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Research Paper

Desulfonation and defluorination of 6:2 fluorotelomer sulfonic acid (6:2 FTSA) by *Rhodococcus jostii* RHA1: Carbon and sulfur sources, enzymes, and pathways

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ABSTRACT

6:2 fluorotelomer sulfonic acid (6:2 FTSA) is one per- and poly-fluoroalkyl substances commonly detected in the environment. While biotransformation of 6:2 FTSA has been reported, factors affecting desulfonation and defluorination of 6:2 FTSA remain poorly understood. This study elucidated the effects of carbon and sulfur sources on the gene expression of *Rhodococcus jostii* RHA1 which is responsible for the 6:2 FTSA biotransformation. While alkane monooxygenase and cytochrome P450 were highly expressed in ethanol-, 1-butanol-, and n-octane-grown RHA1 in sulfur-rich medium, these cultures only defluorinated 6:2 fluorotelomer alcohol but not 6:2 FTSA, suggesting that the sulfonate group in 6:2 FTSA hinders enzymatic defluorination. In sulfur-free growth media, alkanesulfonate monooxygenase was linked to desulfonation of 6:2 FTSA; while alkane monooxygenase, haloacid dehalogenase, and cytochrome P450 were linked to defluorination of 6:2 FTSA. The desulfonation and defluorination ability of these enzymes toward 6:2 FTSA were validated through heterologous gene expression and *in vitro* assays. Four degradation metabolites were confirmed and one was identified as a tentative metabolite. The results provide a new understanding of 6:2 FTSA biotransformation by RHA1. The genes encoding these desulfonating- and defluorinating-enzymes are potential markers to be used to assess 6:2 FTSA biotransformation in the environment.

1. Introduction

Per- and poly-fluoroalkyl substances (PFAS) are a class of more than 5000 synthetic compounds that have been widely used in numerous industries and consumer products for decades (EPA, 2020). In 2006, eight major companies in the PFAS industry voluntarily agreed to phase out production of perfluorooctanoic acid (PFOA) and PFOA-related long-chain precursors by 2015 due to their toxicity, bioaccumulation potential, and recalcitrance in the environment (EPA, 2017). 6:2 Fluorotelomer sulfonic acid (6:2 FTSA) has been used directly in surface treatment of metal and plastic components, as a processing aid in emulsion polymerization of fluoropolymers (Poulsen et al., 2011), and as one of ingredient in aqueous film-forming foams (AFFF) (Houtz et al., 2013; Schultz et al., 2004). The environmental occurrence of 6:2 FTSA can be contributed directly through long-term applications of AFFF during firefighting and/or training or through the transformation of PFAS precursors. For example, 6:2 FTSA has been reported as a biotransformation metabolite from precursors such as 6:2 fluorotelomer thioether amido sulfonate (6:2 FtTAoS), a common ingredient in AFFF (Harding-Marjanovic et al., 2015; Place and Field, 2012). Not surprisingly, 6:2 FTSA has been widely detected in groundwater, rivers, and wastewater effluents ranging from 1.6 to 37.9 ng/L (Ahrens et al., 2009; Schultz et al., 2004; Wang et al., 2016), in landfill leachate (582 ng/L) (Eggen et al., 2010) and in AFFF-contaminated soil (612–2101 ng/g) (Karrman et al., 2011). Although low toxicity and risk of 6:2 FTSA on aquatic organisms have been reported (NASF, 2019), several studies showed that 6:2 FTSA can cause liver and kidney damage in Wistar rats (NASF, 2019).

Biotransformation of 6:2 FTSA has been investigated under aerobic and anaerobic conditions. Nearly no anaerobic/anoxic biotransformation of 6:2 FTSA was observed in sediments and wetland slurry after 100–116 days of incubation (Yin et al., 2019; Zhang et al., 2016). Aerobic biotransformation of 6:2 FTSA was observed in river sediment, activated sludge, soil and leachate, with 5:3 fluorotelomer carboxylic

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acid (5:3 FTCA, ($F(CF_2)_5CH_2CH_2COOH$)), perfluoropentanoic acid (PFPeA, ($F(CF_2)_4COOH$)), and perfluorohexanoic acid (PFHxA, ($F(CF_2)_5COOH$)) as major stable terminal metabolites (Hamid et al., 2020a, 2020b; Harding-Marjanovic et al., 2015; Wang et al., 2011; Zhang et al., 2016). Interestingly, a much longer half-life of 6:2 FTSA was observed in aerobic activated sludge (2 years) than that observed in the aerobic sediment (5 days) (Wang et al., 2011; Zhang et al., 2016). Recent studies reported aerobic biotransformation of 6:2 FTSA by pure strains, *Pseudomonas* sp. strain D2 and *Gordonia* sp. NB4–1Y, only occurred when these strains were cultivated under sulfur-limited conditions (Key et al., 1998; Shaw et al., 2019; Van Hamme et al., 2013). These studies suggested that the low expression levels of monooxygenases (Zhang et al., 2016) and the repression of expression of enzymes for desulfonation of 6:2 FTSA (Van Hamme et al., 2013) might explain the lack of 6:2 FTSA biotransformation. Alkanesulfonate monooxygenase has been suggested to be responsible for the desulfonation of 6:2 FTSA (Van Hamme et al., 2013). Alkanesulfonate monooxygenase is an $FMNH_2$-dependent monooxygenase and known for its ability to cleave the carbon-sulfur bond in a wide range of alkanesulfonates (van der Ploeg et al., 2001). $FMNH_2$ is the reduced 1,5-dihydro form of flavin mononucleotide. Under sulfur-limited conditions, certain bacteria can express alkanesulfonate monooxygenase to acquire sulfide from organosulfur for biosynthetic processes (Kertesz, 2000). However, in the presence of sulfate, sulfite, sulfide, or cysteine, the expression of alkanesulfonate monooxygenase is repressed. Despite the previous suggestions, the desulfonating enzymes responsible for 6:2 FTSA desulfonation remain unidentified and unknown.

Microbial defluorination is essential for removing fluoride ion from 6:2 FTSA; however, enzymes responsible for the defluorination remain unknown. Many dehalogenases and oxygenases can remove halogens from halogenated aliphatic compounds. For example, haloalkane dehalogenase can degrade a variety of chlorinated, brominated, and iodinated chemicals (Keuning et al., 1985; Kulakova et al., 1997; Marek et al., 2000). Haloacid dehalogenase is capable of degrading a wide range of chlorinated, brominated, and fluorinated compounds (Chan et al., 2010; Sallis et al., 1990; Smith et al., 1990; van der Ploeg et al., 1991), and cytochrome P450 monooxygenase is involved in the biotransformation of fluorinated drugs (Kharasch and Thummel, 1993; Murphy, 2016). Also, the defluorination of fluoroacetate catalyzed by fluoroacetate dehalogenase has been reported previously (Donnelly and Murphy, 2009). The ability to cleave the carbon-fluoride bonds implied that these enzymes have the potential to defluorinate 6:2 FTSA. Oxygenases that can defluorinate are of particular interest. Previous studies have reported that butane monooxygenase and alkane monooxygenase expressed by *Pseudomonas butanovora* and *Pseudomonas oleovorans* are capable of defluorinating 4:2, 6:2, and 8:2 FTOHs and 6:2 polyfluoroalkyl phosphates (6:2 PAPs) (Kim et al., 2014, 2012; Lewis et al., 2016). However, more studies are needed on dehalogenases and oxygenases to elucidate their ability to biotransformation PFAS such as 6:2 FTSA.

Rhodococcus jostii RHA1 (designated as RHA1 hereafter) is a well-characterized Gram-positive bacterial strain isolated from γ-hexachlorocyclohexane-contaminated soil (Masai et al., 1995). RHA1 is known for its ability to express different oxygenases to degrade a wide range of persistent pollutants such as 1,4-dioxane, triclosan, and trichloroethylene (TCE) through co-metabolic mechanisms (Hand et al., 2015; Lee and Chu, 2013). RHA1 harbors not only numerous oxygenases with co-metabolic degradation abilities but also desulfonating enzymes and dehalogenases with unknown functions in its genome (McLeod et al., 2006). Previous studies have suggested butane monooxygenase to be responsible for the defluorination of FTOHs (Kim et al., 2014, 2012; Lewis et al., 2016). Interestingly, 1-butane-grown RHA1 can also express butane monooxygenase; however, no study has explored the potential of butane-grown RHA1 to degrade FTOHs or other fluorotelomer-based PFAS such as 6:2 FTSA. Given that RHA1 also contains genes encoding desulfonating enzymes in its genome, we thus hypothesize that RHA1 can biodegrade 6:2 FTSA when both desulfonating and defluorinating genes are expressed.

To date, several possible 6:2 FTSA biotransformation pathways in mixed microbial communities and by pure cultures have been proposed (Shaw et al., 2019; Wang et al., 2011; Zhang et al., 2016). However, little is known about the enzymes responsible for the 6:2 FTSA biotransformation and the conditions that promote desulfonation and defluorination of 6:2 FTSA are still not fully understood. To fill these knowledge gaps, the objective of this study was to determine the factors that affect the biotransformation of 6:2 FTSA in RHA1. Specifically, the effects of carbon (C) source and sulfur (S) source on biotransformation of 6:2 FTSA were examined. Additionally, genes encoding enzymes responsible for desulfonation and defluorination of 6:2 FTSA were identified and their expression levels were quantified in this study. Biotransformation products of 6:2 FTSA under synergistic effects of carbon and sulfur source were further analyzed to confirm our hypothesis.

2. Materials and methods

2.1. Chemicals

1 H, 1 H, 2 H, 2 H-Perfluorooctanesulfonic acid (6:2 FTSA, CAS# 27619-97-2, 98% pure) was obtained from Synquest Laboratories (Alachua, FL, USA). 1 H, 1 H, 2 H, 2 H-perfluorooctanol (6:2 FTOH, CAS # 647–42–7, 97% pure) was obtained from Alfa Aesar (Lancashire, UK). 1-Butanol (99.4% pure) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). n-Octane (97% pure) and ethanesulfonic acid sodium salt monohydrate ($CH_3CH_2SO_3Na{\cdot}H_2O$, ETSA, 97% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA). FastRNA Pro blue kit was purchased from MP Biomedicals (Santa Ana, CA, USA). OneStep Ahead RT-PCR kit was obtained from Qiagen (Hilden, Germany). Power SYBR Green PCR Master Mix was purchased from Applied Biosystems (Waltham, MA, USA). A stock solution of 6:2 FTSA (5 g/L) was prepared in 100% pure ethanol.

2.2. Bacterial strain and cultivation

Rhodococcus jostii RHA1 was kindly provided by Dr. Bill Mohn, University of British Columbia, Canada. The strain RHA1 was cultured in one of two different growth media, sulfur-rich (S-rich) or sulfur-free (S-free) mineral salt medium, as described in the following sections. The recipes of S-rich and S-free media are available in the Supporting Information.

2.3. Biotransformation tests of 6:2 FTOH and 6:2 FTSA using resting cells of RHA1 grown on different carbon sources in S-rich medium

Experiments were designed to investigate whether resting cells of RHA1 can biodegrade 6:2 FTOH and 6:2 FTSA via co-metabolic (i.e., non-growth-associated) reactions. The biotransformation tests were conducted in a series of glass vials containing 6:2 FTOH (or 6:2 FTSA) and resting cells of RHA1 that were cultured on different carbon sources. All tests were conducted in duplicate. Briefly, RHA1 resting cells were prepared by growing in S-rich medium containing one of four different carbon sources: glucose (0.6 mM or 1% w/v), ethanol (0.9 mM or 0.3% v/v), 1-butanol (5 mM, or 0.046% v/v) or n-octane (0.1 mM or 0.1% v/v). These carbon sources are known to support growth of RHA1. Ethanol, 1-butanol, and n-octane can also serve as inducers of alkane monooxygenase or soluble butane monooxygenase that have been suggested to be responsible for co-metabolic defluorination of FTOHs and fluorotelomer-based compounds such as 6:2 PAPs (Kim et al., 2014, 2012; Lewis et al., 2016). Thus, glucose-grown RHA1 resting cells were used in control tests. When cell density reached optical density (OD_{600}) 0.7–0.8, a subset of the cell suspension (15 mL) were harvested for RNA extraction and analysis as described in Section 2.6. The remaining cell

suspension (15 mL) was pelleted at 10,000 *g* for 20 min. The pellet was washed with phosphate-buffered saline (PBS) and then resuspended in PBS for experimental use. The biotransformation tests were initiated by spiking one μL of pure 6:2 FTOH or 40 μL of 6:2 FTSA stock solution into 25-mL glass vials containing 10 mL of the resting cell suspension in PBS (with optical density of 1.1–1.2). The initial concentrations of 6:2 FTOH and 6:2 FTSA were 168 mg/L (0.46 mM) and 20 mg/L (0.05 mM), respectively. The vials were incubated at 30 °C and 150 rpm overnight before being analyzed for fluoride measurements.

2.4. Expression levels of genes coding presumptive desulfonating and defluorinating enzymes in RHA1 grown on different S- and C-sources

Batch experiments were designed to investigate effects of S- and C-sources on expression levels of genes encoding presumptive desulfonating and defluorinating enzymes identified in the bacterial genome of RHA1. Briefly, RHA1 was inoculated into a series of 120-mL glass serum bottles containing one of four carbon sources and one of three sulfur sources in 15 mL of S-free medium. The same four carbon sources: glucose (1% v/v), ethanol (0.3% v/v), 1-butanol (5 mM) and n-octane (0.1% v/v), as described in Section 2.2 were used. The three sulfur sources were sulfate (K_2SO_4, 13 mM), ethanesulfonic acid (ETSA, 10 mM), and 6:2 FTSA (40 μM). Each condition was conducted in duplicate. Following inoculation, the bottles were incubated at 30 °C and 150 rpm. Cells were harvested at OD_{600} 0.7–0.8 and stored at −20 °C before used for RNA extraction and molecular analysis. To determine if defluorination of 6:2 FTSA had occurred during the cultivation, liquid samples from those supplied with 6:2 FTSA as the sole S-source were collected for fluoride measurement. Methods regarding fluoride measurement are provided in supporting materials.

2.5. Tests to examine synergistic effects of S- and C-sources on 6:2 FTSA biotransformation

Experiments were designed to investigate synergistic effects of S- and C-sources on the biotransformation of 6:2 FTSA by RHA1. The best two S- and C-sources linked to high expression levels of the presumptive desulfonating and defluorinating enzymes were used in these time-course experiments. The experiments were set up similarly as described in Section 2.3, except that 6:2 FTSA was added at the beginning. Briefly, a series of 120-mL glass serum bottles were spiked with 6:2 FTSA and one of the two C-sources in S-free or in S-rich medium. To remove the ethanol in the 6:2 FTSA stock solution, a known amount of 6:2 FTSA stock solution was first spiked into the bottle without a cap to allow for ethanol to be completely evaporated (the time required to complete evaporation of ethanol was determined previously in our lab). The corresponding liquid medium (15 mL) was then added into the bottle to results in an initial concentration of 40 μM 6:2 FTSA. After inoculating with RHA1, the bottles were incubated at 150 rpm and 30 °C. At time zero, early exponential (OD_{600} = 0.2; 40–48 hr), mid-exponential (OD_{600} = 0.8–1; 64–72 hr), and stationary phase (OD_{600} = 1.2–1.4; 130 hr), two replicate bottles were sacrificed for 6:2 FTSA metabolites analysis and fluoride measurements. A parallel set was used to monitor cell growth based on optical density measured at OD_{600}.

2.6. Identification and quantification of the expression level of genes encoding presumptive desulfonating and defluorinating enzymes in RHA1

In the bacterial genome of RHA1(GenBank: CP000431.1), we have identified genes encoding these enzymes: alkane monooxygenase (encoded by RHA1_ro02534), haloalkane dehalogenase (encoded by RHA1_ro02879), haloacid dehalogenase (encoded by RHA1_ro00230), alkanesulfonate monooxygenase (encoded by RHA1_ro01768), and cytochrome P450 CYP254 (encoded by RHA1_ro00377). Experiments were designed to examine the expression levels of these presumptive desulfonating and defluorinating enzymes in RHA1 grown on different S- and C-sources using quantitative reverse transcription PCR (RT-qPCR) analyses. The size and sequence of qPCR products were confirmed via electrophoresis and sequencing. Details of primers, RNA extraction, cDNA and RT-qPCR analysis are provided in supporting information.

2.7. LC-TOF-MS analysis

6:2 FTSA and metabolites in liquid samples were extracted with acetonitrile as described previously (Kim et al., 2014, 2012; Lewis et al., 2016). Briefly, 20 mL of acetonitrile was added to each bottle and incubated at 50 °C and 150 rpm for 3 days. After the extraction, the aqueous phase was centrifuged at 10,000 g for 20 min, and the supernatant was passed through 0.22 μm-pore sized sterile cellulose acetate syringe filter (VWR, Radnor, PA, USA). All extracts were store at −20 °C until analysis.

6:2 FTSA and its biotransformation metabolites in the extracts were identified via non-targeted analysis using an Agilent 1100 series HPLC interfaced with a 6210 series high-resolution, accurate-mass time-of-flight mass spectrometer (LC-TOF-MS) (Agilent Technologies, Palo Alto, CA) as described previously (Strynar et al., 2015). Mass spectral analyses were conducted using electrospray ionization (ESI) negative mode. Two reference compounds (purine [*m/z* 119.0363] and the acetate adduct of hexakis (1 H,1 H,3 H-tetrafluoropropoxy) phosphazine [*m/z* 980.0164]) was applied to correct any drift in the mass accuracy of the TOF continuously via dual-ESI sprayer. An Eclipse Plus C8 column (2.1 mm × 50 mm, 3.5 μm; Agilent) was used for chromatographic separation. The separation gradient method was as follows: 0.2 mL/min flow rate; column at 30 °C; mobile phases: A: ammonium formate buffer (0.4 mM) and DI water/methanol (95:5 v/v), and B: ammonium formate (0.4 mM) and methanol/DI water (95:5 v/v); gradient: 0−15 min linear gradient from 75:25 A/B to 15:85 A/B, with a 4-min post time for equilibration. The negative mass defect property of poly- and per-fluorinated compounds was used to identify suspected 6:2 FTSA biotransformation metabolites.

2.8. Statistical analyses

The F-test was first used to check the equality of variance before using Student's *t*-test. The Student's *t*-test was then used to evaluate if there was a significant difference between treatment groups and controls (glucose-grown RHA1). The statistical analysis of data was performed using the software Excel (Microsoft, USA).

3. Results

3.1. Defluorination of 6:2 FTOH and 6:2 FTSA by resting cells of RHA1 grown on different carbon sources in sulfur-rich medium

RHA1 resting cells grown on four different carbon sources (glucose, ethanol, 1-butanol, and n-octane) in S-rich medium showed different defluorination capacity toward 6:2 FTOH, based on fluoride concentrations released in the liquid medium (Fig. 1A). A relatively low fluoride concentration (700 μg/L) was observed in glucose-grown RHA1, while elevated fluoride concentrations (approximately 1.5–2.3 times higher than that of glucose-grown RHA1) were detected in samples with ethanol-, n-octane-, and 1-butanol-grown RHA1 (i.e., 1027, 1306 and 1628 μg/L of fluoride ion, respectively). Unlike those observed for 6:2 FTOH defluorination tests, the fluoride concentrations in samples with 6:2 FTSA were all below the fluoride detection limit (50 μg/L) (Fig. 1B), suggesting that the resting cells RHA1 grown on all tested carbon sources in S-rich medium could not defluorinate 6:2 FTSA.

3.2. Effects of carbon and sulfur sources on production of presumptive desulfonating and defluorinating enzymes in RHA1

In addition to inorganic sulfate, RHA1 was able to grow on ethanol as

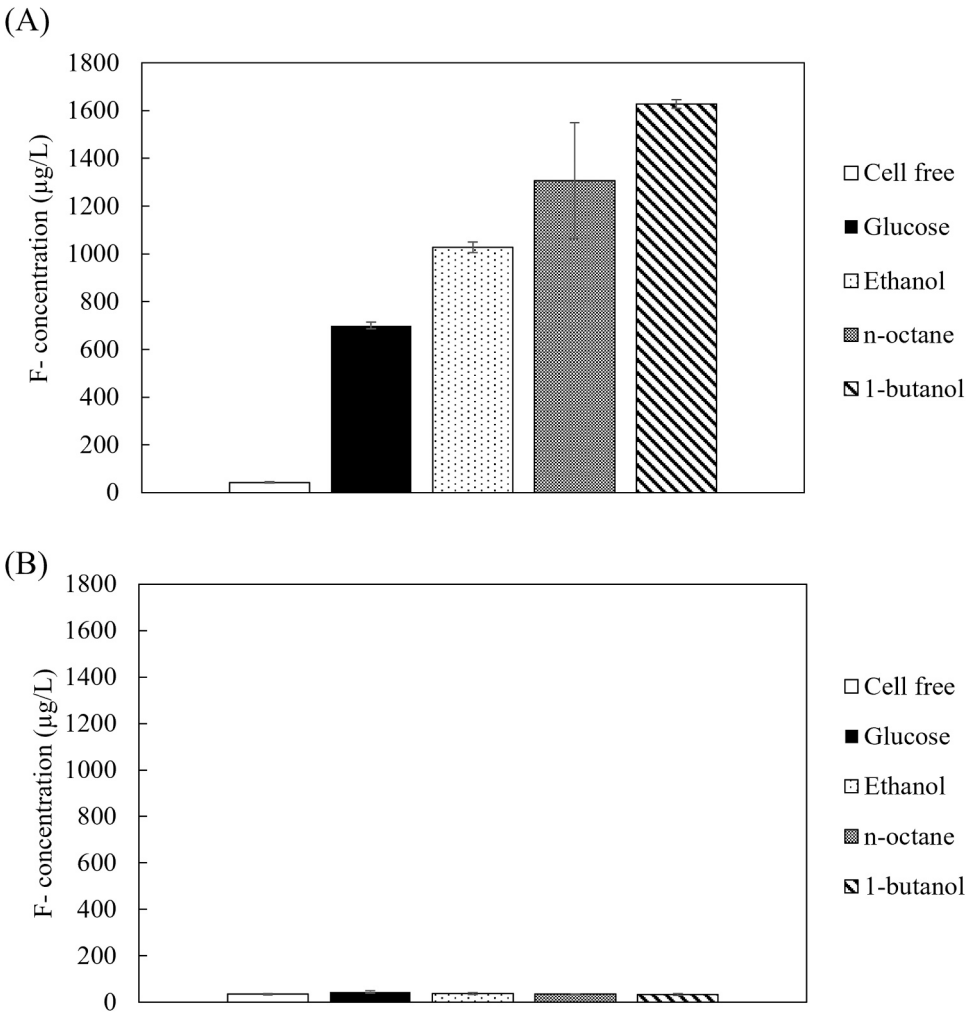

Fig. 1. Defluorination of 6:2 FTOH (A) and 6:2 FTSA (B) by resting cells of *Rhodococcus jostii* RHA1 grown on one of four carbon sources: glucose (1% v/v), ethanol (0.3% v/v), n-octane (0.1% v/v), or 1-butanol (5 mM) in sulfate-containing medium. Each condition was performed in duplicate (n = 2). The bars represented the ranges of the duplicates.

a C-source and ETSA or 6:2 FTSA as a sole S-source. The doubling times for RHA1 cultured with different C- and S-sources, from shortest to longest, were 11.4 h (glucose + sulfate) < 11.6 h (ethanol + sulfate) < 11.7 h (ethanol + ETSA) < 15.5 h (ethanol + 6:2 FTSA) < 15.7 h (1-butanol+ sulfate) < 44.7 h (n-octane + sulfate) (Fig. S1). The ability of RHA1 to use ETSA or 6:2 FTSA as a sole S-source for growth is linked to significantly higher ($p < 0.05$) expression levels of alkanesulfonate monooxygenase (i.e., ETSA [1.60 ± 0.06 folds] and 6:2 FTSA [1.57 ± 0.11 folds]) compared to that observed in RHA1 grown on glucose and sulfate.

Based on RT-qPCR results, the expression levels of alkanesulfonate monooxygenase in RHA1 were different and appeared to be C- and S-source dependent. When sulfate was available, much lower ($p < 0.05$) expression levels of alkanesulfonate monooxygenase were detected in n-octane-grown RHA1 (0.32 ± 0.07 times) compared to that in glucose-grown RHA1 (Fig. 2, first row).

Regardless of S-source, carbon sources affected the expression levels of haloalkane dehalogenase, haloacid dehalogenase, alkanemonooxygenase, and cytochrome P450 in RHA1 (second to fifth rows in Fig. 2). When sulfate was used as the sole S-source, the expression levels of haloalkane dehalogenase and haloacid dehalogenase in glucose- and ethanol-grown RHA1 were similar, followed by slightly lower levels observed in 1-butanol-grown RHA1 and lowest levels in n-octane-grown RHA1 (Fig. 2, second and third rows). For alkane monooxygenase, higher expression levels ($p < 0.05$, statistically significant) were observed in ethanol- and 1-butanol grown RHA1. The expression levels (from high to low) were ethanol- > 1-butanol- > n-octane- > glucose-grown RHA1 (i.e., 2.69 ± 1.30 folds, 2.41 ± 0.87 folds, 1.71 ± 0.52 folds, and 1.00 ± 0.21 folds, respectively). The expression level for cytochrome P450 CYP254 was slightly increased in n-octane-grown (1.29 ± 0.09 folds) and 1-butanol-grown (1.33 ± 0.14) RHA1 than those in ethanol- and glucose-grown RHA1. Interestingly, when 6:2 FTSA was the S-source, higher expression levels ($p < 0.05$, statistically significant) of haloacid dehalogenases, alkane monooxygenases, and cytochrome P450 were observed in the ethanol-grown RHA1 (Fig. 2, see the third, the fourth, and the fifth rows). However, expression levels for haloalkane dehalogenase were relatively low in ethanol-grown RHA1 using ETSA or 6:2 FTSA as a S-source (0.2 and 0.7-fold, respectively), comparing to ethanol-grown RHA1 using sulfate as a S-source (1-fold).

The high expression levels of alkane monooxygenase and the low expression levels of alkanesulfonate monooxygenase in ethanol-, 1-butanol-, and n-octane-grown RHA1 might explain why RHA1 grown on these three carbon sources could defluorinate 6:2 FTOH but not 6:2 FTSA as shown in Fig. 1.

Additional experiments were conducted to further validate the correlation of the gene expression and defluorination activity, enzyme inhibition tests for three presumptive enzymes (alkane monooxygenases, haloacid dehalogenases, and cytochrome P450) were conducted, and the genes encoding these enzymes were cloned, expressed and tested for their defluorination activity toward 6:2 FTOH and 6:2 FTSA (see details

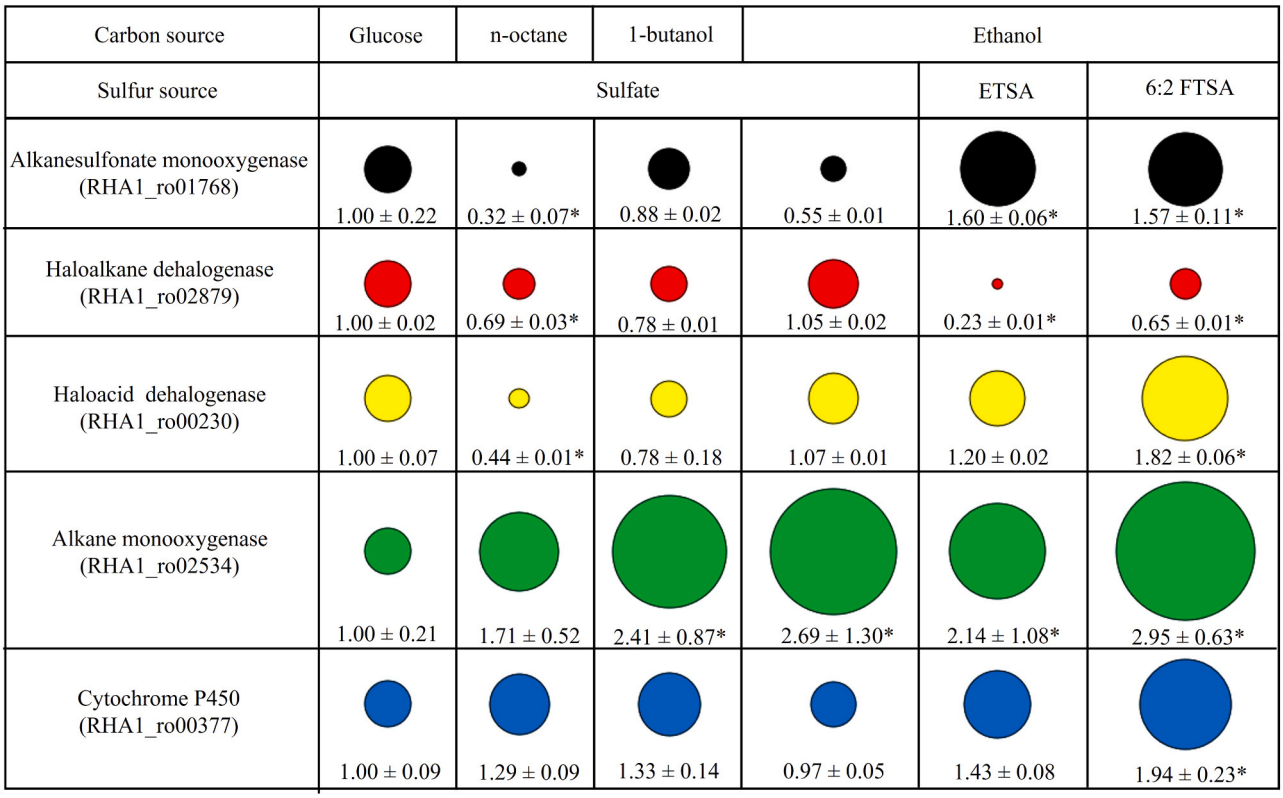

Fig. 2. (a) Variation of expression levels of genes coding a known desulfonating enzyme (RHA1_ro01768 coding alkanesulfonate monooxygenase) and four presumptive defluorinating enzymes (RHA1_ro02879 for haloalkane dehalogenase; RHA1_ro00230 for haloacid dehalogenase; RHA1_ro02534 for alkane monooxygenase; and RHA1_ro00377 for cytochrome P450) in RHA1 grown on different carbon and sulfur sources. (b) Diameters of the bubbles, listed below the bubbles, indicated the fold change of each expression level of gene relative to those expressed in the RHA1 cells grown on glucose as carbon source and sulfate as sulfur source. Asterisk (*) was labeled for those data which has significant difference ($p < 0.05$) comparing to control (RHA1 grown in glucose and sulfate). Three technical replicates (n = 3) for qPCR were performed. Standard deviations were presented for the three technical replicates (n = 3).

in supporting materials). Much higher levels of fluoride were detected in the liquid samples in the absence of enzyme inhibitors compared to those observed in the presence of enzyme inhibitors. The results indicated that these enzymes were involved in the defluorination of 6:2 FTOH (Fig. S3-S5). These three genes were also cloned and expressed in host strain *Rhodococcus opacus* PD631. The engineered PD631 strains were examined for their ability to defluorinate 6:2 FTOH and 6:2 FTSA. Similarly, higher fluoride levels were observed in the engineered PD631 containing cloned vectors compared to those observed in the wild type PD631 (PD631 WT) and PD631 with pTipQC2 (negative controls) (Figs S6–7). These results further support our observation of gene expression that these three presumptive enzymes might involve in the defluorination of 6:2 FTOH.

Similarly, to determine if alkanesulfonate monooxygenase could desulfonate 6:2 FTSA, the genes (ssuD/ssuE) encoding alkanesulfonate monooxygenase was cloned and expressed in *Rhodococcus opacus* PD631 and *E coli*. High levels of SO_3^- (60–100 μM) was detected in solution containing crude enzymes of SsuD/SsuE and substrates (hexanesulfonate (500μM) or 6:2 FTSA (500 μM)) within 1 h of incubation at 30 °C. The results confirmed that alkanesulfonate monooxygenase was responsible for the desulfonation of 6:2 FTSA (see methods and Figs S8 in supporting information).

3.3. Synergistic effects of carbon and sulfur sources on 6:2 FTSA biotransformation

Based on results shown in Figs. 1 and 2, we hypothesized that removal of the sulfonate ($R\text{-}SO_3^-$) of 6:2 FTSA is necessary prior to the occurrence of defluorination of 6:2 FTSA. To test this hypothesis, time-course experiments were conducted to understand the synergistic effects of carbon and sulfur sources on 6:2 FTSA biotransformation by RHA1.

RHA1 was able to grow when supplied with 6:2 FTSA, one of two best performing carbon sources (ethanol [0.3%, v/v] or 1-butanol [5 mM]) in S-free or S-rich medium. The growth curves of RHA1 cultivated with ethanol + sulfate + 6:2 FTSA, ethanol + 6:2 FTSA, and 1-butanol + 6:2 FTSA are shown in Fig. S2. Fluoride release in the growth medium was monitored at different growth stages as a means to assess defluorination of 6:2 FTSA. No fluoride (i.e., < fluoride detection limit) was detected from early exponential phase (OD_{600} = 0.2) to stationary phase (OD_{600} = 1.4) of ethanol-grown RHA1 in S-rich medium (Fig. 3).

However, fluoride concentrations increased as RHA1 continued to grow on ethanol or 1-butanol with 6:2 FTSA in the S-free medium. Ethanol-grown RHA1 in S-free medium not only could use 6:2 FTSA as a sole S-source, but also released fluoride ion, increasing from 0.15 mol F^-/mol 6:2 FTSA at early exponential phase (OD_{600} = 0.2) to 1.2 mol F^-/mol 6:2 FTSA at stationary phase (OD_{600} = 1.2). A similar trend was observed for 1-butanol-grown RHA1 in S-free medium, releasing 0.07 mol F^-/mol 6:2 FTSA at early exponential phase (OD_{600} = 0.2) to 1.3 mol F^-/mol 6:2 FTSA at stationary phase (OD_{600} = 1.4). These results supported our hypothesis that desulfonation is necessary prior to the defluorination of 6:2 FTSA.

3.4. Identification of 6:2 FTSA degradation metabolites

Non-targeted, high-resolution MS analysis was used to screen for possible metabolites produced during biotransformation of 6:2 FTSA by RHA1 grown on ethanol or 1-butanol in S-rich or S-free medium. The negative mass defect property of highly fluorinated compounds was used to identify the suspected 6:2 FTSA metabolites. Metabolites with larger peak areas were considered as higher concentrations.

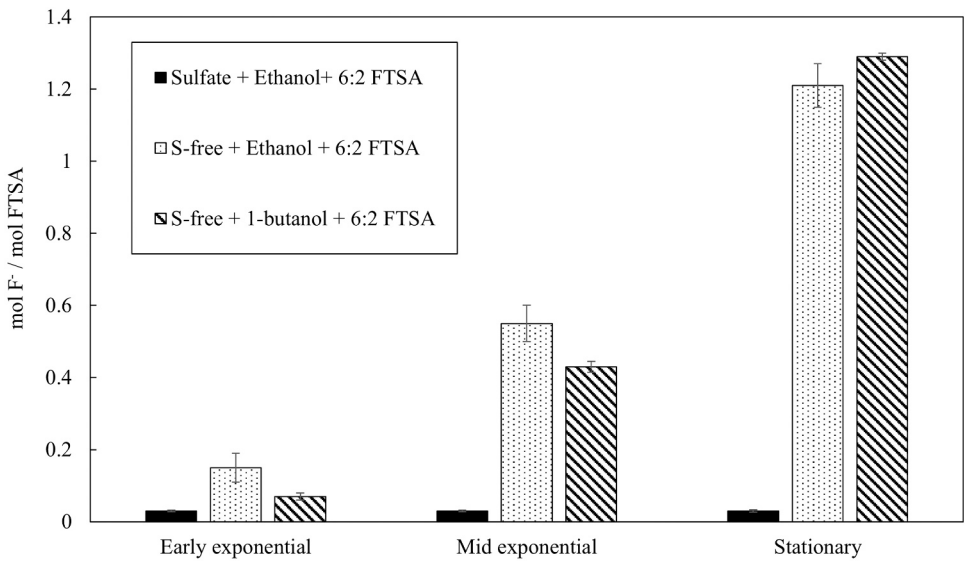

Fig. 3. Synergistic effects of carbon- and sulfur-sources on 6:2 FTSA defluorination by RHA1 at different growth stages. No fluoride was released from RHA1 grown on sulfate, ethanol and 6:2 FTSA. Increased fluoride concentrations were observed from RHA1 grown on ethanol and 6:2 FTSA, or 1-butanol and 6:2 FTSA, in S-free medium. The corresponding time of early exponential phase for the two ethanol-grown RHA1 was 40 hrs. The corresponding time of the early exponential phase for 1-butanol-grown RHA1 was 48 hrs. The corresponding times of mid-exponential and stationary phase for the ethanol- and butanol-grown RHA1 were 64 hrs and 144 hrs, respectively. The unit on y-axis, mol F^- / mol of 6:2 FTSA, was calculated by dividing the amount of fluoride measured by the theoretical amount of F- in the 6:2 FTSA spiked initially. Each condition was performed in duplicate (n = 2). The bars represented the ranges of the duplicates.

Under S-rich conditions, the 6:2 FTSA in ethanol-grown RHA1 was not changed significantly throughout 144 hrs of incubation (Fig. 4A), suggesting that there was no biotransformation of 6:2 FTSA. Not surprisingly, no transformation products were found. On the other hand, under S-free conditions, more than 99% of 6:2 FTSA was degraded in ethanol-grown RHA1 within 64 hrs (Fig. 4B). By a coincidence of this declined trend of 6:2 FTSA, 6:2 fluorotelomer carboxylic acid (6:2 FTCA, [$F(CF_2)_6CH_2COOH$]) was observed at low levels in the 40-hr samples and then declined quickly in the subsequent samples. In the meantime, two peaks corresponding to 6:2 fluorotelomer unsaturated carboxylic acid (6:2 FTUCA, [$F(CF_2)_5CF{=}CHCOOH$]), α-hydroxy-5:3 saturated fluorotelomer carboxylate (α-OH 5:3 FTCA, [$F(CF_2)_5CH_2CH(OH)COOH$]) emerged dramatically in the 40-hr samples and then remained unchanged toward to the end of the experiment, suggesting that 6:2 FTUCA and α-OH 5:3 FTCA are two major metabolites. A peak corresponding to perfluoroheptanoic acid (PFHpA, [$F(CF_2)_6COOH$]) emerged at relatively low levels in 40-hr samples and also remained unchanged toward to the end of the experiment, indicating that PFHpA is a minor metabolite. Interestingly, an intermediate, 6:2 fluorotelomer unsaturated sulfonic acid (6:2 FTUSA, ($F(CF_2)_6CH{=}CHSO_3H$), was tentatively detected with low frequency and abundance in the ethanol-grown RHA1 40-hr samples only, and then quickly disappeared in the 64-hr samples (data not shown in Fig. 4B).

A similar trend was observed in the 1-butanol-grown RHA1 samples, except that no 6:2 FTUSA was detected (Fig. 4C). It was possible that the degradation of 6:2 FTUSA might be faster in 1-butanol-grown RHA1 than that in ethanol-grown RHA1, if 6:2 FTUSA was confirmed in future study. The formation and disappearance of this tentatively identified intermediate, 6:2 FTUSA, along with the production of three shorter-chain metabolites once again supported our hypothesis that the occurrence of desulfonation proceeded the defluorination of 6:2 FTSA. However, given the low detection frequency and abundance of 6:2 FTUSA observed only in the ethanol-grown RHA1 samples, more future work is needed to confirm the formation of 6:2 FTUSA during 6:2 FTSA biotransformation by RHA1.

3.5. A proposed biotransformation pathway for 6:2 FTSA

Based on observed metabolites (Fig. 4) and previous 6:2 FTSA biotransformation studies (Kim et al., 2014, 2012; Shaw et al., 2019; Van Hamme et al., 2013; Wang et al., 2011; Zhang et al., 2016), a 6:2 FTSA biotransformation pathway by RHA1 was proposed (in Fig. 5). Similar to FTOH biotransformation pathways proposed previously (Kim et al., 2014, 2012), the biotransformation of 6:2 FTSA follows a similar upper FTOH biotransformation pathway before branching into major and minor pathways. As shown in Fig. 5, 6:2 FTSA was potentially transformed to 6:2 FTUSA (a tentative metabolite identified only in the ethanol-grown RHA1 samples). Then the sulfonate (SO_3^-) was released from 6:2 FTUSA to form 6:2 FTOH which subsequently entered the 6:2 FTOH degradation pathways (Kim et al., 2014, 2012). The transformation of 6:2 FTOH ($F(CF_2)_6CH_2CH_2OH$) to 6:2 FTAL ($F(CF_2)_6CH_2CHO$) and then to 6:2 FTCA ($F(CF_2)_6CH_2COOH$) are fast. While the method employed in this study would not detect 6:2 FTOH and 6:2 FTAL, 6:2 FTCA was detected as a metabolite in this study.

As shown in the proposed pathway, 6:2 FTCA ($F(CF_2)_6CH_2COOH$) is the branching point to enter one of two lower pathways, leading to production of 6:2 FTUCA, α-OH 5:3 FTCA, and PFHpA observed in this study.

Formation of 6:2 FTUCA and α-OH 5:3 FTCA are two defluorination processes that resulted in release of fluoride. Adapting from previously proposed pathways, we proposed that α-OH 5:3 FTCA was produced from 5:3 FTCA and/or 5:3 FTUCA, despite that the latter two compounds were not detected in this study. Given a longer incubation time, it is likely that α-OH 5:3 FTCA will be further transformed into 4:3 acid and shorter PFAS through one carbon removal pathway (Wang et al., 2012).

4. Discussion

Carbon sources that not only can support the growth of microbial cultures but also can serve as inducers to degradative enzymes of interest have been successfully demonstrated for biodegradation of both priority and emerging environmental pollutants. For example, propane- and 1-butanol-grown (or induced) RHA1 have been shown to express propane monooxygenase and butane monooxygenase to cometabolically dechlorinate trichloroethyene (TCE) and/or 1,2,3-trichloropropane (TCP) (Hand et al., 2015; Wang and Chu, 2017). Similarly, a previous study on co-metabolic degradation of triclosan (an antibiotic) by RHA1 has suggested that propane monooxygenase, alkane monooxygenase, and biphenyl dioxygenase were involved in the dechlorination of triclosan (Lee and Chu, 2013). Additionally, both mixed and pure microbial strains have been reported to co-metabolically defluorinatate 6:2 FTOH and 6:2 polyfluoroalkyl phosphates (6:2 PAPs) (Kim et al., 2014, 2012; Lewis et al., 2016). Specifically, n-octane-grown *Pseudomonas oleovorans* and 1-butanol-grown *Pseudomonas butanovora* were able to biotransform and biodefluorinate FTOHs, suggesting that alkane monooxygenase and butane monooxygenase might be responsible for

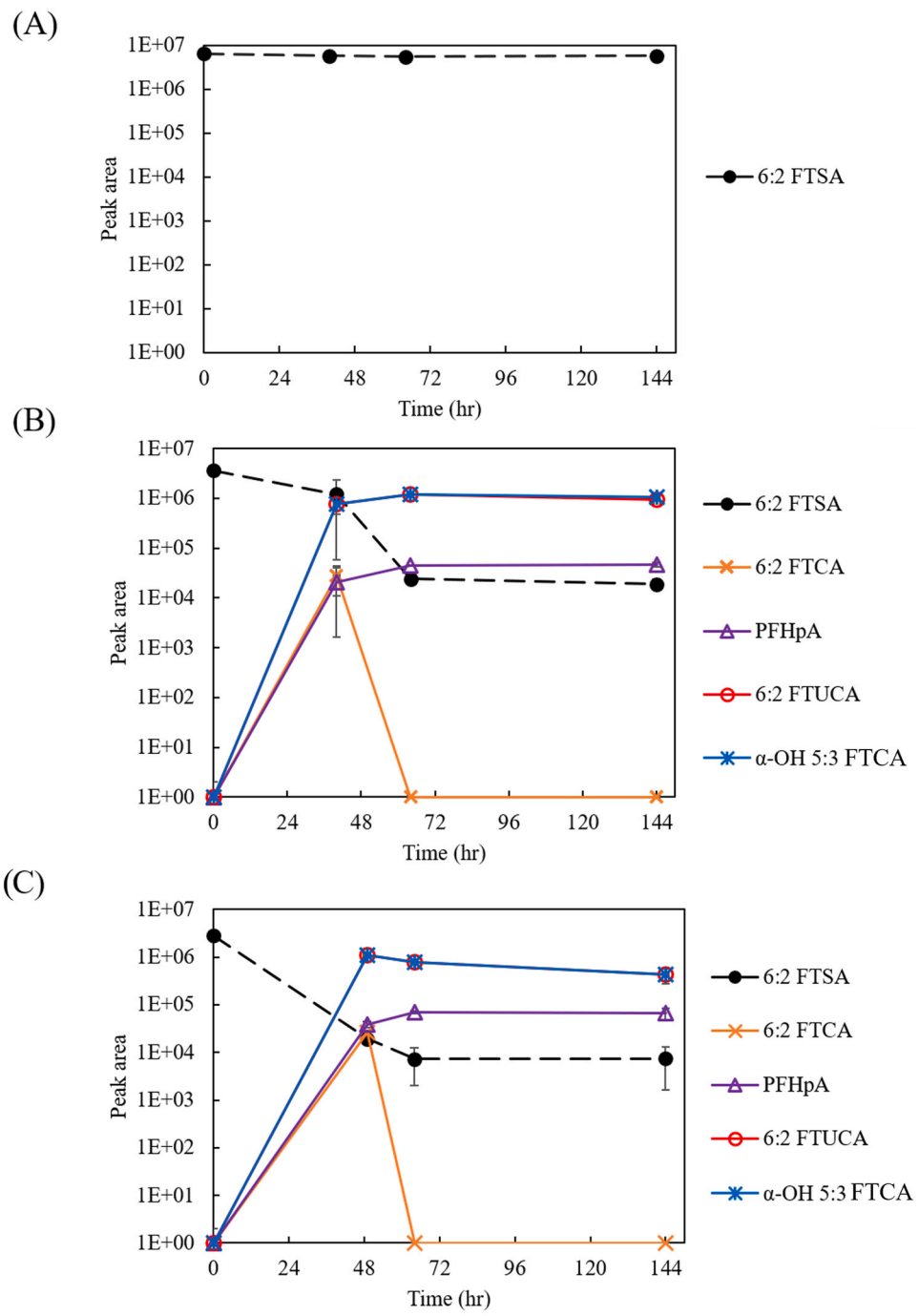

Fig. 4. Changes of 6:2 FTSA and its transformation products by RHA1 grown (A) with ethanol and sulfate; (B) with ethanol in S-free medium; (C) with 1-butanol in S-free medium. No transformation products were found in RHA1 grown in ethanol and sulfate. Two major transformation products (6:2 FTUCA and α-OH 5:3 FTCA) and two minor transformation products (6:2 FTCA and PFHpA) were detected in RHA1 grown in S-free medium with ethanol and 1-butanol respectively. The samples collected at 40 hr and 48 hr corresponded to the early exponential phase for two ethanol-grown RHA1 and 1-butanol-grown RHA1, respectively. The samples collected at 64 hr and 144 hr corresponding to the mid-exponential and stationary phases for ethanol- or 1-butanol-grown RHA1, respectively. Each condition was performed in duplicate (n = 2). The bars represented the ranges of the duplicates.

the defluorination (Kim et al., 2012). Mostly, higher numbers of gene copies of *alk*B (alkane monooxygenase) have been linked to high levels of fluoride release during 6:2 FTOH and 6:2 PAPs biotransformation by enrichment cultures and activated sludge amended with ethanol, 1-butanol and n-octane as carbon sources (Lewis et al., 2016). In this study, the results in Figs. 1A and 2 provide direct evidence to link the role of alkane monooxygenase to co-metabolic defluorination of 6:2 FTOH by ethanol-, n-octane- and 1-butanol-grown RHA1. The detection of low-level fluoride in samples containing 6:2 FTOH and glucose-grown RHA1 was surprising, suggesting some unknown enzymes might have low defluorination activity toward 6:2 FTOH. Additionally, our results suggested that cytochrome P450 induced by 1-butanol and n-octane might also play a role in the defluorination of 6:2 FTOH.

Despite the high expression levels of alkane monooxygenase and cytochrome P450 in the ethanol-, n-octane- and 1-butanol-grown RHA1, no 6:2 FTSA defluorination was observed, suggesting that other factors, perhaps due to the difference of functional group between 6:2 FTOH and 6:2 FTSA, prevented the occurrence of the defluorination of 6:2 FTSA. Accordingly, this aspect was further examined in the following experiments to determine whether the sulfonic group in 6:2 FTSA hinders the enzymatic defluorination activity in the RHA1 resting cells.

Sulfur is an essential element in bacteria specifically for amino acids and vitamins synthesis. In the absence of inorganic sulfate, certain bacteria are capable of utilizing sulfide from organosulfur such as taurine and alkanesulfonates. Two enzymes, taurine dioxygenase and alkanesulfonate monooxygenase, are expressed to catalyze the desulfonation. However, in the presence of other sulfur sources such as sulfate, sulfite, sulfide, or cysteine, these enzymes are repressed. The

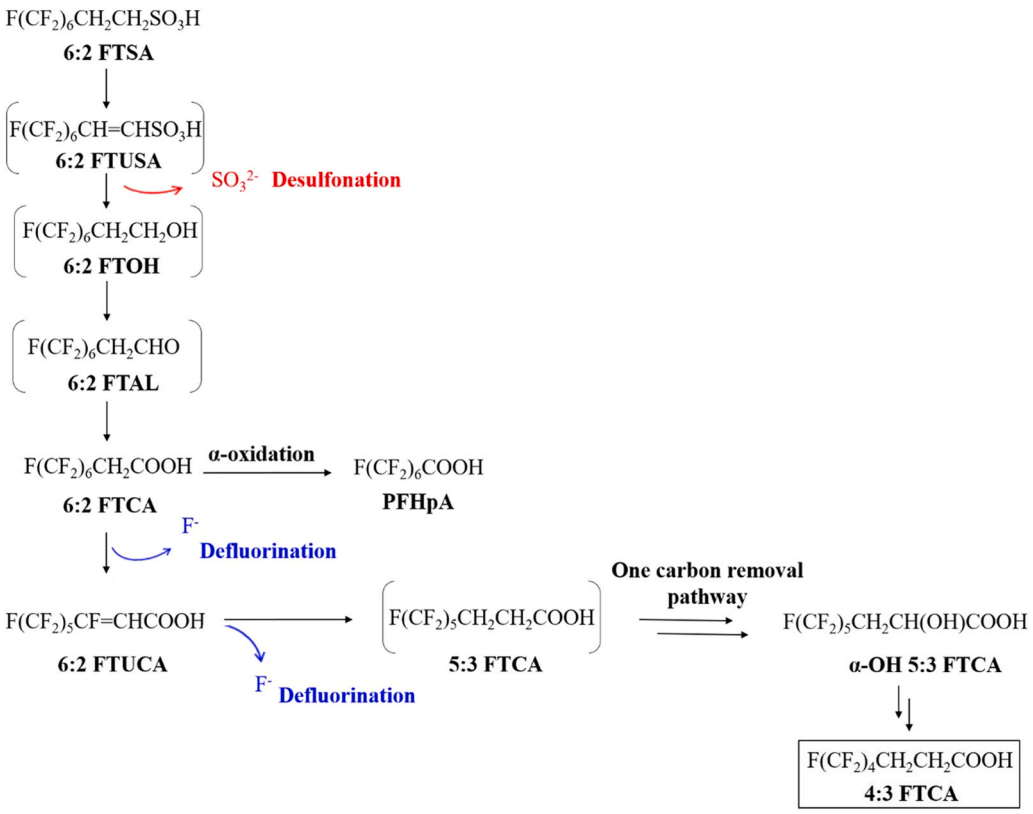

Fig. 5. Proposed 6:2 FTSA biotransformation pathway by RHA1 grown on ethanol or 1-butanol in S-free medium. Chemicals in brackets, except the tentatively identified metabolite 6:2 FTUSA, are assumed metabolites that were not detected in this study. Double arrows indicate multiple steps in the pathway. Chemical shown in the box was adapted from Wang et al., 2012

desulfonation role of alkanesulfonate monooxygenase in bacteria has been well studied (van der Ploeg et al., 2001). The deletion of this gene cassette (*ssuEADCB*) resulted in the loss of the ability to utilize alkanesulfonates as a sulfur source. In this study, higher expression level of alkanesulfonate monooxygenase was observed in ETSA and 6:2 FTSA treatment but not in the inorganic sulfate treatment, suggesting that this enzyme plays an important role in desulfonation of 6:2 FTSA.

Bacterial dehalogenases are unique enzymes which are capable of catalyzing the cleavage of the carbon-halogen bonds through a nucleophilic substitution by water to yield an alcohol. Haloalkane dehalogenase purified from *Rhodococcus rhodochrous* NCIMB 13064, *Sphingomonas paucimobilis* UT26, and *Xanthobacter autotrophicus* GJ10 showed capacity to degrade a variety of chlorinated, brominated, and iodinated compounds (Keuning et al., 1985; Kulakova et al., 1997; Marek et al., 2000). However, no defluorination activity of haloalkane dehalogenase were reported in these studies. This might explain why the mRNA level of haloalkane dehalogenase (RHA1_ro02879) targeted in this study were not highly expressed in 6:2 FTSA treatment. The haloacid dehalogenase isolated from *Pseudomonas putida* AJ1/23, *Rhodococcus erythropolis* Y2, and *Xanthobacter autotrophicus* GJ10 demonstrate a wide range of reactivity to chlorinated and brominated compounds (Sallis et al., 1990; Smith et al., 1990; van der Ploeg et al., 1991). Due to the strong C-F bond, with a dissociation energy of up to 130 kcal/mol, only the fluoroacetate dehalogenases and a few haloacid dehalogenases have defluorination activity (Chan et al., 2010). Fluoroacetate dehalogenase purified from *Moraxella* sp. B, *Pseudomonas fluorescens* DSM 8341, and *Burkholderia* sp. FA1 was able to defluorinate fluoroacetate (Donnelly and Murphy, 2009; Ichiyama et al., 2004; Kurihara et al., 2003). The enzymatic defluorination requires enzymes containing ‘‘a halide pocket’’ (Chan et al., 2011). This precise region provides electrostatic contacts or hydrogen bonds to stabilize the small fluoride ion. Interestingly, the haloacid dehalogenase (RHA1_ro00230) targeted in this study not only shares 70% % amino acid sequence similarity to the fluoroacetate dehalogenase from *Pseudomonas fluorescens* DSM 8341, but also demonstrates defluorination activity reported by Chan et al. suggested that this enzyme might contain a halide pocket and might have the ability to defluorinate other halogens (Chan et al., 2010). In this study, higher mRNA level of haloacid dehalogenase observed only in 6:2 FTSA treatments provides strong evidence that this enzyme in RHA1 plays an important role in defluorination of 6:2 FTSA. Whether haloacid dehalogenases have the ability to defluorinate other PFAS still remains unknown. Further research is needed to characterize its defluorination role in other PFAS species.

When sulfate was present in the growth medium, ethanol- or 1-butanol- or n-octane-grown RHA1 was unable to defluorinate 6:2 FTSA. However, when 6:2 FTSA was supplied as a sole sulfur source in the growth medium, ethanol- and 1-butanol-grown RHA1 were able to biotransform 6:2 FTSA via desulfonation and defluorination. These results indicate that desulfonation is the rate limiting step of the 6:2 FTSA biotransformation. The sulfonate group in 6:2 FTSA hinders the enzymatic defluorination even if these three enzymes (alkane monooxygenase, cytochrome P450, and haloacid dehalogenase) are highly induced. It also indicates that the presence of inorganic sulfate repressed the expression of enzymes responsible for extraction of sulfide from 6:2 FTSA. These results provide strong evidence in support of the suggestion of a previous study - desulfonation of 6:2 FTSA needs to occur before defluorination (Wang et al., 2011).

In this study, PFHpA was detected though at a low level during 6:2 FTSA biotransformation, suggesting that a new branched 6:2 FTSA biotransformation pathway might be driven by α-oxidation by removing one -CH_2- from 6:2 FTCA to form PFHpA. The formation of PFHpA has not been observed in the biotransformation of 6:2 FTSA by pure cultures nor in activated sludge (Shaw et al., 2019; Wang et al., 2011) but the formation has been reported in aerobic sediments (Zhang et al., 2016).

Additionally, PFHpA has been detected as a trace metabolite in the biotransformation of 6:2 polyfluoroalkyl phosphates (PAPs) in activated sludge (Lee et al., 2010). Results of our and previous studies supported that α-oxidation is a possible minor pathway in biotransforming 6:2 FTCA to PFHpA.

Gordonia sp. strain NB4–1Y has shown an ability to biotransform 6:2 FTUCA to a major metabolite 5:2 ketone via a so-called major pathway, and to a minor metabolite 5:3 FTCA via a so-called minor pathway (Shaw et al., 2019). However, in our study, the employed analytical method could not detect 5:2 ketone, making it is unclear whether 5:2 ketone and its metabolites had been formed. The discrepancy might be also explained by the use of different bacteria species in the previous and this studies. Additionally, different carbon sources used in cultivation induce different degradative enzymes that in turn select for different degradation pathways and production of metabolites. This phenomenon has been previously reported by our previous study on FTOH biotransformation using various pure strains grown with different carbon sources and inducers (Kim et al., 2012). For example, n-octane-grown *Pseudomonas oleovorans* transformed 6:2 FTOH using both the major pathway (the pathway that led to the formation of 5:2 ketone and finally to perfluorocarboxylic acids (PFCA)) and minor pathway (the pathway that led to the formation of X:3 acids). However, 1-butanol-grown *Pseudomonas butanovora* only used the major pathway to degrade 6:2 FTUCA to 5:2 ketone (Kim et al., 2012). When different nutrient and carbon source conditions were used, lactate-grown *Pseudomonas butanovora* was able to use both pathways to produce diverse metabolites (Kim et al., 2014). From the bioremediation point of view, metabolites such as PFPeA and PFHxA from the major pathway were not favorable. Metabolites produced from the minor pathway such as 5:3 FTCA and α-OH 5:3 FTCA are preferred, as these compounds can be further transformed into 4:3 FTCA and 3:3 FTCA or shorter PFAS through the removal of -CF_2 group, implying a potential for complete mineralization. In this study, α-OH 5:3 FTCA is the major metabolite detected in ethanol-grown and 1-butanol-grown RHA1 under S-free conditions, suggesting that cultivating RHA1 under these tested conditions can be further developed to serve as a new strategy for effective bioremediation of 6:2 FTSA-contaminated soil and water.

5. Conclusion

In this study, we demonstrated that 6:2 FTSA can only be biotransformed by RHA1 under S-free conditions. Observations of (i) the tentatively identified intermediate of 6:2 FTUSA, (ii) the confirmed intermediate of 6:2 FTCA, and (iii) the higher expression levels of alkanesulfonate monooxygenase in RHA1 grown in S-free medium confirm that desulfonation of 6:2 FTSA is a necessity prior to its defluorination. PFHpA detected in 6:2 FTSA biotransformation indicated α-oxidation is a possible biotransformation pathway. Enzymes including haloacid dehalogenase, alkane monooxygenase, and cytochrome P450 play important roles in the defluorination of 6:2 FTSA. This is the first study examining genes that are responsible for 6:2 FTSA desulfonation and defluorination. Overall, the results of this study provide a new understanding of 6:2 FTSA biotransformation. The metabolites and biotransformation pathway are useful in predicting the fate of 6:2 FTSA. The gene expression of desulfonating enzymes and different specific and non-specific defluorinating enzymes can serve as molecular markers to assess 6:2 FTSA biotransformation in the environment.

Statement of novelty

This study examined the factors that affect the biotransformation of 6:2 FTSA in a well-characterized bacterial strain *Rhodococcus jostii* RHA1. While biodegradation of 6:2 FTSA has been reported, 6:2 FTSA is persistent in the environment and the factors affecting desulfonation and defluorination of 6:2 FTSA are still not understood. This is the first study reporting the effects of carbon and sulfur source to the level of enzymes expression and biotransformation of 6:2 FTSA in the RHA1. Our results provide a new understanding of 6:2 FTSA biotransformation. These enzymes are potential markers to assess 6:2 FTSA biotransformation in the environment.

CRediT authorship contribution statement

Shih-Hung Yang: Investigation, Methodology, Data curation, Visualization, Writing – original draft preparation. **Ying Shi**: Investigation, Methodology, Data curation. **Mark Strynar**: Investigation, Methodology, Data curation, Writing – review & editing. **Kung-Hui Chu:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.127052.

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Supporting Information

for

Desulfonation and Defluorination of 6:2 Fluorotelomer Sulfonic Acid (6:2 FTSA) by *Rhodococcus jostii* RHA1: Carbon and Sulfur Sources, Enzymes, and Pathway

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This supporting information includes text describing methods, two table, eight figures and references.

Acronym

Methods

Sulfur rich (S-rich) and Sulfur free (S-free) mineral salt medium media
Fluoride measurement
Primer design
RNA extraction, cDNA synthesis, and RT-qPCR assays
Enzyme inhibition tests
Construction of plasmids with genes encoding presumptive defluorinating and desulfonating enzymes, gene expression, and enzyme activities.

Tables

Table S1. Primer sets used for RT-PCR and qPCR analysis in this study. The primers were designed to target genes encoding presumptive desulfonating- and defluorinating-enzymes in RHA, as well as the 16S rRNA gene of *Rhodococcus jostii* RHA1.

Table S2. Primer sets used for constructing plasmid pTipQC2 and pET11a with presumptive defluorinating and desulfonating genes: alkane monooxygenase, haloacid dehalogenase, cytochrome P450, alkanesulfonate monooxygenase, and NADPH:flavin oxidoreductase. Characters labeled in red refer to the restriction digest sites.

Figures

Figure S1. Growth curves of *Rhodococcus jostii* RHA1 supplied different C- and S-sources in S-free medium. Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.

Figure S2. Growth curves of *Rhodococcus jostii* RHA1 grown on 6:2 FTSA and ethanol in S-free medium (red dots), on 6:2 FTSA and butanol in S-free medium (black dots), or on ethanol in S-rich medium (blue dots). Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.

Figure S3. **(A)** Enzyme inhibition tests of alkane monooxygenase in hexane-induced *Rhodococcus jostii* RHA1. The known inhibitors, acetylene (ACT), allylthiourea (ATU) or both, was used to inhibit the alkane monooxygenase activity. The activity of alkane monooxygenase was confirmed by the decrease of hexane in samples without addition of inhibitors, compared to little or no hexane degradation in the presence of inhibitors, ATU, ACT, and ATU+ACT. **(B)** 6:2 FTOH and 6:2 FTSA were spiked into the hexane-induced cells in the presence and absence of inhibitors. After 40 hrs of incubation, samples containing 6:2 FTOH but no enzyme inhibitors released a significant amount of fluoride compared to the samples amended with inhibitors. No fluoride was released from samples containing with 6:2 FTSA, regardless the presence of the inhibitors. The results confirmed that alkane monoxygenase involved in defluorination of 6:2 FTOH. Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.

Figure S4. **(A)** Enzyme inhibition assay of haloacid dehalogenase in fluoroacetate-induced *Rhodococcus jostii* RHA1. The known enzyme inhibitor, CuSO4, was used to inhibit the haloacid dehalogenase activity. Defluorination of fluoroacetate (NaFAc) activity was confirmed in samples without the inhibitor and release of corresponding fluoride in liquid medium. No fluoride was detected in the killed treatment and the $CuSO_4$ treatment. **(B)** The ability of defluoriantion of 6:2 FTOH and 6:2 FTSA by haloacid dehalogenase were determined based on fluoride release detected after 64 hrs of incubation. In the absence of inhibitor, high fluoride levels were detected in samples containing 6:2 FTOH but not in the sample containing 6:2 FTSA. Much lower fluoride levels were detected in the 6:2 FTOH samples amended with the inhibitor (CuSO4). No fluoride released was detected in 6:2 FTSA samples amended with the inhibitor. Overall, the results linked the role of haloacid dehalogenase in defluorination of 6:2 FTOH. Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.

Figure S5. **(A)** Enzyme inhibition assay of cytochrome P450 in 1-butanol-induced *Rhodococcus jostii* RHA1. The known inhibitor, 1-aminobenzotriazole (ABT), was used to inhibit the cytochrome P450 activity. Allylthiourea (ATU) were used to rule out the effects of alkane monooxygenase. Degradation of 1-butanol were analyzed as the positive control for the enzyme activity and the efficiency of inhibitors. 6:2 FTOH and 6:2 FTSA were tested to confirm the defluorination activity of cytochrome P450 at 55 hrs. A significant degradation of 1-butanol was observed in RHA1 resting cell. The presence of either ATU or ABT did not completely inhibit the degradation of 1-butanol. However, in the presence of both ATU and ABT,

degradation of 1-butanol was strongly inhibited. **(B)** In the absence of inhibitor, significant levels of fluoride were released from 6:2 FTOH by the 1-butanol-induced RHA1 cells. Both ATU and ABT inhibited the activity of cytochrome P450, with better inhibitory effects when both ATU and ABT were added together. Once again, no defluorination of 6:2 FTSA was observed in all treatments. The results clearly linked the defluorination ability of cytochrome P450 toward 6:2 FTOH. Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.

Figure S6. **(A)** An agarose gel showed PCR products of presumptive defluorinating genes: alkane monooxygenase gene (ALK, 1233 bp), haloacid dehalogenase gene (HAD, 765 bp), and cytochrome P450 gene (P450, 1254 bp). (**B)** An agarose gel showed PCR products of presumptive desulfonating genes: alkanesulfonate monooxygenase (ssuD, 1071 bp) and NADPH:flavin oxidoreductase (ssuE, 630 bp).

Figure S7. Defluorination of 6:2 FTOH and 6:2 FTSA by engineered PD631 strains harboring plasmids (pALK, pHAD, and p450). About 220 to 230 ug/L of fluoride was detected in wild type PD631 and PD631 with a pTipQC2 empty vector. However, slightly higher concentration of fluoride was observed in PD631 with pALK, pHAD, and p450. No fluoride (below fluoride detection limit) was detected in all 6:2 FTSA treatments. Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.

Figure S8. Desulfonation of hexanesulfonate (HxSA) and 6:2 FTSA by crude enzymes of SsuD and SsuE. Compared to those with an empty vector, higher concentrations of SO_3^{2-} were released from two sulfonate substrates, HxSA and 6:2 FTSA, in SsuD/SsuE treatments. The results provided direct evidence that SsuD/SsuE were responsible for the desulfonation of 6:2 FTSA. Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.

Acronym

α-OH 5:3 FTCA	α-hydroxy-5:3 Fluorotelomer carboxylic acid	$F(CF_2)_5CH_2CH(OH)COOH$
4:3 FTCA	4:3 Fluorotelomer carboxylic acid	$F(CF_2)_4CH_2CH_2COOH$
5:3 FTCA	5:3 Fluorotelomer carboxylic acid	$F(CF_2)_5CH_2CH_2COOH$
6:2 FTSA	6:2 Fluorotelomer sulfonic acid	$F(CF_2)_6CH_2CH_2SO_3H$
6:2 FTUSA	6:2 Fluorotelomer unsaturated sulfonic acid	$F(CF_2)_6CH=CHSO_3H$
6:2 FTOH	6:2 Fluorotelomer alcohol	$F(CF_2)_6CH_2CH_2OH$
6:2 FTAL	6:2 Fluorotelomer aldehyde	$F(CF_2)_6CH_2CHO$
6:2 FTCA	6:2 Fluorotelomer carboxylic acid	$F(CF_2)_6CH_2COOH$
6:2 FTUCA	6:2 Fluorotelomer unsaturated carboxylic acid	$F(CF_2)_5CF=CHCOOH$
PFCA	Perfluorocarboxylic acids	$F(CF_2)_nCOOH$
PFHpA	Perfluoroheptanoic acid	$F(CF_2)_6COOH$
PFHxA	Perfluorohexanoic acid	$F(CF_2)_5COOH$
PFPeA	Perfluoropentanoic acid	$F(CF_2)_4COOH$
PFOA	Perfluorooctanoic acid	$F(CF_2)_8COOH$
PFOS	Perfluorooctanesulfonic acid	$F(CF_2)_8SO_3H$

Methods

Sulfur rich (S-rich) and Sulfur free (S-free) mineral salt medium. Sulfate was the sole sulfur source in the S-rich medium consisting of Na_2HPO_4 (0.87 g/L), $(NH_4)_2SO_4$ (0.78 g/L), K_2SO_4 (0.17 g/L), $MgSO_4 \cdot 7H_2O$ (0.04 g/L), $CaSO_4 \cdot 2H_2O$ (0.01g/L), $FeSO_4 \cdot 7H_2O$ (0.02 g/L) and 0.1% (v/v) trace mineral solution. The trace mineral solution contains $Na_2EDTA \cdot 2H_2O$ (0.5 g/L), $MnSO_4 \cdot 7H_2O$ (0.3 g/L), $CoSO_4 \cdot 7H_20$ (1.1 g/L), KI (0.2 g/L), H_3BO_3 (0.1 g/L), and $ZnSO_4 \cdot 7H_2O$ (0.6 g/L). For S-free medium, it was prepared as follows: K_2HPO_4 (3.5 g/L), KH_2PO_4 (1.5 g/L), NH_4Cl (0.5g/L), $MgCl_2 \bullet 6H_2O$ (0.15 g/L), NaCl (0.62 g/L) and 0.1 % (v/v) S-free trace mineral solution. The S-free trace solution contains $FeCl_3 \bullet 6H_2O$ (0.24 g/L), $MnCl_2 \bullet 4H_2O$ (0.03 g/L), $CoCl_2 \bullet 6H_2O$ (0.04 g/L), $CuCl_2 \bullet H_2O$ (0.04 g/L), $ZnCl_2$ (0.15 g/L), and $Na_2MoO_4 \bullet 2H_2O$ (0.03 g/L).

Fluoride measurement. Concentrations of fluoride in liquid samples were determined as described previously (Huang et al., 2018; Lewis et al., 2016). Briefly, the liquid samples were first centrifuged at 13,000 rpm for 10 min and then filtered through a 0.45 μm-pore size sterile syringe filter (VWR, Radnor, PA, USA) to remove cell debris before being used for fluoride measurement. The fluoride ion concentrations were determined by using a fluoride ion selective probe Orion 96-09BNWP (Thermo Scientific, Beverly, MA, USA) following the manufacture's instruction. The detection limit for fluoride of this method is 50 μg/L.

Primer design. Online PrimerQuest tool program (https://www.idtdna.com/PrimerQuest/Home/Index) was used to design primers targeting these genes RHA1_ro01768 (encoding alkanesulfonate monooxygenase), RHA1_ro02879 (encoding haloalkane dehalogenase), RHA1_ro00230 (encoding haloacid dehalogenase), RHA1_ro02534 (encoding alkane monooxygenase), and RHA1_ro00377 (encoding cytochrome P450 CYP254). The designed primer sets were listed in Table S1 in supporting materials. All the primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The primers are listed in Table S1.

RNA extraction, cDNA synthesis, and RT-qPCR assays. Total RNA was extracted from the biomass sample (two replicate) using FastRNA Pro blue kit and the extracted RNA was used for cDNA synthesis using OneStep Ahead RT-PCR kit according to the manufacturer's instruction. Diluted cDNA (250 times dilution) was then used as a template in real-time quantitative PCR (RT-qPCR) for these targeted genes and 16S rRNA (as the housekeeping gene). Briefly, the 25-μL reaction mixture contained 1 μL of diluted cDNA, 10 μL of PowerSYBR Green PCR Master Mix, 0.5 μM of each of the primer sets and nuclease-free water to 20 μL. The RT-qPCR was conducted using an IQ5 multicolor real-time PCR detection system (Bio-rad, Hercules, CA, USA). The cycling condition was as follows: 1 cycle of denaturation at 95 °C for 10 min, followed by 35 three-step cycles of amplification (95 °C for 30 sec, 54 °C for 45 sec and 72 °C for 45 sec). The fluorescence intensity was measured at the end of each of the three-step cycles. Three technical repeats were conducted for qPCR. The Ct value, defined as the PCR cycle number that crosses an arbitrary fluorescence signal threshold, was used to calculate the expression level using the double delta Ct method as described in the previous study (Winer et al., 1999). The fold changes of the expression levels of targeted genes in RHA1 grown under different conditions were normalized to those in RHA1 grown on glucose and sulfate (as baseline). Standard deviation was represented as errors.

Whole cells enzyme inhibition tests. Enzyme inhibition tests were designed and conducted to confirm whether the presumptive defluorinating enzymes are responsible for the defluorination of 6:2 FTOH and 6:2 FTSA. Acetylene (ACT) and allylthiourea (ATU) were known inhibitors of alkane monooxygenase (Curry et al., 1996; Hamamura et al., 2001). Cu^{2+}($CuSO_4$) is a known inhibitor of haloacid dehalogenase (Zhang et al., 2014). 1-aminobenzotriazole (ABT) is a known inhibitor of cytochrome P450 (Steffan et al., 1997). RHA1 cells were pregrown in R2A medium to desired optical densities, followed by incubating with one of three specific substrates to induce desired enzyme expression in the RHA1 cells. Hexane, fluoroacetate and 1-butanol were used as an enzyme inducer for alkane monooxygenase, haloacid dehalogenase and cytochrome P450, respectively. These three substrates were also used in inhibition assays as positive controls, i.e., without inhibitors. The inhibition tests were conducted in duplicate as follows.

(i) Inhibition tests for alkane monooxygenase

RHA1 cells were prepared by growing in R2A medium to optical density (OD_{600}) 0.7-0.8 before harvesting by centrifugation, washed and resuspend in PBS. Hexane, as an inducer for alkane monooxygenase, was then added to the cell suspension which was then incubated at 30 ºC and 150 rpm for 12 hrs. The hexane-induced cells were then collected by centrifugation, washed and resuspended in PBS for experimental use. Ten milliliters of the cell suspension were allocated in a series of 30-ml serum vials. The vials were then amended with one of the substrates (Hexane (5.7 mg/L), 6:2 FTOH (0.46 mM), 6:2 FTSA (0.05 mM)) with and without enzyme inhibitors. Enzyme inhibitors, ATU (1mM), or ACT (0.1%) or both, were used. The vails were then incubated at 30 ºC and 150 rpm. Samples were collected at 24 hr and 40 hr for fluoride and hexane measurements. Fluoride concentrations were determined as described above. Hexane concentrations were determined by injecting 50 ul of head space into Gas Chromatography (GC)/Flame Ionization Detection (FID) (Agilent Technologies 6890N) as described in previous study (Hand et al., 2015). Calibration curves for hexane were generated from hexane solutions, ranging from 0.2 to 7.6 mg hexane/L in aqueous phase. Henry's law was used to convert headspace hexane concentrations into hexane concentrations in liquid medium.

(ii) Inhibition tests for haloacid dehalogenase

RHA1 cells were prepared as described above. Sodium fluoroacetate were added as inducer for haloacid dehalogenase in RHA1 cells. After incubating at 30 ºC at 150 rpm for 12 hrs, the induced cells were harvested for experimental use as described above. Enzyme inhibition tests were conducted in a series of 30-ml serum vials containing ten milliliters of cell suspension, one of three substrates (Fluoroacetate (4.9 mg/L), 6:2 FTOH (0.46 mM), 6:2 FTSA (0.05 mM)) with and without enzyme inhibitor, $CuSO_4$ (10 mM). A set of vials without adding $CuSO_4$ was also used. The vails were then incubated at 30 ºC and 150 rpm for 64 hrs. Liquid samples were collected over time and used for fluoride measurements as described above.

(iii) Inhibition tests for cytochrome P450

RHA1 cells were prepared as described above, and induced with 1-butanol, a known inducer for cytochrome P450, at 30 ºC and 150 rpm for 12 hrs. As 1-butanol not only induce cytochrome P450 but also alkane monooxygenase (Figure 2), two different enzyme inhibitors ATU (1 mM) and ABT (10 mM) were used to delineate the role of alkane monooxygenase during 6:2 FTOH defluorination. Similarly, cell suspension (10 mL) was allocated into each of a series of 30-ml serum vials, which were dosed with ATU, ABT or both. A parallel set of vials without adding

inhibitors was used. One of three different substrates –1-butanol (300 mM), 6:2 FTOH (0.46 mM), 6:2 FTSA (0.05 mM) – was spiked to the vails. The vails were incubated at 30 ºC and 150 rpm for 55 hrs. Time course samples were collected and analyzed for fluoride release and 1-butanol measurement. Fluoride concentrations were measured as described above. The concentrations of 1-butanol was determined by injecting 50 ul of headspace gas into GC/FID as the described above. Calibration curve for 1-butanol was generated from 1-butanol solutions, ranging from 20 to 500 mM in aqueous phase. Standard curves and Henry's law were then used to determine 1-butanol concentrations.

Construction of plasmids with genes encoding presumptive defluorinating and desulfonating enzymes, gene expression, and enzyme activities.

(i) PCR reaction, ligation, and transformation for the recombinant plasmids

A 25-μL of PCR reaction - including 12.5 μL of 2X PCR Master Mix (Promega, Madison, WI, USA), 1 μL of RHA1 genome DNA, 1 μL each of 10 μM forward and reverse primers (Table S2), and 9.5 μL of nuclease-free water - was used. The PCR reaction was carried out as follows: denaturing at 95 °C for 2 min, followed by 35 cycles at 95 °C for 45 sec, 54 °C for 45 min, and 72 °C for 1 min and 20 sec, and then a final extension cycle at 72 °C for 10 min. The PCR product for alkane monooxygenase gene, haloacid dehalogenase gene, and cytochrome P450 gene, alkanesulfonate monooxygenase gene, and NADPH:flavin oxidoreductase gene (ssuE) was clearly observed as a band of 1233, 765, 1254, 1071, and 630 base pairs (bp) on the agarose gel respectively (Figure S6). The PCR product was purified by QiAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

The PCR product of the alkane monooxygenase gene was cloned into a pTipQC2 plasmid (AIST Japan, Tokyo, Japan) using the NdeI-EcoRI cloning site. The PCR products of the haloacid dehalogenase gene, and cytochrome P450 gene, and alkanesulfonate monooxygenase gene were cloned into a pTipQC2 plasmid using the NdeI-BamHI cloning site. The NADPH:flavin oxidoreductase gene was cloned into a pET11a plasmid using the NdeI and NheI cloning site. The 20-μL of ligation mixture included 3 μL of linearized vector (about 50 ng), 2 μL of 10X T4 DNA ligase buffer, 1 μL of purified PCR product (a molar ratio of 1:3 vector to insert), 1 μL of T4 DNA ligase, and 13 μL of deionized water. The mixture was incubated at 16 °C for 16 hrs, followed by inactivation of enzymes at 65 °C for 10 min. A 2-μL of reaction mixture was used for chemical transformation into competent NEB 5-alpha *Escherichia coli* cells (Product No. C2987H, New England Biolabs, Ipswich, MA, USA) by heat-shock, according to the manufacturer's instructions. The transformed culture was plated on LB agar with 100 mg/L of ampicillin (AMP) as a selective marker. The candidate plasmid constructs were prepared by QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and were confirmed by verification of plasmid sequences (Eton Bioscience, San Diego, CA, USA). These inducible plasmids containing the alkane monooxygenase gene, alkanesulfonate monooxygenase gene, haloacid dehalogenase gene, and cytochrome P450 gene are designated as pALK, pSUD (and pSUE), pHAD, and p450, respectively. The recombinant plasmids pALK, pSUD, pHAD, and p450 were used for transformation into electrocompetent cells of *R. opacus* PD631 according to the previous study (Hwangbo et al., 2021). The pSUE plasmid was used for transformation into *E. coli* BL21 (DE3) as described in the previous study (Hwangbo et al., 2019).

(ii) Expression and activity of genes responsible for defluorination of 6:2 FTOH and 6:2 FTSA.

The engineered strains PD631 harbored pALK, pHAD, p450 were first grown in LB medium containing 35 mg/L of CAM at 200 rpm at 30 °C, respectively. The optical density (OD_{600}) of the cell suspension was monitored over time. When reaching the exponential growth phase (OD_{600} ~0.6), 3 μg/mL of thiostrepton was added to induce the expression of genes in pALK, pHAD, and p450. 6:2 FTOH (0.46 mM) and 6:2 FTSA (0.05 mM) were spiked at the same time to cell suspensions, respectively to examine the defluorination activity of expressed proteins. PD631 wild type and PD631 carrying pTipQC2 were used as two different types of negative controls. The cells were harvested after 16 hrs incubation at 200 rpm and 30 °C. Fluoride released was measured as described above.

(iv) Expression and activity of genes encoding enzymes responsible for desulfonation of 6:2 FTSA.

The engineered *Rhodococcus* strain harboring pSUD was first grown in LB medium containing 35 mg/L of chloramphenicol at 200 rpm and 30 °C. When reaching the exponential growth phase (OD_{600} ~0.6), 3 μg/mL of thiostrepton was added to induce the expression of the genes in pSUD. Cells were harvested after 16 hrs incubation at 200 rpm and 30 °C. The engineered strain *E. coli* BL21 (DE3) harboring pSUE was grown in LB medium containing 100 mg/L of AMP at 200 rpm at 37 °C. When reaching the exponential growth phase (OD_{600} ~0.4), isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce the expression of the gene in pSUE. Cells were harvested after 16 h incubation at 200 rpm at 23 °C.

Crude enzymes of alkanesulfonate monooxygenase (SsuD) and NADPH:flavin oxidoreductase (SsuE) were collected as described in Hwangbo et. al., 2019. Alkanesulfonate monooxygenase activity was assessed using Ellman's reagent. The reaction mixture (1.5 mL) consisted of 500 μM NADPH, 3 μM FMN, 500 μM sulfonate substrate (hexanesulfonic acid sodium salt or 6:2 FTSA) with SsuD to SsuE at a ratio of 1:3 in 25 mM potassium phosphate buffer. Crude enzymes expressed from cells harboring an empty vector (pET11a) were used as negative controls. The reaction was initiated by adding SsuD into the mixture and then incubated at 30 °C for 1 hr. The sulfite production was determined after the addition of 100 μL Ellman's reagent (1 mg/mL in 100 mM sodium phosphate buffer, pH 7.0). The colorimetric reaction was allowed to develop at room temperature for 2 min and the absorbance was measured at 412 nm.

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Table S1. Primer sets used for RT-PCR and qPCR analysis in this study. The primers were designed to target genes encoding presumptive desulfonating and defluorinating enzymes in RHA, as well as the 16S rRNA gene of RHA1.

Target gene	Protein	Primers	Sequence	PCR Product length (bp)	Tm (°C)
RHA1_ro01768	alkanesulfonate monooxygenase	ssuD-F ssuD-R	5'-CGAAGATCGCGGACATGAA-3' 5'-GTCGCGCACGATGATGTA-3'	92	54
RHA1_ro02879	haloalkane dehalogenase	halk-F halk-R	5'-TATTGGACCTGGTCGAGGTAA-3' 5'-GATGCCGACGCACATCTT-3'	147	54
RHA1_ro00230	Haloacid dehalogenase	hacid-F hacid-R	5'-CAGGTTTGTATTTCCGGTTGATG-3' 5'-AACGGCAACACCTCACTT-3'	97	54
RHA1_ro02534	alkane monooxygenase	alk-F alk-R	5'-TCAATGCCTGGCTCATGTC-3' 5'-CGAGGAGAGAGAAACCGTAGA-3'	111	54
RHA1_ro00377	cytochrome P450 CYP254	cyt-F cyt-R	5'-GATGAGCAGCAGGAAGAACA-3' 5'-GATCATCAACACACCGATCCT-3'	88	54
RHA1_ro01467	16S rRNA	16S-F 16S-R	5'-AACCGCCTACGAACTCTTTAC-3' 5'-AAGAAGCACCGGCTAACTAC-3'	96	54

Table S2. Primer sets used for constructing plasmid pTipQC2 and pET11a with presumptive defluorinating and desulfonating genes: alkane monooxygenase, haloacid dehalogenase, cytochrome P450, alkanesulfonate monooxygenase, and NADPH:flavin oxidoreductase. Characters labeled in red refer to the restriction digest sites.

Target gene	Protein	Primers	Sequence	PCR Product length (bp)	Tm (°C)
RHA1_ro00230	Haloacid dehalogenase	HAD-F HAD-R	5'-TTAGGTCATATGTCACCTGAACCCTGTCGATC-3' 5'-AATAATGGATCCATGGCTGGTGTGCCGTTC-3'	765	54
RHA1_ro02534	alkane monooxygenase	ALK-F ALK-R	5'- TTGGATCATATGGTGACGACGTCGAATATCAG -3' 5'- ATTAATGAATTCTCACCGAACTCCGCCGCTGT -3'	1233	54
RHA1_ro00377	cytochrome P450 CYP254	450-F 450-R	5'-TTTAATCATATGTCACAGTTCCGGAGTGAACG-3' 5'-AATAATGGATCCGTGGCTTTCGAAGGGTGTTC-3'	1254	54
RHA1_ro01768	alkanesulfonate monooxygenase	SUD-F SUD-R	5'-TTAGGTCATATGATGCACTTCGGCTACTGGAC-3' 5'-AATAATGGATCCTTAACCGCCCGTGGTTGACA-3'	1071	54
RHA1_ro06436	NADPH:flavin oxidoreductase	SUE-F SUE-R	5'-TAATTGCATATGATGTCACAGACCAACGTTCTCG-3' 5'-TTATTTGCTAGCTCAGGCGTCGACGAGCTG-3'	630	54

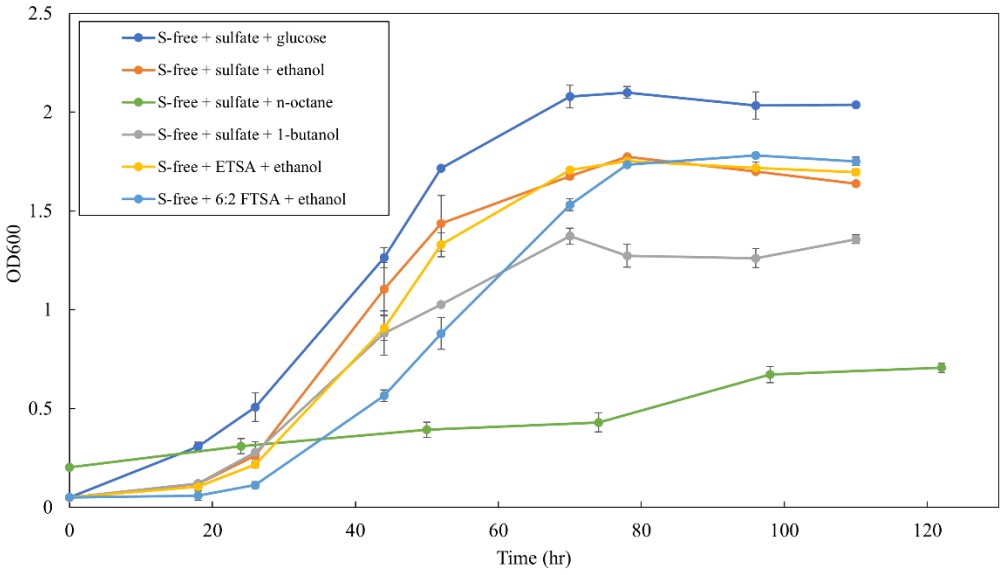


Figure S1. Growth curves of *Rhodococcus jostii* RHA1 grown on different C- and S-sources in S-free medium. Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.

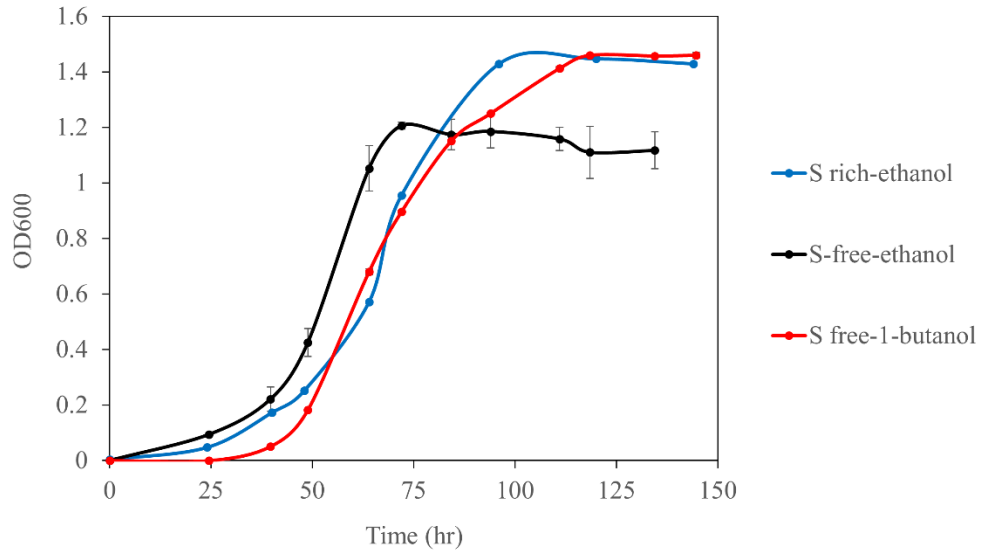


Figure S2. Growth curves of *Rhodococcus jostii* RHA1 grown on 6:2 FTSA and ethanol in S-free medium (red dots), on 6:2 FTSA and butanol in S-free medium (black dots), or on ethanol in S-rich medium (blue dots). Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.

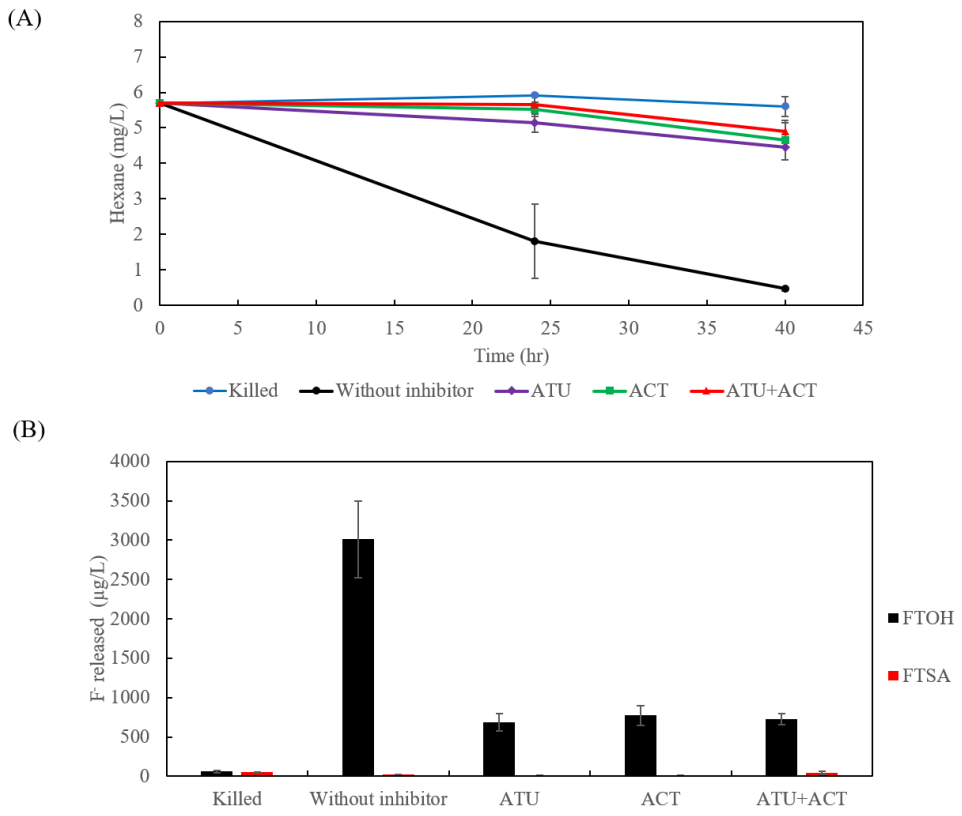


Figure S3. **(A)** Enzyme inhibition tests of alkane monooxygenase in hexane-induced *Rhodococcus jostii* RHA1. The known inhibitors, acetylene (ACT), allylthiourea (ATU) or both, was used to inhibit the alkane monooxygenase activity. The activity of alkane monooxygenase was confirmed by the decrease of hexane in samples without addition of inhibitors, compared to little or no hexane degradation in the presence of inhibitors, ATU, ACT, and ATU+ACT. **(B)** 6:2 FTOH and 6:2 FTSA were spiked into the hexane-induced cells in the presence and absence of inhibitors. After 40 hrs of incubation, samples containing 6:2 FTOH but no enzyme inhibitors released a significant amount of fluoride compared to the samples amended with inhibitors. No fluoride was released from samples containing with 6:2 FTSA, regardless the presence of the inhibitors. The results suggested that alkane monooxygenase might involve in defluorination of 6:2 FTOH. Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.

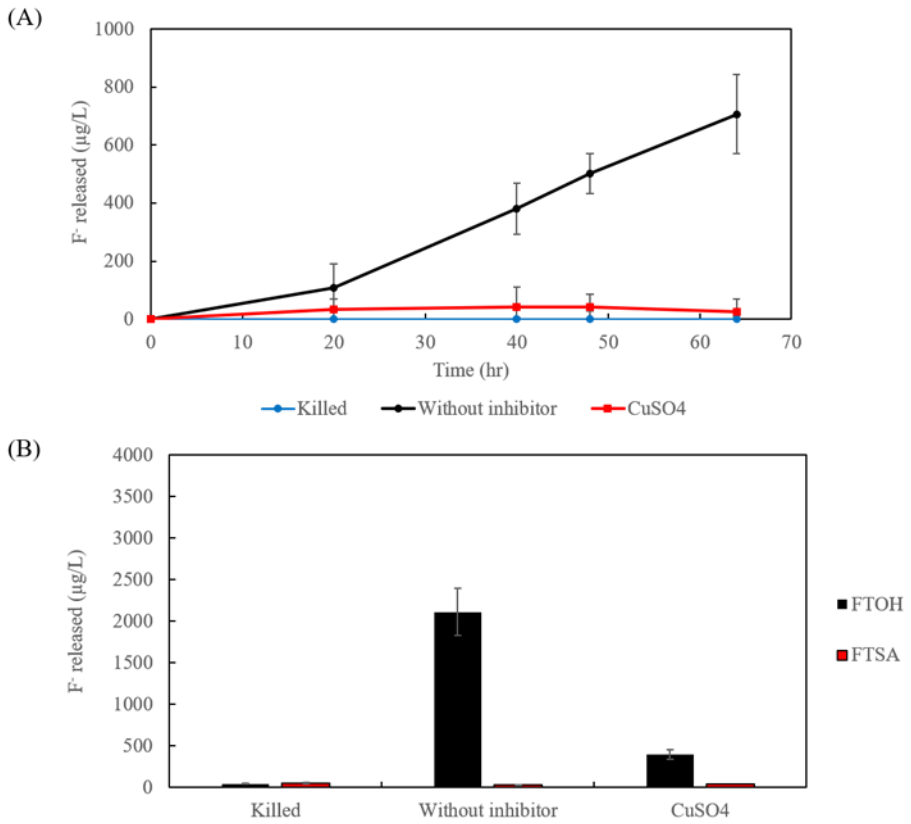


Figure S4. **(A)** Enzyme inhibition assay of haloacid dehalogenase in fluoroacetate-induced *Rhodococcus jostii* RHA1. The known enzyme inhibitor, $CuSO_4$, was used to inhibit the haloacid dehalogenase activity. Defluorination of fluoroacetate (NaFAc) activity was confirmed in samples without the inhibitor and release of corresponding fluoride in liquid medium. No fluoride was detected in the killed treatment and the $CuSO_4$ treatment. **(B)** The ability of defluoriantion of 6:2 FTOH and 6:2 FTSA by haloacid dehalogenase were determined based on fluoride release detected after 64 hrs of incubation. In the absence of inhibitor, high fluoride levels were detected in samples containing 6:2 FTOH but not in the sample containing 6:2 FTSA. Much lower fluoride levels were detected in the 6:2 FTOH samples amended with the inhibitor ($CuSO_4$). No fluoride released was detected in 6:2 FTSA samples amended with the inhibitor. The results support the gene expression data that haloacid dehalogenase might play the role in defluorination of 6:2 FTOH. Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.

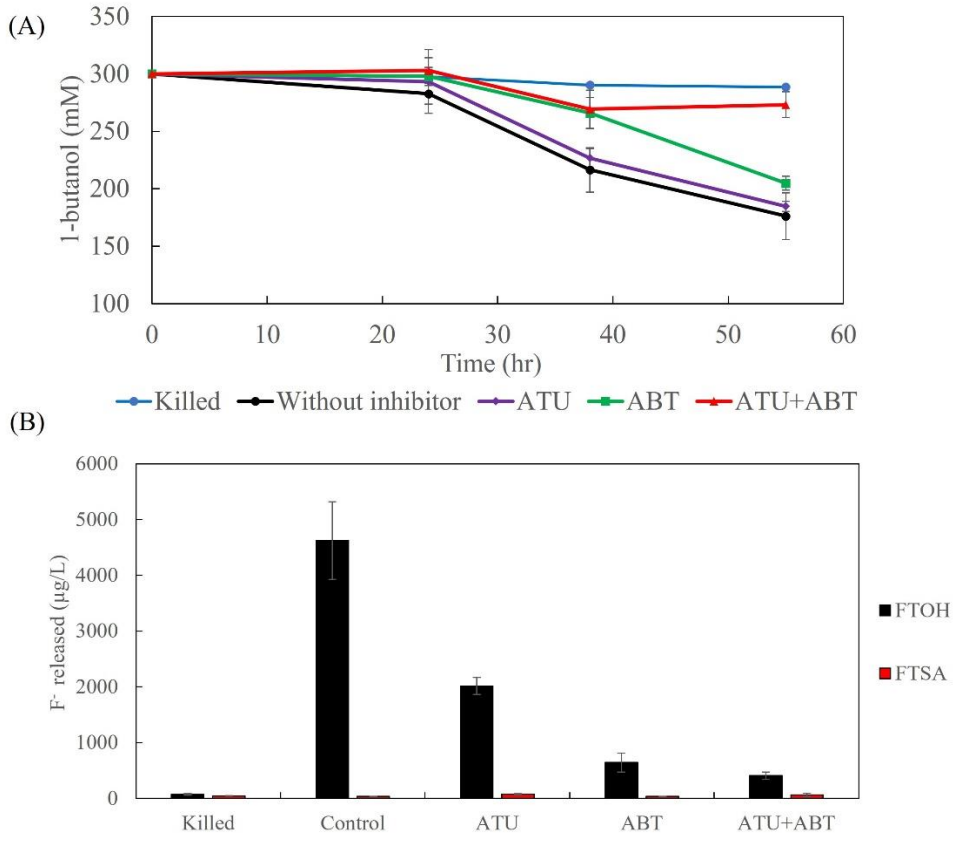
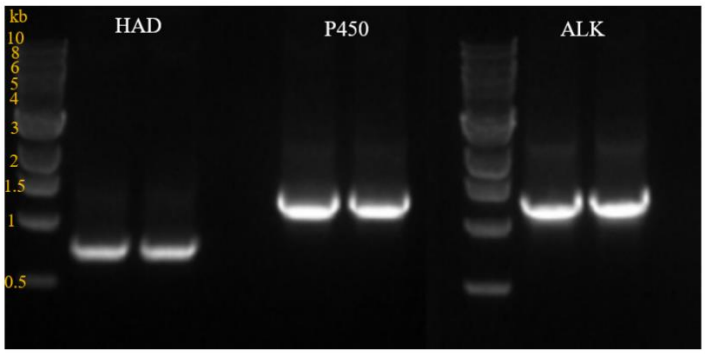


Figure S5. **(A)** Enzyme inhibition assay of cytochrome P450 in 1-butanol-induced *Rhodococcus jostii* RHA1. The known inhibitor, 1-aminobenzotriazole (ABT), was used to inhibit the cytochrome P450 activity. Allylthiourea (ATU) were used to rule out the effects of alkane monooxygenase. Degradation of 1-butanol were analyzed as the positive control for the enzyme activity and the efficiency of inhibitors. 6:2 FTOH and 6:2 FTSA were tested to confirm the defluorination activity of cytochrome P450 at 55 hrs. A significant degradation of 1-butanol was observed in RHA1 resting cell. The presence of either ATU or ABT did not completely inhibit the degradation of 1-butanol. However, in the presence of both ATU and ABT, degradation of 1-butanol was strongly inhibited. **(B)** In the absence of inhibitor, significant levels of fluoride were released from 6:2 FTOH by the 1-butanol-induced RHA1 cells. Both ATU and ABT inhibited the activity of cytochrome P450, with better inhibitory effects when both ATU and ABT were added together. Once again, no defluorination of 6:2 FTSA was observed in all treatments. The results implied the defluorination ability of cytochrome P450 toward 6:2 FTOH. Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.

(A)



(B)

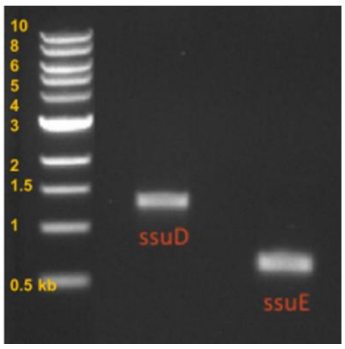


Figure S6. **(A)** An agarose gel showed PCR products of presumptive defluorinating genes: alkane monooxygenase gene (ALK, 1233 bp), haloacid dehalogenase gene (HAD, 765 bp), and cytochrome P450 gene (P450, 1254 bp). (**B)**An agarose gel showed PCR products of presumptive desulfonating genes: alkanesulfonate monooxygenase (ssuD, 1071 bp) and NADPH:flavin oxidoreductase (ssuE, 630 bp).

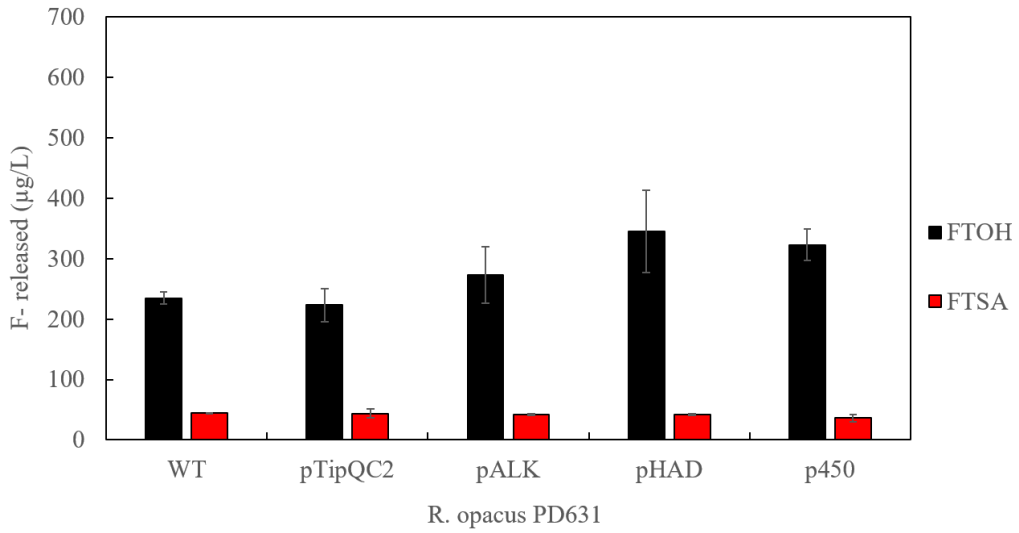


Figure S7. Defluorination of 6:2 FTOH and 6:2 FTSA by engineered PD631 strains harboring plasmids (pALK, pHAD, and p450). About 220 to 230 ug/L of fluoride was detected in wild type PD631 and PD631 with a pTipQC2 empty vector. However, slightly higher concentration of fluoride was observed in PD631 with pALK, pHAD, and p450. No fluoride (below fluoride detection limit) was detected in all 6:2 FTSA treatments. Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.

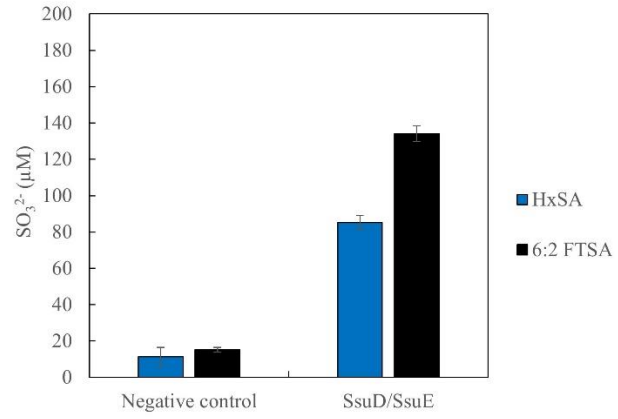


Figure S8. Desulfonation of HxSA and 6:2 FTSA by crude enzymes of SsuD and SsuE. Compared to those with an empty vector, higher concentrations of SO_3^{2-} were released from two sulfonate substrates, hexanesulfonate (HxSA) and 6:2 FTSA, in SsuD/SsuE treatments. The results provided direct evidence that SsuD/SsuE were responsible for the desulfonation of 6:2 FTSA. Each condition was performed in duplicate (n=2). The bars represented the ranges of the duplicates.