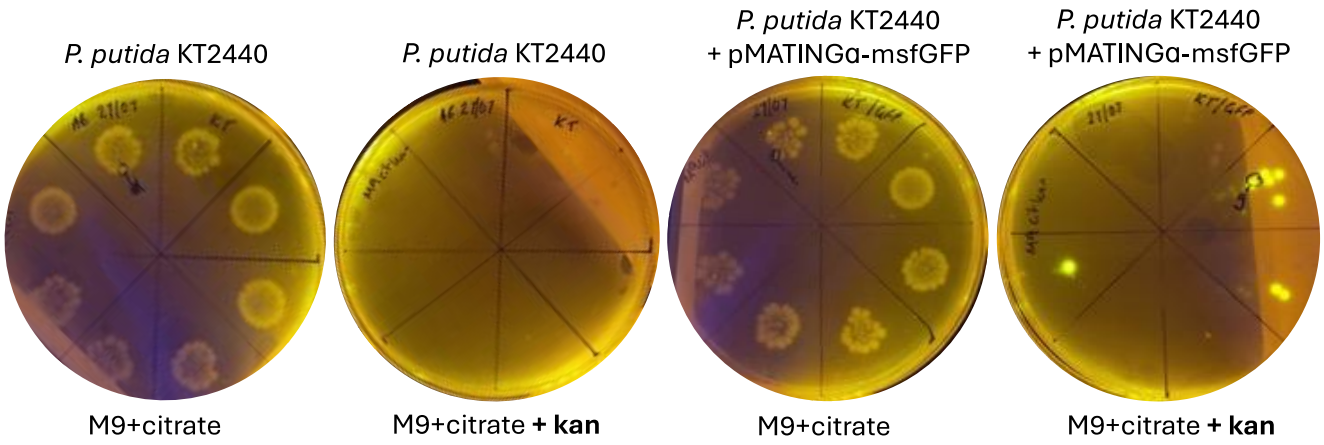

Figure 14. Dilution series of *P. putida* conjugations with and without *E. coli*_pMATINGα-msfGFP plated onto M9+citrate with and without kanamycin supplementation. Colonies expressing msfGFP are visible where the conjugation mixture was plated onto selective medium supplemented with kanamycin (right).

4 Conclusions

The pSEVA221-lindane and atrazine plasmids have been successfully constructed and modified with additional activities, and delivered to *P. putida* KT2440. The inclusion of lindane in culture medium to a maximum concentration of 5 mM does not have a negative impact on growth of the wild-type strain, however, solubility the pesticides – and especially lindane – is low in water and the use of common and biocompatible surfactants did not significantly improve the bioavailability of the substrates, leaving room for better formulations of culture media with more appropriate solvents. The initial set of engineered *P. putida*_ pSEVA221-lindane did not show evidence of growth using lindane as a sole carbon source or atrazine as nitrogen sources. However, when lindane was assessed in a nanoparticle assay designed for hydrophobic substrates, there was preliminary evidence of assimilation of the substrate albeit this is inconclusive.

No lindane related enzymatic activities was detected in the initial design of the *lin* plasmid while the *atz* construct exhibited evident clearing halos indicating removal of atrazine. This prompted the construction of a second generation of plasmids with additional genes for both substrates. These constructs are in the process of validation, but the new *atz* strains shows great promise as demonstrated by its growth using cyanuric acid, an intermediate of atrazine degradation, as sole nitrogen source. These newly generated strains will be sent to UBU in the coming days for their testing in microcosm experiments.

In parallel to the biodegradation of molecules relevant to BIOSYSMO, we have validated the use of the RP4 propagation machinery present in the pMATING plasmid series. This has been tested using an *E. coli* strain carrying the pMATINGα-msfGFP plasmid which results in msfGFP expression and kanamycin resistance in successfully engineered individuals. *P. putida* KT2440 was successfully engineered using conjugation without the need for a helper strain. The efficiency of the conjugation was not particularly high, as can be observed by comparing the relative abundance of msfGFP fluorescent colonies in the plated transformations on plates with and without kanamycin. Upgraded lindane and atrazine pathways will be transferred to this system once their functionality has confirmed, for the genetic augmentation of natural communities.

5 References

1. Vega, M., Romano, D. & Uotila, E. Lindane (persistent organic pollutant) in the EU.

2. Nagata, Y. *et al.* Complete Genome Sequence of the Representative ⁄-Hexachlorocyclohexane-Degrading Bacterium Sphingobium japonicum UT26.

3. Nagata, Y., Endo, R., Ito, M., Ohtsubo, Y. & Tsuda, M. Aerobic degradation of lindane (gamma-hexachlorocyclohexane) in bacteria and its biochemical and molecular basis. *Appl Microbiol Biotechnol* **76**, 741–752 (2007).

4. Shapir, N. *et al.* Evolution of Catabolic Pathways: Genomic Insights into Microbial s -Triazine Metabolism. *J Bacteriol* **189**, 674–682 (2007).

5. Martínez-García, E. & De Lorenzo, V. Pseudomonas putida as a synthetic biology chassis and a metabolic engineering platform. *Current Opinion in Biotechnology* **85**, 103025 (2024).

6. Martínez-García, E. *et al.* SEVA 3.0: an update of the Standard European Vector Architecture for enabling portability of genetic constructs among diverse bacterial hosts. *Nucleic Acids Research* **48**, D1164–D1170 (2020).

7. de Lorenzo, V. & Timmis, K. N. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Methods Enzymol* **235**, 386–405 (1994).

8. Finan, T. M., Kunkel, B., De Vos, G. F. & Signer, E. R. Second symbiotic megaplasmid in Rhizobium meliloti carrying exopolysaccharide and thiamine synthesis genes. *J Bacteriol* **167**, 66–72 (1986).

9. Coupland, G. M., Brown, A. M. C. & Willetts, N. S. The origin of transfer (oriT) of the conjugative plasmid R46: Characterization by deletion analysis and DNA sequencing. *Mole Gen Genet* **208**, 219–225 (1987).

10. Esquirol, L. *et al.* Structural and biochemical characterization of the biuret hydrolase (BiuH) from the cyanuric acid catabolism pathway of Rhizobium leguminasorum bv. viciae 3841. *PLOS ONE* **13**, e0192736 (2018).