Providing octane degradation capability to *Pseudomonas putida* KT2440 through the horizontal acquisition of oct genes located on an integrative and conjugative element

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Abstract
The extensive use of petrochemicals has produced serious environmental pollution problems; fortunately, bioremediation is considered an efficient way to fight against pollution. In line with Synthetic Biology is that robust microbial chassis with an expanded ability to remove environmental pollutants are desirable. *Pseudomonas putida* KT2440 is a robust lab microbe that has preserved the ability to survive in the environment and is the natural host for the self-transmissible TOL plasmid, which allows metabolism of toluene and xylenes to central metabolism. We show that the *P. putida* KT2440 (pWW0) acquired the ability to use octane as the sole C-source after acquisition of an almost 62-kb ICE from a microbial community that harbours an incomplete set of octane metabolism genes. The ICE bears genes for an alkane monooxygenase, a PQQ-dependent alcohol dehydrogenase and aldehyde dehydrogenase but lacks the electron donor enzymes required for the monooxygenase to operate. Host rubredoxin and rubredoxin reductase allow metabolism of octane to octanol. Proteomic assays and mutants unable to grow on octane or octanoic acid revealed that metabolism of octane is mediated by redundant host and ICE enzymes. Octane is oxidized to octanol, octanal and octanoic acid, the latter is subsequently acylated and oxidized to yield acetyl-CoA that is assimilated via the glyoxylate shunt; in fact, a knockout mutant in the aceA gene, encoding isocitrate lyase was unable to grow on octane or octanoic acid.

INTRODUCTION
Over the last century, the chemical industry has been based on the exploitation of petrochemicals as fuels for combustion and as raw materials for the synthesis of a wide range of compounds. Many of these chemicals reach the environment, and while some are degraded others remain in soils or water either because of their recalcitrance or their rate of deposit is still far superior to that of removal (Danso et al., 2019; Mori & Kanaly, 2021; Nagata et al., 2019). Pollution related to petrochemical use has led to global warming and the search is now on to replace polluting chemicals with new green alternatives (Calero & Nikel, 2019; García-Franco et al., 2021; Godoy et al., 2021; Ramos & Duque, 2019); although the new (bio)chemistry for production of chemicals is still developing (Brooks & Alper, 2021; Liu et al., 2020; Reed & Alper, 2018).

Bioremediation is an efficient way to fight against pollution and to warrant environmental performance, biodegradation platforms that are able to remove multiple pollutants are of interest. In the selection of such
microorganisms, it must be noted that some pollutants are intrinsically toxic (Beites & Mendes, 2015; Ramos et al., 2015; Whyte et al., 1997). Hence, the selection of biodegradation platforms should be based not only on the basic metabolic potential of the microorganisms but also on their endurance and stress responses. Current legislation limits the use of recombinant microbes in the environment and for this reason microbes that have naturally acquired catabolic genes for removal of pollutants should be considered as a preferential way to build new pathways to expand their metabolic potential (Ramos & Timmis, 1987; Reineke & Knackmuss, 1988).

The soil bacterium *Pseudomonas putida* survives well in soils and is highly tolerant to hydrocarbons and xenobiotic compounds. Thus, it has the potential to serve as a robust platform for the degradation of chemicals. Pangenome analysis of the species has revealed a series of core genes that provide it with a versatile central metabolism, and which enable it to metabolize natural and xenobiotic compounds. In addition, strains of this species are endowed with a series of efflux pumps that extrude toxic chemicals from the cytoplasm, the periplasm and the outer membrane to the surrounding medium to provide a defensive mechanism against toxic chemicals (reviewed by Udaondo et al., 2012; Ramos et al., 2015; Udaondo et al., 2016). Furthermore, their metabolic potential allows bacteria of this species to adapt to rapidly changing conditions (e.g. oxidative stress, temperature challenges and sudden osmotic perturbations) (Cuenca et al., 2016b; Matilla et al., 2007; Molina-Santiago et al., 2014; Nikel & de Lorenzo, 2013; Nikel & de Lorenzo, 2014; Nogales et al., 2020). As such, the core functions define the *P. putida* species as a robust chassis for environmental survival and operation (Belda et al., 2016; Molina et al., 2000; Nogales et al., 2020; Udaondo et al., 2016). Analysis of *P. putida* accessory genes also revealed a number of catabolic properties shared by two or more strains but not all, for example, chromosomally encoded pathways for degradation of aromatic hydrocarbons—the *tod* pathway (Zylstra & Gibson, 1989) or pathways linked to self-transmissible plasmids—the TOL plasmid for degradation of toluene via the toluene monoxygenase pathway (Worsey & Williams, 1975).

*Pseudomonas putida* KT2440 strain is a derivative of *P. putida* mt-2, which was isolated as a degrader of 3-methylbenzoic acid from a soil sample in Japan in 1960 (Nakazawa, 2002). This metabolic property was linked to the presence of the catabolic self-transmissible TOL plasmid pWW0, which encodes a set of enzymes that enable *P. putida* to grow on several aromatic hydrocarbons such as toluene, *m*-xylene and *p*-xylene (Bagdasarian et al., 1981; Worsey & Williams, 1975). The strain has also been subjected to mutagenesis and subsequent selection to isolate clones able to degrade recalcitrant chemicals such as *p*-ethylbenzoate (Ramos et al., 1987). *Pseudomonas putida* KT2440 strain is a well-characterized member of this species and has become a model laboratory microbe for bioremediation. It has also retained its ability to survive and thrive in edaphic and aquatic environments despite a long history in the laboratory (Molina et al., 2000). The strain was included as part of a bacterial consortium to remove petroleum-based hydrocarbons from soils (Pizarro-Tobias et al., 2015). In this niche it is resilient; field tests revealed that the strain could survive for months in the roots of plants (Molina et al., 2000; Niqui-Arroyo et al., 2013; Ronchel & Ramos, 2001). *In silico* analysis of the 6,181,873 bp long genome sequence of *P. putida* KT2440 (Belda et al., 2016; Nelson et al., 2002) revealed the lack of any virulence factor in the genome, which was the basis of the early designation of the strain as a generally safe host for recombinant DNA constructs and use in the environment (Kampers et al., 2019; Nikel & de Lorenzo, 2018; Poblete-Castro et al., 2013; Poblete-Castro et al., 2020; Timmis, 2002). The genome of KT2440 encodes a broad array of enzymes that can remove oxygen-free radicals, i.e. superoxide dismutase, peroxidases and other enzymes capable of dealing with oxidative agents.

In a previous study, Niqui-Arroyo et al. (2013) described a consortium of microorganisms that grew well with a wide range of linear (i.e. octane, decane, dodecane) and branched hydrocarbons present in wastewaters generated by cleaning airport runways. Analysis of the 16S rRNA amplicons of this consortium revealed a wide range of microbes belonging to genus *Acinetobacter*, *Brevibacterium*, *Sphingobium*, *Cupridiaviridae*, *Yersinia*, *Alteromonas* and others. Unfortunately, certain limitations prevent the direct use of the consortium as a bioremediation agent. Namely, some of the microbes have potentially pathogenic characters and certain bacteria could not be recovered and cultured on solid agar plates. To overcome these limitations, we hypothesized that it would be possible to rescue the catabolic potential of the bacteria in hydrocarbon-enriched samples (i.e. the ability to degrade linear hydrocarbons) by incorporating the corresponding genes via natural gene transfer into the KT2440 (pWW0) chassis (Nogales et al., 2020). This was carried out to enhance the ability of KT2440 to catabolize and degrade pollutants.

**RESULTS AND DISCUSSION**

**Achieving octane degradation in *Pseudomonas putida* KT2440 (pWW0)**

Because some of the octane-degrading microorganisms in the consortium did not grow on solid medium (Niqui-Arroyo et al., 2013) we chose to carry out liquid culture matings to transfer genes to KT2440 and enable
the strain to grow on linear hydrocarbons. We set up liquid mating by mixing 1 ml of KT2440 (pWW0) grown with toluene as the sole C-source and 1 ml of the consortium supplied by Bio-Iliberis R&D. The mixed cultures were kept at 30°C with soft agitation. To counter select the KT2440 (pWW0) capable of degrading octane, we spread 0.1 ml of the mating culture on M9 minimal medium plates with octane as the sole C-source and 30 μg ml⁻¹ of rifampicin—an antibiotic that KT2440 is resistant to but that none of the consortium bacteria is resistant to. The rate of appearance of Rif⁺ Oct⁺ colonies on these plates was 1 out 10⁸ per recipient cell.

To test if the colonies on the plates were derived from KT2440 (pWW0), we performed a series of quick metabolic tests which consisted of checking that the cells used three carbon sources: (i) toluene, which is assimilated through the upper and meta cleavage pathway of the TOL plasmid present in the original recipient cells (Worsey & Williams, 1975); (ii) citrate, a tricarboxylic acid that P. putida is able to transport into the cytoplasm and is used as a sole carbon and energy source (Herrero et al., 1990); and (iii) the newly acquired property of degrading octane. In all cases all strains were positive. To further assure that KT2440 was the host, we used the 27F (5'-AGAGTTTATCCTGCTGAGG-3') and 1492R (5'-GTCCTCAGCGGCCGCGG-3') primers and amplified the 16S rRNA gene. The sequences we obtained matched >99% to those of P. putida KT2440, which led us to believe that KT2440 (pWW0) had acquired the genes involved in the degradation of octane. Because matings were kept for 48 h, we cannot discard siblings and for this reason a random colony, which we named EM2-4, was chosen for further characterization. The EM2-4 strain was able to grow exponentially on octane with duplication times of 105–120 min and yields of about 0.36–0.4 CDW g⁻¹ octane. Surprisingly the strain did not use any other medium-chain or branched hydrocarbons.

It is known that P. putida cells exposed to hydrocarbons fortify their membranes by isomerization of both C16 and C18 cis fatty acids to the trans forms. In agreement with this observation is that the cis:trans ratio of EM2-4 changed from about 5.9–7.8 in glucose grown cells to 1.04–1.14 in cells growing on octane (Table S1). Similar results have been reported before for several Pseudomonas strains (Chen et al., 1995). As such, the acquisition of the ability to grow on octane at a high rate does not preclude one of the toxic chemical stress responses of the P. putida KT2440 strain.

Sequence analysis of the genome of P. putida EM2-4

We sequenced the genome of strain EM2-4 using Illumina and a genome coverage of 200×. The 6.2 Mb genome was comprised of 78 contigs with sizes ranging between 43,286 and 549,539 bp. We found a contig of 66,272 bp that contained a 61,974 bp insert with flanking chromosomal DNA, which allowed us to identify that the set of genes was on the chromosome and not on the TOL plasmid. In fact, the genetic element gained was located between PP_4491 (phhB), which encodes a pterin-4-alpha-carbinolamine dehydratase involved in the folate biosynthetic pathway, and PP_4492 (yhsS, which encodes a carbohydrate efflux transporter) (Figure 1). Sequence analysis supported that the acquired DNA with a G+C of 64.86% and encoding 58 ORFs corresponded to an integrative conjugative element (ICE) confirmed by using ICEberg v2.0 database (https://db-mml.sjtu.edu.cn/CEfinder.html) (Johnson & Grossman, 2015; Liu et al., 2019). The online tool from ICEberg database, ICEfinder detects recombination and conjugation modules in the query DNA sequence using Hidden Markov Models and then looks for the oriT region, then it performs a pattern-based colocalization of gene groups in the element (Li et al., 2018). Further analysis comparing the ICE acquired by P. putida against other elements in the ICEberg dataset revealed that several ICE modules of the ICE genes are present on chromosomes or plasmids of multiple microorganisms from different taxa. It appears that the ICE present in KT2440 gained successive sets of genes that most likely originated from Burkholderia, Delftia, Aeromonas, Ralstonia, Shewanella, Paraburkholderia and others (see Table 1). The set of ORFs present on the ICE was distributed into three defined regions (Table 1; Figure 1). The first one is flanked by an integrase (ORF1 of the ICE) and a series of IS5, IS6, IS21 and IS478 sequences (ORFs 33–39 in Table 1). This region was populated by genes that encoded proteins of unknown function, and an efflux pump (ORF23-25). Downstream, a second region contains seven genes putatively involved in the degradation of alkanes, namely: ORF41, which encodes an alkane 1-monoxygenase that could oxidize octane into octanol; ORF42, a quinone-dependent alkan-1-ol dehydrogenase that transforms octanol to the corresponding aldehyde; ORF44, an aldehyde dehydrogenase that transforms octanol to octanoic acid; and ORF43, a putative acyl-CoA ligase that permits the entry to the acylated fatty acid to the β-oxidation cycle (Table 1), and two transcriptional regulators that belong to the LysR and AraC families. In addition, we found 13 proteins (from ORF46 to ORF58) which encode a series of transfer functions of the P-type conjugative system (i.e. TrbCBJKLFGI).

Alkane monoxygenases of Pseudomonas GPO1 that hydroxylate medium-chain hydrocarbons usually comprise three components: (i) the AlkB monoxygenase (51% identity with ORF41 in the ICE acquired by EM2-4), (ii) a soluble NADH-rubredoxin reductase and (iii) the soluble transfer protein rubredoxin (Alonso & Roujeinikova, 2012; Chen et al.,
The \textit{alk} cluster in EM2-4 is atypical in the sense that genes encoding electron donors of the mono-oxygenase, such as the rubredoxin oxidoreductase or rubredoxin, were not present. Therefore, we assumed that host proteins would participate in ALK reaction electron transfers (Li et al., 2019; Rojo, 2009).

More concretely, the genome of \textit{P. putida} KT2440 contains a gene (PP\_5315 or \textit{rubA}) that encodes a rubredoxin-I and two genes (\textit{alkT} or PP\_5314 and PP\_5371) encoding two rubredoxin-NAD(+) reductases; enzymes responsible of the reduction of oxidized rubredoxins. Therefore, the acquisition of the octane degradation capability by \textit{P. putida} KT2440 appears to be the result of a new pathway made of acquired environmental genes and host genes, which work coordinately.

We tested if the ICE element was stable and if it can be transferred to other strains. We grew EM2-4 cells for 100 generations in LB and then cells were spread on M9 with toluene as sole C-source, M9 with octane as the sole C-source, or M9 with citrate as the sole C-source. All of the cells were able to grow in each condition, suggesting stable inheritance of the ICE element.

To test if the ICE can be transferred horizontally to other \textit{Pseudomonas} strains, we mated EM2-4 with \textit{P. putida} PSC303 and PSC2078, two KmR derivatives of KT2440 marked with a mini-Tn5, that grow on citrate as a C-source but not on octane (del Castillo et al., 2008). Donor and recipient cells were harvested in the mid logarithmic phase or in the late stationary phase and the mattings were done on solid LB medium plates, M9 minimal medium plates with glucose as a C-source and in liquid M9 medium for up to 24 h. No KmR Oct+ clones were recovered indicating that the rate of transfer of the ICE element under these conditions is below $10^{-10}$ per recipient.

Proteomic analysis reveals how octane is channelled to central metabolism in this strain

Given that EM2-4 grew exponentially with octane and that the ICE was genetically stable, we endeavoured to further characterize the phenotype. We carried out proteomic analysis to define the route through which the cells metabolize the linear hydrocarbon.

For this, three replicates were performed in two different growth conditions: M9 with glucose (control), and M9 with octane (0.5\%, vol./vol.) as sole carbon source (octane metabolism). Doubling times in the exponential phase with glucose (97 ± 3 min) and octane (110 ± 5 min) were similar, and cells were harvested by centrifugation at the mid-log phase (14,000 g 5 min) when cultures had reached a turbidity of about 0.8 at OD$_{660}$. Cell pellets were frozen at $-80^\circ$C until used and lysed as previously described (Molina et al., 2019). The proteins were digested with trypsin after reduction of disulfur bridges with 100 mM Tris-carboxyethyl-phosphine, then alkylated with 200 mM chloroacetamide (see Supplementary Experimental procedures) and finally TMT-6plex labelled (Altelaar et al., 2013). Then, peptides were identified by high-throughput-tandem mass spectrometry and quantified as described in the Supplementary Experimental procedures. We compared the control condition (glucose) with cells grown in octane as sole carbon source in terms of protein enrichment using the T-fold method of PROTEOBIOTICS, as recommended by the Proteomic Facility of CNB. A total of 2677 different quantification peptide groups were confidently assigned (Table S2; data are hosted on figshare and available via \url{https://figshare.com/s/d5bc13bcb41bda951fc} and DOI: 10.6084/m9.figshare.17061629). Their relative quantities were
| ORF | Amino acids | Function | Organism | ORF | Query cover | Percent intent | E-value |
|-----|-------------|----------|----------|-----|-------------|----------------|--------|
| 0   | 119         | Pterin-4-alpha-carbinolamine dehydratase | *Pseudomonas putida* | PP_491 | 99          | 96.33          | 0.0     |
| 1   | 401         | Integrase | *Burkholderia cenocepacia* | A3203_15290 | 99          | 96.33          | 0.0     |
| 2   | 355         | Nuclease domain-containing protein | *Delftia lacustris* | I6G47_25115 | 100         | 93.02          | 0.0     |
| 3   | 170         | DNA repair protein RadC | *Pseudomonas aeruginosa* | EIP87_28665 | 100         | 95.69          | 0.0     |
| 4   | 117         | Mlr6156 protein | *Pseudomonas putida* | E6B08_23585 | 100         | 97.72          | 4.00E-169 |
| 5   | 93          | Hypothetical protein | *Pseudomonas putida* | E6B08_23580 | 100         | 95.34          | 6.00E-121 |
| 6   | 84          | Hypothetical protein | *Aeromonas sp.* | C2U47_04775 | 100         | 98.81          | 4.00E-122 |
| 7   | 277         | UPF0380 proteins YafZ and homologues | *Burkholderia cenocepacia* | A3203_15330 | 100         | 96.99          | 0.0     |
| 8   | 688         | Putative plasmid stabilization protein, ParB | *Aeromonas caviae* | WP3S18E02_07940 | 100         | 92.89          | 0.0     |
| 9   | 105         | y4eB gene homologue | *Pseudomonas aeruginosa* | K0E62_28125 | 100         | 98.1           | 2.00E+152 |
| 10  | 111         | Transcriptional regulator, Xre family | *Pseudomonas aeruginosa* | MCN99_21695 | 98          | 96.34          | 1.00E-149 |
| 11  | 117         | Putative lipoprotein | *Burkholderia cenocepacia* | A3203_15355 | 100         | 96.58          | 0.0     |
| 12  | 60          | Hypothetical protein | *Burkholderia cenocepacia* | CP015036 (3492922…3493101) | 100         | 99.44          | 4.00E-71 |
| 13  | 257         | Hypothetical protein | *Burkholderia cenocepacia* | A3203_15360 | 100         | 94.81          | 0.0     |
| 14  | 95          | Helix–turn–helix domain-containing protein | *Delftia lacustris* | I6G47_25120 | 100         | 96.14          | 1.00E-127 |
| 15  | 286         | Plasmid replication initiator protein | *Ralstonia solanacearum* | RSc2606 | 100         | 95.45          | 0.0     |
| 16  | 73          | Hypothetical protein | *Enterobacter sp.* | CU081_17605 | 100         | 97.65          | 3.00E-119 |
| 17  | 213         | ParA-like protein | *Pseudomonas aeruginosa* | CWI20_06980 | 100         | 94.21          | 0.0     |
| 18  | 91          | ParB-like protein | *Thioalkalivibrio sulfidophilus* | Tgr7_1880 | 100         | 93.41          | 3.00E-109 |
| 19  | 186         | Hypothetical protein | *Pseudomonas aeruginosa* | EIP87_28580 | 100         | 94.99          | 0.0     |
| 20  | 200         | S26 family signal peptidase | *Sphingomonas sp.* | EIK56_27525 | 93          | 92.50          | 0.0     |
| 21  | 43          | VirD2 components relaxase | *Variorax paradoxus* | CP091716 (1641334…1641462) | 100         | 90.77          | 1.00E-39 |
| 22  | 665         | VirD2 components relaxase | *Burkholderia cenocepacia* | A3203_15395 | 100         | 97.46          | 0.0     |
| 23  | 296         | Efflux RND transporter periplasmic adaptor subunit, mdtA | *Shewanella chilensis* | Gli14_05840 | 100         | 95.5           | 0.0     |
| 24  | 1108        | Efflux RND transporter permease subunit, mdtB | *Thauera sp.* | Tmz1t_2073 | 100         | 93.8           | 0.0     |
| 25  | 495         | Efflux transporter outer membrane subunit, outer membrane factor (OMF) lipoprotein | *Thauera sp.* | Tmz1t_2072 | 100         | 93.36          | 0.0     |

(Continues)
| ORF | Amino acids | Function | Organism | ORF | Query cover | Percent intent | E-value |
|-----|-------------|----------|----------|-----|-------------|---------------|---------|
| 26  | 583         | Ubiquinone biosynthesis protein UbiB | Diaphorobacter sp. | I3K84_20315 | 93 | 93.8 | 0.0 |
| 27  | 325         | Patatin-like phospholipase family protein | Variovorax paradoxus | L3V85_07690 | 100 | 99.79 | 0.00E+00 |
| 28  | 254         | Octaprenyl diphosphate synthase | Hydrogenophaga pseudoflava | HPF_12290 | 100 | 96.33 | 0.0 |
| 29  | 898         | Processive diacylglycerol beta-glucosyltransferase | Hydrogenophaga pseudoflava | HPF_12295 | 100 | 96.51 | 0.0 |
| 30  | 234         | CerR family C-terminal domain-containing protein | Hydrogenophaga pseudoflava | HPF_12300 | 100 | 99.72 | 0.0 |
| 31  | 203         | Hypothetical protein | Hydrogenophaga pseudoflava | HPF_12305 | 100 | 99.51 | 0.0 |
| 32  | 236         | Hypothetical protein | Hydrogenophaga pseudoflava | HPF_12310 | 100 | 98.59 | 0.0 |
| 33  | 113         | Transposase | Variovorax paradoxus | L3V85_07650 | 100 | 100.00 | 8.00E-176 |
| 34  | 107         | IS66 family insertion sequence element accessory protein TnpB | Variovorax paradoxus | L3V85_07645 | 100 | 100.00 | 3.00E-149 |
| 35  | 511         | IS66 family transposase | Variovorax paradoxus | L3V85_07640 | 100 | 100.00 | 0.0 |
| 36  | 418         | IS5 family transposase | Acidovorax sp. | BSY15_3119 | 100 | 90.91 | 0.0 |
| 37  | 515         | IS21 family transposase | Acidovorax sp. | Ajs_3594 | 100 | 93.72 | 0.0 |
| 38  | 246         | IS21-like element helper ATPase lstB | Comamonas thiooxydans | LCH15_25240 | 100 | 94.04 | 0.0 |
| 39  | 94          | IS1478 transposase | Acidovorax sp. | BSY15_166 | 91 | 92.66 | 9.00E-100 |
| 40  | 371         | AraC family transcriptional regulator | Variovorax paradoxus | L3V85_07635 | 100 | 99.91 | 0.0 |
| 41  | 754         | Alkane-1 monoxygenase | Variovorax paradoxus | L3V85_07630 | 100 | 99.96 | 0.0 |
| 42  | 559         | Alkan-1-ol dehydrogenase, PQD-dependent | Variovorax paradoxus | L3V85_07625 | 100 | 100.00 | 0.0 |
| 43  | 542         | 3-Methylmercaptopropionyl-CoA ligase | Variovorax paradoxus | L3V85_07620 | 100 | 99.94 | 0.0 |
| 44  | 580         | Aldehyde dehydrogenase | Variovorax paradoxus | L3V85_07615 | 100 | 99.94 | 0.0 |
| 45  | 315         | Transcriptional regulator, LysR family | Variovorax paradoxus | L3V85_07610 | 100 | 100.00 | 0.0 |
| 46  | 93          | Entry exclusion lipoprotein TrbK | Variovorax paradoxus | L3V85_07605 | 100 | 100.00 | 1.00E-142 |
| 47  | 678         | Conjugal transfer protein TraG | Variovorax paradoxus | L3V85_07600 | 100 | 100.00 | 0.0 |
| 48  | 159         | CopG family transcriptional regulator | Variovorax paradoxus | L3V85_07595 | 100 | 100.00 | 0.0 |
| 49  | 355         | P-type conjugative transfer ATPase TrbB | Variovorax paradoxus | L3V85_07590 | 100 | 100.00 | 0.0 |
| 50  | 130         | Conjugative transfer protein TrbC, TrbC/VirB2 | Variovorax paradoxus | L3V85_07585 | 100 | 100.00 | 0.00E+00 |
| 51  | 91          | Family type IV secretion system protein, TrbB/VirB3 | Variovorax paradoxus | L3V85_07580 | 100 | 100.00 | 0.00E+00 |
| 52  | 825         | Conjugative transfer protein TrbE | Pseudomonas aeruginosa | PERCYII40_2689 | 100 | 91.53 | 0.0 |
| 53  | 252         | Conjugative transfer protein TrbJ | Comamonas sp. | FOZ74_04005 | 100 | 93.83 | 0.0 |
estimated for each condition based on their respective spectral counts and normalized spectral abundance and the whole-cell proteomes were compared on the basis of their detection in the three replicates. Data are reported in Table S2. Figure 2 shows a general overview of the functional categories of the whole cell proteome, weighted by the Normalized Spectral Abundance Factor (Zybailov et al., 2006) of the identified proteins in all conditions tested. Proteins involved in central metabolism comprised nearly 67% of total proteins in terms of quantities of the whole cell proteomes. Proteins involved in translation and transcription, cell envelope biogenesis and cell motility and secretion represented each about 10% of total proteins (see Figure 2). These ratios are similar to those reported previously for Pseudomonas with other C-sources (Cuenca et al., 2016a). This global view of P. putida EM2-4 protein content indicates no specific bias in the proteomic strategy and points to central metabolism as key octane-related functional categories for analysis.

Using a log 2-fold change threshold equal or above 1.5 and a stringent statistical level of confidence (q-value <0.05), a list of 89 proteins was identified as statistically significant more abundant in the octane grown than in the glucose grown (Table S3), while 37 proteins were less abundant in the whole-cell proteome in cells growing with octane (Table S4).

**Insights into octane metabolism based on proteomic analysis**

Octane needs to enter into EM2-4 cells for oxidation and we identified an uncharacterized porin PP_2662 that was 25-fold more abundant in octane grown than in glucose grown cells and two porins, OprD and OpdD, involved in amino-acid uptake that were induced five-fold. It is likely that octane enters into the periplasmic space through these porins (Table S3, Doncheva et al., 2019). Octane is then oxidized to octanol and subsequently to octanal at the membrane level, and eventually to octanoic acid.

Regarding oxidation of octane by EM2-4, we wish to emphasize that host proteins may support the initial oxidation steps up to octanal and of the latter to octanoic as we found that a PQQ-dependent alcohol dehydrogenase (PP_2679) and an aldehyde dehydrogenase (PP_2680) were induced. This agrees with the notion that octane is being oxidized to octanoic acid.

Among the proteins with the higher fold change were enzymes related to acylation of medium-chain fatty acids, the β-oxidation cycle and the glyoxylate shunt (Table S3 and Figure 3). We identified a set of enzymes involved in the potential acylation of octanoic acid and its subsequent β-oxidation. The corresponding proteins were induced between 10- and 86-fold, with the highest induction found for two acyl-CoA dehydrogenase (PP_4948, PP_0370) (see Table S3) which are involved in each cycle of β-oxidation and yield a trans-double bond between C2 and C3, which is the substrate of β-keto-thiolases (PP_2137 and PP_3754) that are also induced (7- to 18.4-fold) (Table S3). In addition, acyl-CoA synthetases PP_2351 and PP_4487 were induced 4.7- and 8.7-fold, respectively. Figure 4 shows a STRING-derived interactome. This figure shows that, among the proteins highly induced in the presence of octane, there were a set of membrane proteins (PP_2662, PP_2663, PP_2667, PP_2669, PP_2674, PP_2675, PP_2678, PP_2679 and PP_2680).
The second highest fold change (40-fold) was isocitrate lyase (PP_4116) (Table S3), a protein that is involved in central metabolism through its role in the glyoxylate shunt, where glyoxylate is subsequently converted to malate and, as expected, malate synthase (PP_0356, GcbB) was induced (7.1-fold) (Figure 3, Table S3). Concomitantly, isocitrate dehydrogenase and oxo-glutarate dehydrogenase, two key enzymes of the Krebs cycle were strongly repressed, which supports blockage of the TCA cycle and full operation of the glyoxylate shunt (Table S4). To further confirm the role of the glyoxylate shunt in octane assimilation we generated an aceA mutant by homologous recombination using a KmR cassette that interrupted the aceA gene (see Supplementary Experimental procedures). The KmR aceA mutant failed to grow on octane and butanol as expected. We also found induced 4.5- to 7-fold enzymes that are involved in polyhydroxyalkanoate (PHA) synthesis (PP_5007 and PP_5008), suggesting that excess carbon is stored in the form of PHA. This was confirmed by transmission electron microscopy of cells growing on octane as the sole C source (data not shown).

PP_4203, an electron transfer flavoprotein-ubiquinone oxidoreductase, a cytochrome (PP_2675) and cbb3-2 terminal cytochrome oxidase (PP_4256, PP_4255, PP_4258 and PP_4257) were also induced. The induction of this set of respiratory proteins is concomitant with repression of another ubiquinol oxidase involved in electron transfer (PP_4651) and several cytochromes (PP_0813, PP_0105, PP_4193, PP_4251 and PP_4250). This suggests that octane utilization...
leads to optimization of electron flow by synthesis and removal of a series of electron transfer protein from the respiratory chain.

Octane exerts stress in the cells and a number of defence strategies were activated in response to this linear hydrocarbon, for example, the chaperon IbpA was induced 12-fold (Table S3), as well as an efflux pump (PP_2019), and an alkyl-hydro peroxidase induced over fivefold. The histone-like HU protein was also induced fivefold (Table S3) suggesting a potential role in maintenance of internal chromosome structure.

We did not identify an increase in the alkane mono-oxygenase component or the adjacently encoded alcohol dehydrogenase, which suggests that the oct gene cluster may be expressed constitutively. This is in agreement with the absence of specific alkS/alkT regulatory genes, although other regulators were found adjacent to these catabolic genes in the island. Pathway evolution is thought to follow this route: for hydrocarbon degradation it has been proposed that cells first acquire the catabolic capacity and later the regulatory genes (Pérez-Pantoja et al., 2021). This may also be the case with the acquired oct genes.

In the same vein, we did not see changes in the level of the cis-trans isomerase, in spite of the above experimental data showing a cis to trans isomerization of unsaturated fatty acids (Table S1) (Junker & Ramos, 1999). This agrees with previous observations that cti isomerase in several strains of Pseudomonas is expressed constitutively (Junker & Ramos, 1999) and became functional once exposed to different toxic compounds. As such, responses to toxic hydrocarbons through phospholipid modification are an inherent and constitutive function in Pseudomonas, regardless of the biodegradative potential of the strain.

Simultaneous degradation of aromatic and linear hydrocarbons by P. putida EM2-4

Toluene, xylene, and their corresponding alcohols [benzyl alcohol/3-methylbenzyl alcohol (3MBA)] and acids [benzoate/3-methylbenzoate (3MBz)] are degraded by enzymes that are encoded by the TOL plasmid (Worsey & Williams, 1975). However, octane is degraded by a hybrid OCT pathway located on the host chromosome. We devised a way to test whether, in EM2-4, octane can be degraded simultaneously with one of three molecules: (i) glucose, which is metabolized through the Entner–Doudoroff pathway, (ii) the TOL upper pathway substrate 3MBA, or (iii) the TOL lower pathway substrate 3MBz. EM2-4 cells were grown on glucose in the absence and in the presence of hydrocarbons and when cells reached a turbidity of about 1 cells were harvested and suspended at a turbidity OD660 of 10 to prepare resting cells. Degradation assays were set up with 50 ppm octane and 3 mM glucose, 1 mM 3MBA or 1 mM 3MBz. We then measured the C-source concentrations over time. In accordance with the constitutive expression of the OCT pathway we found that regardless of an additional C-source, octane was removed at a rate of 4.1 ± 0.4 to 15.6 ± 2.0 mg L⁻¹ h⁻¹ (Table 2). In fact, octane degradation rate was highest with glucose—an observation that could be
explained by the fact that energy generation from glucose could favour cell metabolism. We also found that TOL pathway substrates were consumed at a rate of 12–24 mg L$^{-1}$ h$^{-1}$ in the absence and in the presence of octane. Therefore, the EM2-4 strain has the ability to degrade several pollutants simultaneously.
Pseudomonas putida (pWW0) acquired an ICE with \textit{oct} genes from a microbial community, that allowed oxidation of octane to octanoic acid, the clone was named EM2-4 and it was characterized. The acquired \textit{oct} cluster was atypical in the sense that it lacked the required electron transfer proteins, which were ‘parasitized’ from the host. Once octanoic acid is made, it is acylated and degraded via β-oxidation to produce high levels of acetyl-CoA, which is metabolized through the glyoxylate shunt (Figure 3). In agreement with this proposal, the set of enzymes involved in β-oxidation and the glyoxylate shunt was induced, and a mutant in aceA failed to grow on octane or octanoic acid. Our proteomic analysis revealed a redundancy of acyl-CoA and β-oxidation enzymes induced in octane growing cells, which agrees with the fact that no mutants unable to grow on octane in this set of enzymes were found. We have previously generated a collection of mutants in KT2440 and found mutants in several of these enzymes. All of them grew on butanol, supporting the referenced redundancy of acylating enzymes related above. Accordingly, with operation of the glyoxylate shunt, isocitrate lyase—a key enzyme of the pathway—was one of the most abundant proteins when octane was used as the sole C. In a previous study, other mutations in the glyoxylate shunt were found, i.e. in malate synthase (GcB), that limited the catabolism of butanol. This mutant failed to use octanoic acid (not shown). Therefore, the set of induced proteins and mutants support that medium-chain alkanes are metabolized and β-oxidized to acetyl-CoA, which is channelled to central metabolism via the glyoxylate shunt, while the Krebs cycle playing a minor role. Meanwhile, respiratory chains adapt to optimize electron flow and growth using octane under aerobic conditions.

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SUPPORTING INFORMATION

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