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Biodefluorination and Biotransformation of Fluorotelomer Alcohols by Two Alkane-Degrading *Pseudomonas* Strains

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ABSTRACT: Fluorotelomer alcohols [FTOHs, $F(CF_2)_nCH_2CH_2OH$, $n = 4$, 6, and 8] are emerging environmental contaminants. Biotransformation of FTOHs by mixed bacterial cultures has been reported; however, little is known about the microorganisms responsible for the biotransformation. Here we reported biotransformation of FTOHs by two well-studied *Pseudomonas* strains: *Pseudomonas butanovora* (butane oxidizer) and *Pseudomonas oleovorans* (octane oxidizer). Both strains could defluorinate 4:2, 6:2, and 8:2 FTOHs, with a higher degree of defluorination for 4:2 FTOH. According to the identified metabolites, *P. oleovorans* transformed FTOHs via two pathways I and II. The pathway I led to the production of *x*:2 ketone [dominant metabolite, $F(CF_2)_xC(O)CH_3$; $x = n - 1$, $n = 6$ or 8], *x*:2 sFTOH [$F(CF_2)_xCH(OH)CH_3$], and perfluorinated carboxylic acids (PFCAs, perfluorohexanoic, or perfluorooctanoic acid). The pathway II resulted in the formation of *x*:3 polyfluorinated acid [$F(CF_2)_xCH_2CH_2COOH$] and relatively minor shorter-chain PFCAs (perfluorobutyric or perfluorohexanoic acid). Conversely, *P. butanovora* transformed FTOHs by using the pathway I, leading to the production of *x*:2 ketone, *x*:2 sFTOH, and PFCAs. This is the first study to show that individual bacterium can biotransform FTOHs via different or preferred transformation pathways to remove multiple $-CF_2-$ groups from FTOHs to form shorter-chain PFCAs.

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KEYWORDS: fluorotelomer alcohols; *Pseudomonas*; biodefluorination; biotransformation pathways

Introduction

Perfluorinated carboxylic acids (PFCAs) and perfluorinated sulfonic acids are persistent compounds that have been detected in the environment, wildlife, and human (Calafat et al., 2007; Higgins et al., 2005; Houde et al., 2006; Loos et al., 2008; Powley et al., 2008; Sinclair and Kannan, 2006; So et al., 2007; Yamashita et al., 2008). The environmental sources of PFCAs have not been fully understood; however, one possible source of PFCAs comes from abiotic and biotic transformation of fluorotelomer alcohols (FTOHs) precursors (Dinglasan et al., 2004; Ellis et al., 2003, 2004).

FTOHs [$F(CF_2)_nCH_2CH_2OH$], consisting of a hydro- and oleo-phobic perfluoroalkyl moiety and an ethanol moiety, are used as raw-materials to manufacture surfactants and polymeric products with water- and oil-repelling properties (Kissa, 2001). The unique properties of FTOH-based products have led to a wide variety of applications including paints, adhesives, waxes, polishes, metals, electronics, caulks, agrochemicals, refrigerants, fire-fighting foams, and lubricants (Kissa, 2001). From 2000 to 2002, the production of FTOH-based products was estimated to be ~6,000 tons of fluorotelomer A equivalent/year in the world (Prevedouros et al., 2006).

FTOHs are volatile, not very soluble in water (carbon chain-length dependent) in the absence of a sorbing medium (Liu and Lee, 2005) and have a tendency to be adsorbed strongly to solid matters such as household dusts (Strynar and Lindstrom, 2008), soils or activated sludge (Liu and Lee, 2005, 2007; Wang et al., 2005a). Field monitoring studies have detected FTOHs in the troposphere at concentrations ranging from 7 to 196 pg/m^3 (Martin et al., 2002) and averaged 87 pg/m^3 (Dreyer et al., 2009) with 6:2 and 8:2 FTOHs in majority. The major source of environmental FTOHs has been postulated to come from the residual unreacted FTOH present in commercial products (Ellis et al., 2003). A recent study suggested that

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current practice of applying sludge from WWTPs to soils is a potential pathway to introduce FTOHs and PFCAs into the environment (Yoo et al., 2010).

Similar to the historical aspect of PCB dechlorination (Field and Sierra-Alvarez, 2008), microbial removal of $–CF_2–$ groups from within FTOHs was considered extremely difficult due to the strength of C–F bonds in the perfluoroalkyl moiety of FTOHs. Early studies had reported biotransformation of 8:2 FTOH in 1,2-dichloroethane enrichment culture (Dinglasan et al., 2004), activated sludge (Wang et al., 2005a,b), and soils (Liu et al., 2007; Wang et al., 2009). By using ^{14}C-labeled 8:2 FTOH, Wang et al. (2005a) reported the first evidence of C–F bond cleavage in 8:2 FTOH by activated sludge. In recent years, Liu et al. (2010b) and Wang et al. (2009) reported more FTOH transformation products from FTOH defluorination and proposed possible transformation pathways for 6:2 FTOH and 8:2 FTOH in aerobic soils. Nevertheless, all these findings were derived from experiments with mixed cultures or microbial consortia, providing little or no knowledge about the specific microorganisms that are responsible for the FTOH biotransformation. Furthermore, it is unclear which transformation pathways are preferred or used by the degradative microorganisms under the study conditions.

Many alkane-degrading microorganisms are known for their degradative enzymes and diverse metabolic pathways, enabling them to utilize or degrade alkanes and other pollutants with different chain lengths (Rojo, 2009; Van Beilen and Funhoff, 2007; Wentzel et al., 2007). Among these alkane-degraders, *Pseudomonas butanovora* and *Pseudomonas oleovorans* are two well-studied middle-chain (C_6–C_{12}) alkane-degrading strains. *P. butanovora* is a butane-oxidizing bacterium originally isolated from activated sludge of an oil-refining company (Takahashi et al., 1980; Takahashi and Perlman, 1980). In addition to butane, *P butanovora* can grow on C_2–C_9 *n*-alkanes and a number of primary alcohols (Takahashi and Perlman, 1980). This strain expresses soluble butane monooxygeanse (sBMO) to oxidize butane to 1-butanol (Arp, 1999), which is further converted to butyraldehyde, and then to butyrate (Vangnai et al., 2002). Previous studies also showed that sBMO can degrade a wide range of chlorinated alkanes and aromatics via cometabolic reactions (Doughty et al., 2005; Halsey et al., 2005; Hamamura et al., 1997). *P. oleovorans* is a soil isolate known for its ability to express alkane monooxygenses to degrade octane to octanoic acid (Baptist et al., 1963) and to oxidize various C_6–C_{12} linear alkanes or alkenes (Witholt et al., 1990). Both sBMO and alkane monooxygenase have a broad substrate specificity. Given that FTOHs are structurally similar to middle-chain alkanes, it is possible that these two middle-chain alkane-degrading strains can use the enzymes expressed for alkane oxidation to transform FTOHs.

In this study, we examined the biodegradability of FTOHs (4:2, 6:2, and 8:2 FTOHs) by the aforementioned two *Pseudomonas* strains. This is the first report to show that individual pure bacterial strain was able to defluorinate FTOHs to form transformation products with shorter carbon-chain length, resulting in the removal of three $–CF_2–$ groups from FTOHs. Furthermore, this work demonstrated that the two individual microbial strains deployed different or preferred pathways to transform FTOHs.

Materials and Methods

Chemicals

1H, 1H, 2H, 2H-heptadecafluoro-*n*-decanol (8:2 FTOH, CAS # 678-39-7, >96% pure) was purchased from TCI America (manufactured in Tokyo, Japan). 1H, 1H, 2H, 2H-perfluorooctanol (6:2 FTOH, CAS # 647-42-7, 97% pure) was obtained from Alfa Aesar (Lancashire, UK). 1H, 1H, 2H, 2H-perfluorohexan-1-ol (4:2 FTOH, CAS # 2043-47-2) was purchased from Aldrich (Shinagawa-Ku, Japan). Hexane (95% pure) was obtained from Acros Organics (Morris Plains, NJ). 1-Butanol (99.4% pure) was purchased from Fisher Scientific (Fair Lawn, NJ) and *n*-octane (97% pure) was from Acros Organics (Geel, Belgium). Acetonitrile (>99% pure) was purchased from Acros Organics, and ethanol (95% pure) was from IBI scientific (Peosta, IA). The experiments were conducted in two parts: (1) 4:2 FTOH transformation test using GC–MS and GC–ECD, and (2) 6:2 and 8:2 FTOH transformation and metabolites identification using LC/MS/MS. For 4:2 FTOH transformation test, the pure compound (in liquid form) was directly used. For experiments with LC/MS/MS analysis to quantify FTOH and potential metabolites, 8:2 and 6:2 FTOHs were prepared in 50% ethanol (ethanol:water = 1:1) as stock solution to be dosed into individual bacterial culture samples.

For LC/MS/MS analysis, perfluorinated carboxylic acid standards were purchased from either Sigma–Aldrich (St. Louis, MO) or Synquest Laboratories (Alachua, FL), 6:2 FTOH was from Fluka (St. Louis, MO), and 5:2 and 7:2 ketones were from TCI America (Portland, OR). [$M+5$] perfluorohexanoic acid (PFHxA) internal standard was from Wellington Laboratories (Guelph, Ontario, Canada). The rest of polyfluorinated acid standards used for quantitative analysis were synthesized by DuPont (Wilmington, DE). All these standards used have a purity of +97%.

Bacterial Strains and Growth Conditions

The *P. oleovorans* was purchased from America Type Culture Collection (ATCC number # 29347). The *P. oleovorans* was grown in P1 medium (Schwartz, 1973) with 1% *n*-octane to an optical density at 660 nm (OD_{660}) of 1.0 before harvested for experimental use. The cells were centrifuged at 10,000*g* for 5 min, washed twice with 0.1 M phosphate buffer (pH 7) and the pellet was then resuspended in P1 medium (100 mL

for the short-term experiments and 250 mL for the long-term experiments) for experimental use. The *P. butanovora* was generously provided by Dr. Daniel J. Arp, of Oregon State University. The cell suspension was prepared similarly as described above, except that *P. butanovora* was pregrown in ATCC1581 medium (Arp, 1999) with 2 mM 1-butanol to $OD_{600} = 0.5–0.7$.

Short-Term Experiments for FTOH Transformation

Transformation experiments with 4:2 FTOH were conducted in a series of 22 mL-glass vials containing 5 mL of cell suspension of *P. oleovorans* (or *P. butanovora*) dosed with 4:2 FTOH. The vials were first sealed with PTFE-faced silicone rubber septa and caps before adding 4:2 FTOH by using a 25 μL-gas tight syringe. One microliter of 4:2 FTOH (as pure liquid) was added into vials to bring the initial concentration to 318 mg/L. A parallel set of vials was used to examine the effects of additional co-substrate on FTOH transformation. *n*-Octane (1% final concentration) or 1-butanol (2 mM) was supplied as a co-substrate into vials containing *P. oleovorans* or *P. butanovora* and 4:2 FTOH. Cell-free controls and killed (sterile) controls were also used to ensure study integrity. For killed controls, 100 μL of concentrate sulfuric acid was used to inactive the resting cells. All samples and controls were in duplicate. The vials were incubated on a shaker at 150 rpm in a 30°C dark room. The vials were sacrificed over different time points (0, 12, and 24 h for 6:2 and 8:2 FTOHs transformation by *P. oleovorans*; 0, 24, and 48 h for 4:2 FTOH transformation by *P. oleovorans*; 0, 1, and 3 days for 4:2, 6:2 and 8:2 FTOHs transformation by *P. butanovora*). At each sampling time point, 2 mL of hexane was added into the vials to stop transformation and to extract the remaining FTOH. After overnight extraction, a gas tight syringe was used to transfer the hexane layer into new capped GC amber vials (1.5 mL), which were then stored at −20°C for later GC/MS analysis. The aqueous layer in the old sample vials (i.e., 22 mL-glass vials) were preserved at 4°C and later used for fluoride ion measurement. Short-term transformation experiments were also conducted for 6:2 FTOH and 8:2 FTOH. Experiments were conducted similarly as described for 4:2 FTOH transformation tests, except using 6:2 FTOH or 8:2 FTOH. Two microliters (or 3,300 μg) of 6:2 FTOH was added. For 8:2 FTOH transformation tests, 5 μL of 8:2 FTOH stock solution (40 g/L in 95% ethanol; or 200 μg) was used.

Long-term Experiments for Determining FTOH Transformation Products

Long-term experiments for determining FTOH transformation products were performed in a series of 120-mL glass bottles containing cell suspension (10 mL), 6:2 FTOH (or 8:2 FTOH), and one carbon source. The cell suspensions were prepared similarly as described in the biotransformation tests for 4:2 FTOH. 0.05% *n*-octane and 2 mM 1-butanol were added for cell suspensions of *P. oleovorans* and *P. butanovora*, respectively. The 120-mL glass bottles were first sealed with butyl rubber septa and aluminum caps before FTOH addition. Ten microliter of 8:2 FTOH stock solution (2 g/L in 50% ethanol) or 6:2 FTOH stock solution (4.125 g/L in 50% ethanol) was added into the bottles using a 25 μL-gas-tight syringe. Killed controls, containing acid-killed cells (by adding 50 μL of concentrate sulfuric acid) and 6:2 or 8:2 FTOH, were also prepared to assess parent compound recovery during FTOH transformation. Live controls, containing cell suspension and one of the additional carbon sources (1-butanol or *n*-octane) and 50% of ethanol, were also used to monitor oxygen condition. Oxygen concentrations in the headspace of live controls were determined using a headspace Oxygen Analyzer Model 905 (Quantek Instruments, Grafton, MA). The bottles were incubated on a shaker at 150 rpm in a 30°C dark room. At each of following sampling points (0, 0.5, 1, 3, 7, 14, and 28 days), two live samples and two killed control bottles were sacrificed for solvent extraction. Twenty milliliters of acetonitrile was added to each of the bottles to extract remaining FTOH and potential metabolites in the 10-mL cell suspension. The extraction was carried out for 3 days at 50°C with ∼150 rpm shaking. After the extraction, each bottle was resealed with a new septum and preserved at −50°C. The spent septum from each bottle was placed into a new glass bottle and extracted twice with 5 mL acetonitrile for 3 days at 50°C. The two extracts from the spent septum were pooled together with the extract from the liquid sample. The combined extract was then filtered through a 0.45-μm pore size filter disk and the filtrate was collected and analyzed by LC/MS/MS. Fluoride concentrations were not determined due to the small quantities of FTOHs applied initially and the limitation of detection limit of the Orion 96-09BNWP ion-selective electrode.

Fluoride Measurement

The aqueous samples from short-term FTOH transformation tests were first filtered through a 0.22 μm-pore size filter to remove the cell debris before use. The filtrate was used to determine fluoride ions concentrations using an Orion 96-09BNWP ion-selective electrode (Thermo Scientific, Beverly, MA) following the manufacturer's protocol. Fluoride standard solutions, ranging from 2 to 20 mg/L, were prepared by diluting a certified fluoride standard solution (100 mg/L, Thermo Scientific) in the corresponding growth media. The detection limit of fluoride probe is 0.1 mg/L.

GC/MS and GC/ECD Analysis

The concentrations of 4:2 FTOH were determined using an Agilent Technologies 6890N gas chromatograph (GC) coupled with a 5973N mass selective detector (Agilent Technologies, Santa Clara, CA) under full-scan positive

electron impact mode. The GC unit was equipped with a DB-5 column (30 m × 0.25 mm × 0.25 μm film thickness, Agilent Technologies). The oven temperature program was modified from Russell et al. (2008) as follows: 50°C for 2 min, with a first ramp at 20°C min^{-1} to 210°C and a second ramp at 50°C min^{-1} to 280°C, and then held at 280°C for 3 min. One microliter hexane extract from each sample was injected to the GC/MS system. The injector temperature was 150°C and the detector temperature was 280°C. Helium was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. 4:2 FTOH applied for transformation test was used to make standard solutions with serial dilution in hexane and the linear calibration curve was obtained for 4:2 FTOH from 0.298 to 2.385 μg ($R^2 > 0.99$). The hexane extracts obtained during the transformation tests were also used for screening metabolites that might be produced during 4:2 FTOH biotransformation. The screening was performed by using an Agilent 6890 series gas chromatograph equipped with a DB-1 MS capillary column (30 m × 0.25 mm × 0.25μm film thickness) and an electron capture detector (ECD). The oven temperature started at 45°C for 2 min, ramping at 10 min^{-1} to 95°C held for 5 min, and ramping at 30°C min^{-1} to 250°C. The injector temperature was 150°C and the detector temperature was 300°C.

LC/MS/MS Analysis

LC/MS/MS analysis was used to identify and quantify possible metabolites produced during 6:2 and 8:2 FTOH biotransformation. Acetonitrile extracts obtained from the 120-mL bottle samples as previously described (Liu et al., 2010a) with some modifications. The LC/MS/MS system (Micromass Quattro Micro and Waters 2795 HPLC) was operated in negative electrospray ionization mode with multiple reaction monitoring. Each extract was spiked at 50 μL mL^{-1} sample of an internal standard solution containing 200 ng mL^{-1} of [1,2-^{13}C] PFHxA ($M+2$ PFHxA) and 5,000 ng mL^{-1} of [1,1,2,2-D; 3-^{13}C] 6:2 FTOH ($M+5$ 6:2 FTOH) for quantification of 6:2 FTOH and metabolites. In particular, [1,2-^{13}C] PFHxA internal standard was used for quantification of perfluorobutyric acid (PFBA), PFPeA, PFHxA, PFHpA, 6:2 FTCA, 6:2 FTUCA, 5:3 acid, 4:3 acid, and 5:3 Uacid and [1,1,2,2-D; 3-^{13}C] 6:2 FTOH internal standard was used for quantification of 6:2 FTOH, 5:2 ketone, and 5:2 sFTOH. The same concentrations of [1,2-^{13}C] PFOA ($M+2$ PFOA) and [1,1,2,2-D; 3-^{13}C] 8:2 FTOH ($M+5$ 8:2 FTOH) was spiked to each sample extract for quantification of 8:2 FTOH and metabolites. The [1,2-^{13}C] PFOA internal standard was used for quantification of PFHxA, PFHpA, PFOA, PFNA, 8:2 FTCA, 8:2 FTUCA, 7:3 acid, and 7:3 Uacid and [1,1,2,2-D; 3-^{13}C] 8:2 FTOH internal standard was used for quantification of 8:2 FTOH, 7:2 ketone, and 7:2 sFTOH. Unlike previous method (Liu et al., 2010a), the extracts were not acidified before LC/MS/MS analysis. The detection limit of FTOH and transformation products and other detailed information on instrumental parameters was included in Tables S2 and S3.

Results and Discussion

Biodefluorination of 4:2 FTOH by *P. oleovorans*

Biotrasnformation of 4:2 FTOH was observed by *P. oleovarans* within 48 h in the absence and presence of *n*-octane (Fig. 1 and Table S1) along with an increase of fluoride concentration in the samples. Currently, no information is available regarding 4:2 FTOH biotransformation pathways and potential transformation products. The extent of 4:2 FTOH biodefluorination and 4:2 FTOH transformation products were not determined due to the lack of metabolite standards. However, based on the amounts of fluoride released, 4:2 FTOH was biodefluorinated more extensively in the absence of *n*-octane (Table S1). The addition of *n*-octane inhibited FTOH defluorination by more than twofold (Table S1). This might be due to that *P. oleovorans* preferred *n*-octane as its carbon source over 4:2 FTOH. Table S1 in the supporting information explains how defluorination was calculated and compares defluorination of 4:2 FTOH by these two strains in the absence and presence of additional carbon sources. Even though *P. oleovorans* can defluorinate 4:2 FTOH, the bacterium cannot grow on 4:2 FTOH as the sole carbon source.

Biodefluorination of 4:2 FTOHs by *P. butanovora*

Similarly, *P. butanovora* showed an ability to transform 4:2 FTOH in the absence and presence of 1-butanol (Fig. 1 and Table S1) to produce fluoride. However, the effects of 1-butanol on 4:2 FTOH defluorination by *P. butanovora* were less pronounced than that of *n*-octane described above.

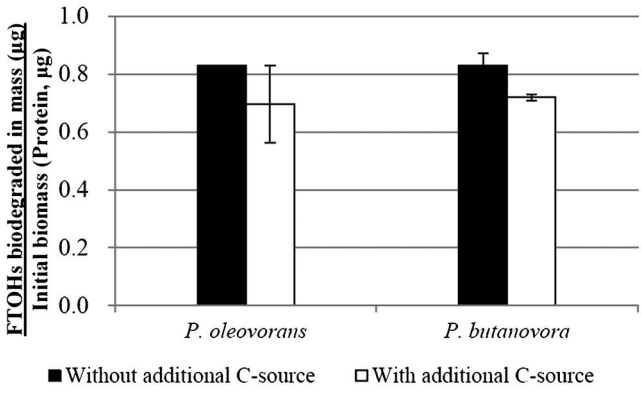

Figure 1. *P. oleovorans* and *P. butanovora* were able to transform 4:2 FTOH in the absence and presence of additional carbon sources (*n*-octane for *P. oleovorans* and 1-butanol for *P. butanovora*). Initial mass of 4:2 FTOH applied is 1,590 μg (6.0 μmol) in 5 mL bacterial cell suspension. The bars represent ranges of duplicate samples.

For example, on day 3, 149.5 μg (7.9 μmol) fluoride was released in the absence of 1-butanol versus 111.5 μg (5.9 μmol) fluoride with the addition of 1-butanol. The amounts of fluoride released corresponded to defluorination potential of 37.3% in the absence of 1-butanol and 32% in the presence of 1-butanol (Table S1).

Potential Metabolites During 4:2 FTOH Biotransformation

GC/ECD analysis was used to screen metabolites formed during 4:2 FTOH biotransformation by these two strains. As shown in Figure S1 in supporting information (SI), in the absence of *n*-octane, two new peaks were detected during 4:2 FTOH biotransformation by *P. oleovorans*. When 4:2 FTOH was biotransformed by *P. butanovora*, five new peaks were observed in the samples without 1-butanol and only three new peaks were detected in the samples with 1-butanol (Fig. S2 in SI). These chromatographs suggest that 4:2 FTOH was transformed by these two strains through different pathways and that the degradative enzymes expressed by these two strains might have different affinities toward 4:2 FTOH and its metabolites. Unfortunately, due to lack of authentic metabolite standards for LC/MS/MS analysis, the identities of these fluorinated and other potential non-fluorinated metabolites were not determined in this study.

Transformation Products for 6:2 and 8:2 FTOHs

Similar to 4:2 FTOH, GC/ECD analysis revealed new peaks (peaks 1 and 2) during 6:2 and 8:2 FTOHs biotransformation by *P. oleovorans* (Figs. S3 and S4 in SI) during the short-term transformation tests. To identify these metabolites, long-term experiments were conducted and samples were analyzed by LC/MS/MS. More 6:2 and 8:2 FTOHs transformation products were identified and quantified by LC/MS/MS analysis (Fig. 2 for *P. oleovorans* and Fig. 3 for *P. butanovora*). Over the course of FTOH biotransformation (28 days), almost a half (~10%) of the initial oxygen in the air was remaining (Figs. 2b and 3b) in live control bottles and 103–109% (6:2 FTOH) and 105–106% (8:2 FTOH) of initially applied FTOHs were still remaining in sterile controls (Figs. 2b and 3b), demonstrating the integrity of the experimental systems. In the live samples (except the study with *P. butanovora* dosed with 6:2 FTOH), the sum of FTOH and transformation products from LC/MS/MS analysis did not add up to 100% of initial FTOH applied. This "missing" of approximately 40–54% initially applied FTOH may be due to two factors. First, bound-residue (up to 25–30% of initial applied FTOH) formed between fluorinated chemicals and biological organic components catalyzed by bacterial enzymes made this fraction non-quantifiable by LC/MS/MS (Liu et al., 2010b; Wang et al., 2009). Second, other potential metabolites formed during FTOH biotransformation were not quantified due to either lack of authentic analytical standards or unknown identities. Nonetheless,

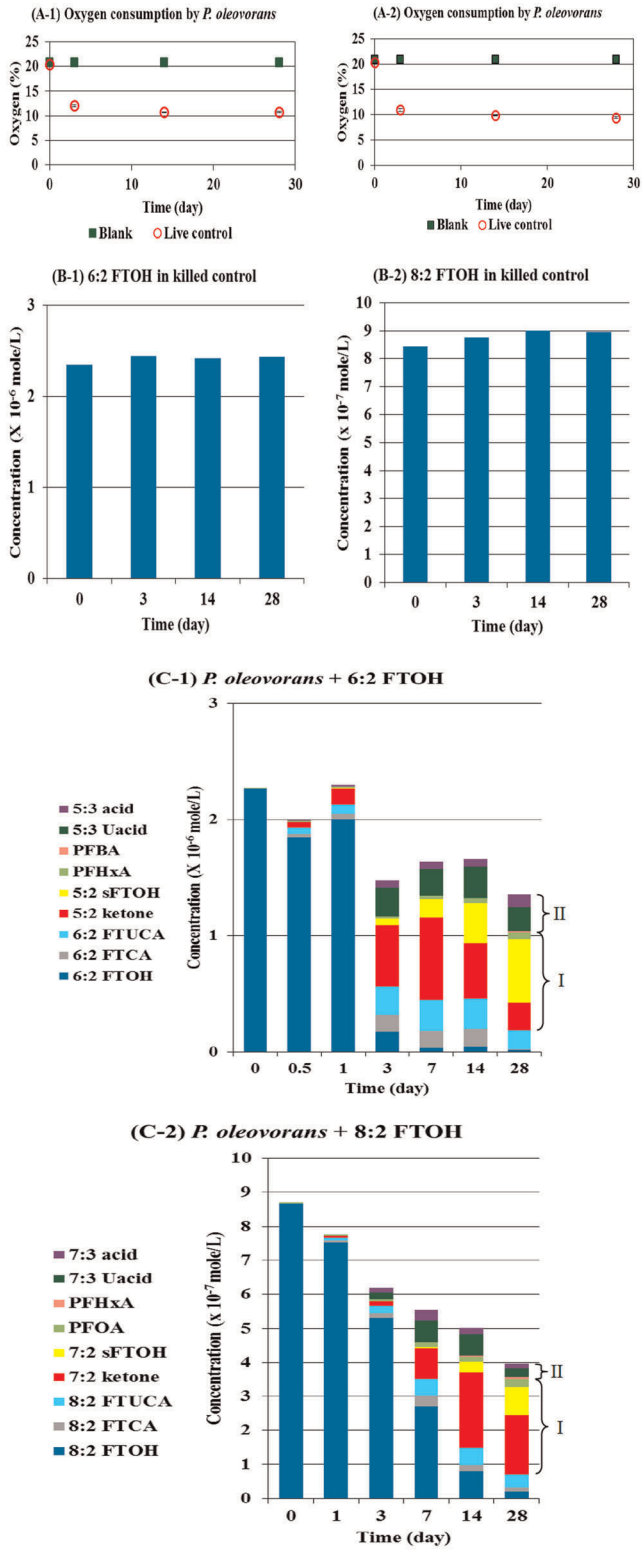

Figure 2. Time trend of metabolite (transformation product) formation during 6:2 and 8:2 FTOH biotransformation by *P. oleovorans*. **a**: Changes of oxygen concentrations during FTOH biotransformation; (**b**) changes of 6:2 and 8:2 FTOH concentrations during 28 days in killed (sterile) controls; (**c**) changes of different metabolite concentrations dosed with 6:2 FTOH or 8:2 FTOH. The transformation products in pathway I and II are grouped together in (c).

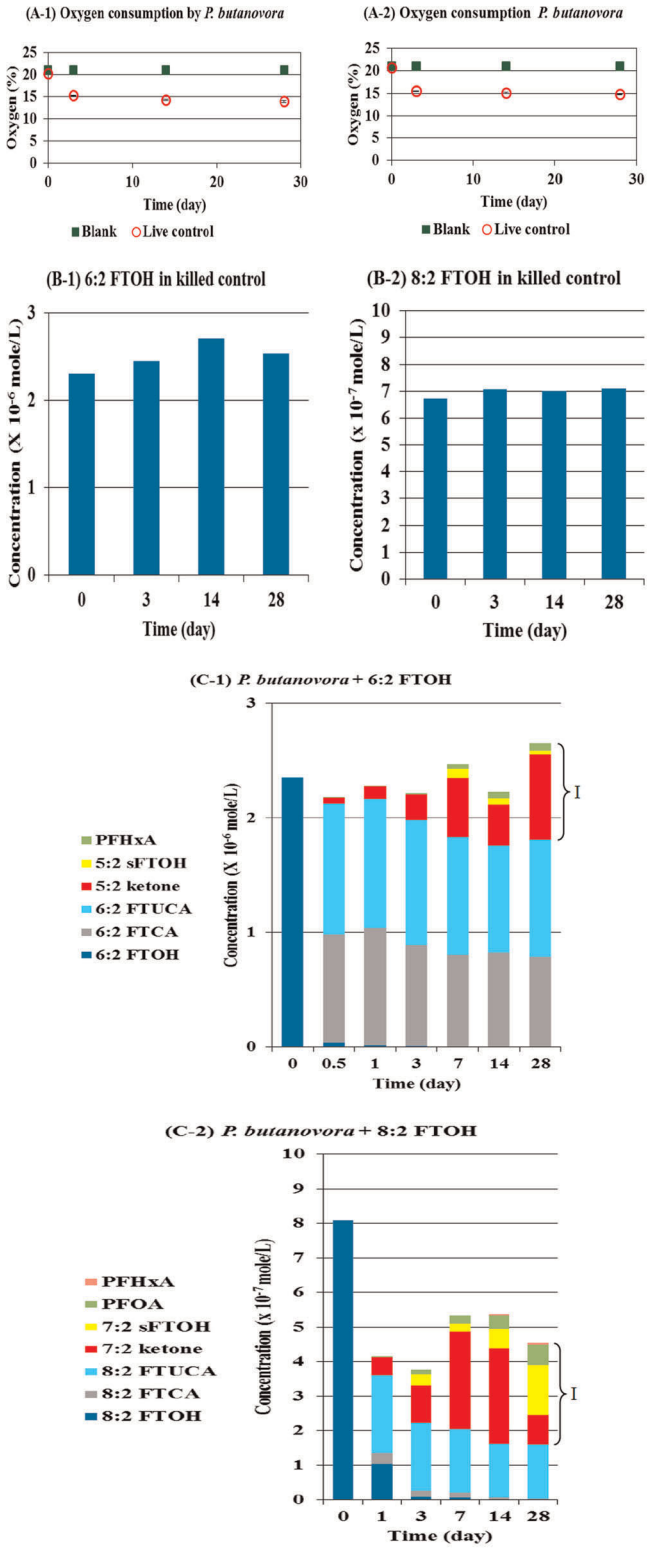

Figure 3. Time trend of metabolite formation during 6:2 and 8:2 FTOH biotransformation by *P. butanovora*. **a**: Changes of oxygen concentrations during FTOH biotransformation; (**b**) changes of 6:2 and 8:2 FTOH concentrations during 28 days in killed controls; (**c**) changes of different metabolite concentrations dosed with 6:2 FTOH or 8:2 FTOH. The transformation products in pathway I and II are grouped together in (c).

the total mass balance of this study is comparable to that of previous studies on FTOHs with microbial consortia (Dinglasan et al., 2004; Liu et al., 2007, 2010a).

Eight different metabolites were detected and quantified during 6:2 and 8:2 FTOHs biotransformation by *P. oleovorans*, respectively (Fig. 2c-1 and c-2). According to the quantity of each metabolite detected over time (Fig. 2c), a general trend of FTOH biotransformation was observed as follows. From day 0 to day 3, *P. oleovorans* quickly transformed 6:2 and 8:2 FTOHs to *n*:2 FTCA [*n*:2 fluorotelomer saturated carboxylic acid, $F(CF_2)_nCH_2COOH$, $n = 6$ or 8), to *n*:2 FTUCA (*n*:2 fluorotelomer unsaturated carboxylic acid, $F(CF_2)_{n-1}CF = CHCOOH$), to *x*:2 ketone [$F(CF_2)_xC(O)CH_3$; $x = n - 1$, where $n = 6$ or 8], and then to *x*:2 sFTOH [*x*:2 secondary alcohol, $F(CF_2)_xCH(OH)CH_3$]. As the 6:2 and 8:2 FTOH concentrations decreased, these four metabolites continued to increase to reach their maximum and remained little or no change until day 14, and then decreased slightly on day 28. Also, on day 3, PFCAs such as PFBA in 6:2 FTOH samples and PFHxA in 8:2 FTOH samples started showing up in trace amounts and increased slightly on day 28, accounting for 0.44 and 0.62 mol% of initially applied 6:2 and 8:2 FTOHs, respectively. The formation of PFBA from 6:2 FTOH or PFHxA from 8:2 FTOH indicates that three $-CF_2-$ groups were removed from 6:2 FTOH or 8:2 FTOH during biotransformation. On the other hand, PFHxA or PFOA is the major stable transformation product from 6:2 FTOH or 8:2 FTOH biotransformation, accounting for 2.8 or 2.6 mol% of initially applied 6:2 or 8:2 FTOH.

The *x*:3 Uacid [*x*:3 unsaturated acid, $F(CF_2)_xCH = CHCOOH$; $x = n - 1$, where $n = 6$ or 8] and *x*:3 acid [$F(CF_2)_xCH_2CH_2COOH$], started to emerge on day 3. The concentrations of *x*:3 Uacid continued to increase slightly to day 14 and then decreased at the end of experiment on day 28, with 5:3 Uacid accounting for 9.1 mol% and 7:3 Uacid for 2.9 mol% of initially applied 6:2 and 8:2 FTOHs, respectively. However, the *x*:3 acid continued to increase on day 28, with 5:3 acid accounting for 4.7 mol% and 7:3 acid accounting for 1.8 mol%, respectively.

Only five and six different metabolites were detected during 6:2 and 8:2 FTOHs biotransformation by *P. butanovora*, respectively (Fig. 3c-1,c-2). Similarly, within one day, *P. butanovora* quickly transformed 6:2 and 8:2 FTOHs to *n*:2 FTCA, *n*:2 FTUCA, and *x*:2 ketone (Fig. 3c). In the case for samples with 6:2 FTOH, the concentrations of 5:2 ketone increased throughout the experiment, whereas in the case for samples with 8:2 FTOH, the concentrations of 7:2 ketone increased five times at day 7 compared with day 1 and then decreased on day 28. On day 3, new metabolites *x*:2 sFTOHs started to emerge and continue to increase on day 28. Also, PFHxA (during 6:2 FTOH transformation) and PFOA (during 8:2 transformation) showed up around day 1 and started to increase slightly from day 7. Interestingly, a trace amount of PFHxA was detected after day 7 during 8:2 FTOH transformation and accounted for 0.45 mol% of initially applied 8:2 FTOH on day 28. Contrary to

P. oleovorans, no PFBA was detected during 6:2 FTOH biotransformation by *P. butanovora*. At the end of experiments on day 28, the observed major intermediates include *n*:2 FTUCA, *x*:2 ketone, and *x*:2 sFTOH whereas PFHxA or PFOA is the major stable transformation product from 6:2 FTOH or 8:2 FTOH biotransformation, accounting for 2.9 or 7.9 mol% of initially applied 6:2 or 8:2 FTOH. In contrast to *P. oleovorans*, no *x*:3 Uacid or *x*:3 acid was detected in *P. butanovora* samples dosed with 6:2 or 8:2 FTOH, suggesting that *P. butanovora* under this study conditions may be lacking enzymes capable of defluorinating *n*:2 FTUCA to *x*:3 Uacid and then of reducing the later to *x*:3 acid.

Biotransformation Pathways for 6:2 and 8:2 FTOHs

Based on the metabolites detected in this study, two possible pathways, I and II, for 6:2 and 8:2 FTOHs transformation were proposed (Fig. 4), with pathway I leading to major PFCAs and pathway II leading to *x*:3 acids and small amounts of shorter-chain PFCAs. As shown in Figure 4, these two pathways shared the same first three transformation steps before diverging into two different paths. In the first step of the transformation, 6:2 and 8:2 FTOHs were transformed into *n*:2 FTAL (not analyzed; an assumed intermediate, Wang et al., 2005a), which was quickly transformed to *n*:2 FTCA (the second step). Then, *n*:2 FTCA was further transformed to *n*:2 FTUCA (the third step), where the first defluorination reaction occurred. This defluorination reaction involved hydrogen fluoride (HF) elimination (Liu et al., 2010a; Wang et al., 2009).

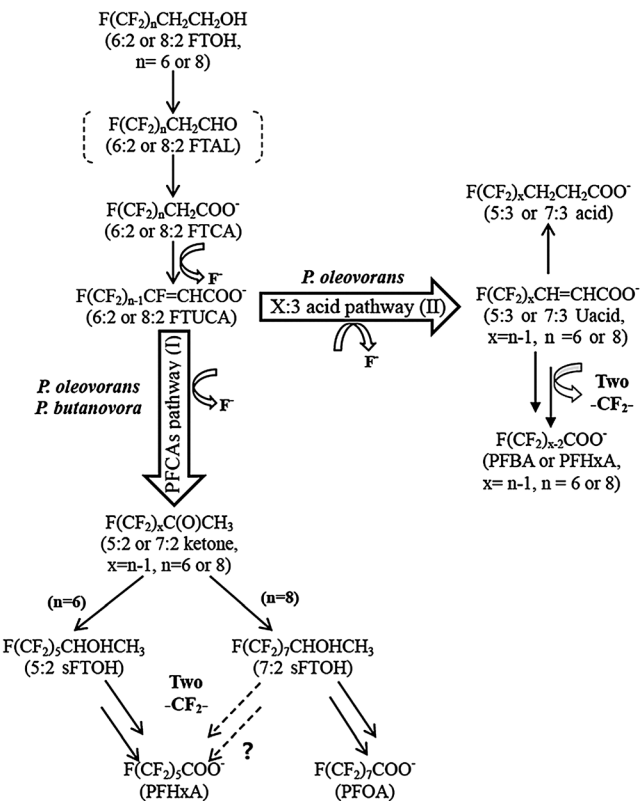

Figure 4. 6:2 FTOH ($n=6$) and 8:2 FTOH ($n=8$) biotransformation pathways by *P. oleovorans* and *P. butanovora*. The pathway I leads to major perfluorinated carboxylic acids (PFCAs) whereas pathway II leads to 5:3 acid or 7:3 acid small amounts of shorter-chain PFCAs. *P. oleovorans* utilized bothboth pathway I and II while *P. butanovora* only utilized pathway I. The compound in the dashed bracket is an assumed intermediate that was not quantified. The double arrows indicate multiple biotransformation steps. Dash arrows indicate pathways that may or may not exist.

The second defluorination reaction occurred when *n*:2 FTUCA was transformed to *x*:2 ketone on pathway I, or to *x*:3 Uacid on pathway II. By following the PFCA pathway I, *x*:2 ketone was further converted to *x*:2 sFTOH, and then to PFCAs via many unknown enzymatic steps. As *n*:2 FTUCA looks similar to long-chain fatty acid, β-oxidation has been previously speculated for the transformation. However, due to the deficiency of protons in the β-carbon position, *n*:2 FTUCA cannot be the direct substrates for the β-oxidation (Wang et al., 2005b). This view is supported by recent finding that PFBA was formed from 5:3 Uacid in activated sludge through one-carbon removal pathways (Wang et al., 2012) and not formed from 5:2 sFTOH (Liu et al., 2010b). Yet, it is still unclear if PFHxA observed in this study with 8:2 FTOH-dosed *P. butanovora* strain was transformed directly from 7:2 sFTOH or from other unknown 8:2 FTOH metabolites. Previous work in mixed bacterial culture and soils (Wang et al., 2005a, 2009) hypothesized that PFHxA was from 8:2 FTUCA or from 7:3 Uacid biotransformation. This work indicates that PFHxA (~0.45 mol% of initially applied 8:2 FTOH) detected in *P. butanovora* could come from 8:2 FTUCA or other unknown metabolites rather than 7:3 Uacid, which was not detected along with PFHxA. The formation of *x*:3 acids via pathway II is rather simple and involved only two enzymatic steps. The *n*:2 FTUCA was first defluorinated to *x*:3 Uacid via a dehalogenase. The *x*:3 Uacid was then converted to *x*:3 acid catalyzed by a dehydrogenase or saturase. The results from this study clearly demonstrated that *P. oleovorans* transformed 6:2 and 8:2 FTOHs using both PFCA pathway I and *x*:3 acid pathway II. On the other hand, *P. butanovora* utilized only the PFCA pathway I for FTOH transformation, which was supported by the lack *x*:3 Uacid and *x*:3 acid in samples dosed with 6:2 or 8:2 FTOH.

The production of PFHxA, resulting from removal of three $-CF_2-$ groups from 8:2 FTOH by *P. oleovorans*, is consistent with previous findings that 8:2 FTOH can be transformed into perfluorohexanoic acid and other perfluorohexyl-containing substances by different microbial consortia (Wang et al., 2005b, 2009). As described earlier, other shorter-chain perfluorinated products might also be formed but not identified in this study due to the lack of authentic standards and unknown identities. This work suggests that a complete enzyme system does exist in a pure microbial strain as in a consortium to remove multiple $-CF_2-$ groups from FTOHs. Future work includes understanding the detailed enzymatic pathways leading to the removal of multiple $-CF_2-$ groups and identifying/isolating individual microbial strains

from environmental matrices (e.g., soil and activated sludge) with enhanced ability to mineralize FTOHs.

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Table S1. The amounts of 4:2 FTOH removed and fluoride released in cell suspension of *P. oleovorans* and *P. butanovora* dosed with 4:2 FTOH. The 4:2 FTOH starting concentration is 318 mg L^{-1} cell culture (1590 µg or 6.0 µmol 4:2 FTOH was added to the 5 mL cell suspension). The samples were extracted for 4:2 FTOH and fluoride analysis 48 h for *P. oleovorans* and 96 h for *P. butanovora* after the initiation of 4:2 FTOH biotransformation experiments. The values were average from duplicate samples.

Bacterial Strains	**4:2 FTOH applied** (µg/5 mL cell suspension)		**4:2 FTOH removed**[a] (µg/5 mL cell suspension)		**Fluoride released** (µg/5 mL cell suspension)		**Defluorination efficiency** (% maximum*)	
P. oleovorans	+ *n*-octane	No *n*-octane	+ *n*-octane	No *n*-octane	+ *n*-octane	No n-octane	+ *n*-octane	No *n*-octane
	1590	1590	991	1180	38	78.5	5.9[§]	10.3
P. butanovora	+ 1-butanol	No 1-butanol	+ 1-butanol	No 1-butanol	+ 1-butanol	No 1-butanol	+ 1-butanol	No 1-butanol
	1590	1590	535	616	111.5	149.5	32	37.3

*: When 1 µmole of 4:2 FTOH (264 µg) is completely mineralized, 171 µg fluoride (9 µmole fluoride × 19 µg molecular weight) would be released. Therefore, the complete mineralization of 4:2 FTOH (a) would result in (0.65 × a) µg fluoride release (production).
§: {38 µg/(0.65×991 µg)} × 100 % = 5.9 %

Table S2. Instrumental method for analysis of 6:2 FTOH and transformation products by LC/MS/MS.

Instrument:	Waters Model 2795 High Performance Liquid Chromatograph with a Waters Quattro Micro Mass Spectrometer equipped with an electrospray source. The mass spectrometer was operated in the negative ion multiple reaction-monitoring mode.
Analytical Column:	Agilent Zorbax RX-C8 (150 mm x 2.1 mm, 5 µm particle size, pore size 80 Å, not end-capped, carbon loading 5.5%)
Column Temperature:	30°C
Mobile Phases:	A: 0.15% acetic acid in nanopure water B: 0.15% acetic acid in acetonitrile

Gradient Profile:

Time (min)	Percentage A	Flow Rate (mL/min)
0.0	90	0.400
1.0	90	0.400
1.1	45	0.400
2.0	45	0.400
7.5	20	0.400
7.6	90	0.400
8.0	90	0.400

Injection Volume: 10 - 20 µL

Monitored Ion Transitions:

Analytes (LOD*)	Ion Transitions	Cone Voltage, V	Collision Energy
PFBA (0.50)	213 > 169	14	8
PFPeA (0.50)	263 > 219	14	8
PFHxA (0.50)	313 > 269	14	8
PFHpA (0.20)	363 > 319	16	10
[M+2] PFHxA	315 > 270	14	8
5:2 ketone (10)	389 > 311	8	10
6:2 FTA (2.1)	377 > 293	16	16
6-2 FTUA (1.0)	357 > 293	16	14
4:3 acid (3.0)	291 > 187	18	13
5:3 u acid (3.0)	339 > 255	16	14
5:3 acid (1.9)	341 > 237	18	13
5:2 sFTOH (11)	373 > 59	12	8
6-2 FTOH (7.0)	423 > 59	12	8
[M+5] 6-2 FTOH	428 > 59	12	8

* LOD: Limit of detection defined as lowest calibration standard in µg L^{-1}.

LC/MS/MS Analog Parameters:

Capillary (kV) = 3.50	Q 1: unit resolution
Extractor (V) = 0	Ion Energy 1 = 0.6
RF Lens (V) = 0	Entrance = -1
Source Temperature (°C) = 120	Exit = 0
Desolvation Temperature (°C) = 250	Q 2: unit resolution
Cone Gas Flow (L/Hr) = 50	Ion Energy 2 = 0.6
Desolvation Gas Flow (L/Hr) = 500	Multiplier (V) = 700

Table S3. Instrumental method for analysis of 8:2 FTOH and metabolites (transformation products) by LC/MS/MS.

Instrument:	Waters Model 2795 High Performance Liquid Chromatograph with a Waters Quattro Micro Mass Spectrometer equipped with an electrospray source. The mass spectrometer was operated in the negative ion multiple reaction-monitoring mode.
Analytical Column:	Agilent Zorbax RX-C8 (150 mm x 2.1 mm, 5 µm particle size, pore size 80 Å, not end-capped, carbon loading 5.5%)
Column Temperature:	30°C
Mobile Phases:	A: 0.15% acetic acid in nanopure water B: 0.15% acetic acid in acetonitrile
Gradient Profile:	Time (min) / Percentage A / Flow Rate (mL/min) 0.0 / 90 / 0.400 1.0 / 90 / 0.400 1.1 / 45 / 0.400 2.0 / 45 / 0.400 7.5 / 20 / 0.400 8.0 / 10 / 0.400 8.5 / 10 / 0.400 9.0 / 90 / 0.400 10.0 / 90 / 0.400
Injection Volume:	10 - 20 µL
Monitored Ion Transitions:	Analytes (LOD*) / Ion Transitions / Cone Voltage, V / Collision Energy PFHxA (0.50) / 313 > 269 / 14 / 8 PFHpA (0.20) / 363 > 319 / 16 / 10 PFOA (0.5) / 413 > 369 / 16 / 10 [M+2] PFOA / 415 > 370 / 20 / 10 PFNA (0.5) / 463 > 419 / 15 / 10 7:2 ketone (10) / 489 > 411 / 8 / 10 8:2 FTA (2.1) / 477 > 393 / 16 / 16 8-2 FTUA (1.0) / 457 > 393 / 16 / 14 7:3 u acid (3.0) / 439 > 369 / 16 / 14 7:3 acid (3.0) / 441 > 337 / 16 / 16 7:2 sFTOH (10) / 473 > 59 / 12 / 20 8-2 FTOH (10) / 523 > 59 / 12 / 20 [M+5] 8-2 FTOH / 528 > 59 / 12 / 20 * LOD: Limit of detection defined as lowest calibration standard in µg L^{-1}.
LC/MS/MS Analog Parameters:	Capillary (kV) = 3.50 Extractor (V) = 0 RF Lens (V) = 0 Source Temperature (°C) = 120 Desolvation Temperature (°C) = 250 Cone Gas Flow (L/Hr) = 50 Desolvation Gas Flow (L/Hr) = 500 Q 1: unit resolution Ion Energy 1 = 0.6 Entrance = -1 Exit = 0 Q 2: unit resolution Ion Energy 2 = 0.6 Multiplier (V) = 700

Table S4. FTOHs remaining and transformation products formed per initially applied FTOHs on 28 day (%)

	P. oleovorans	*P. butanovora*		*P. oleovorans*	*P. butanovora*
6:2 FTOH remaining [a]	0.85	ND	**8:2 FTOH remaining** [a]	2.51	ND
Transformation products formed [b]			**Transformation products formed** [b]		
6:2 FTCA	0.23	33.4	8:2 FTCA	1.21	0.28
6:2 FTUCA	7.26	43.5	8:2 FTUCA	4.3	19.58
5:2 ketone	10.3	31.8	7:2 ketone	20.2	10.4
5:2 sFTOH	24.1	1.21	7:2 sFTOH	9.63	17.9
PFHxA	2.72	2.87	PFOA	2.68	7.3
5:3 Uacid	9.06	ND	PFHxA	0.62	0.61
5:3 acid	4.71	ND	7:3 Uacid	2.9	
PFBA	0.44	ND	7:3 acid	1.78	

a) [(6:2 FTOH remaining on day 28) / (6:2 FTOH initially applied on day 0)] * 100

b) [(transformation products formed on day 28) / (6:2 FTOH initially applied on day 0)] * 100

- ND: not detected

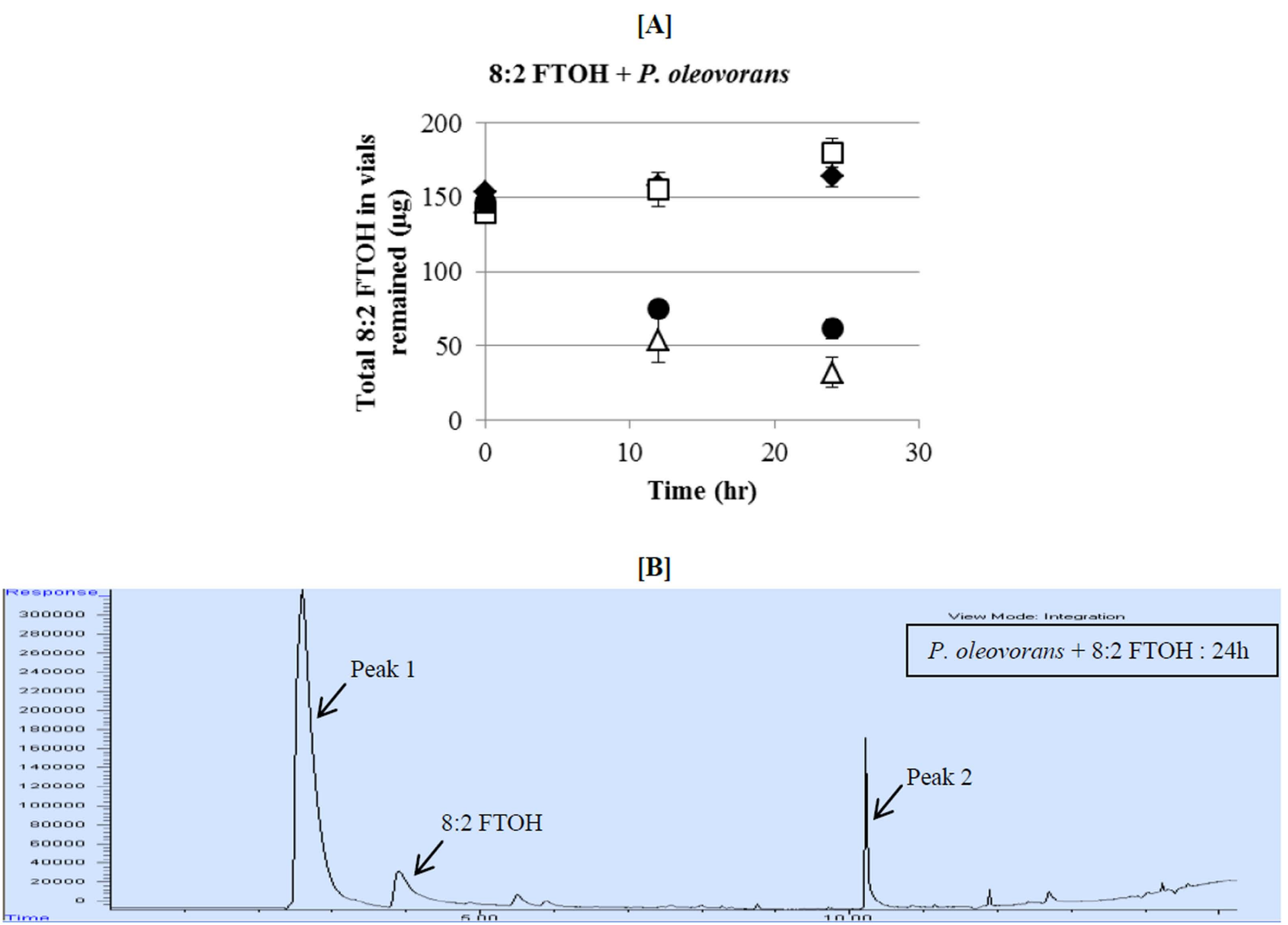

Fig. S4. 8:2 FTOH transformation by *P. oleovorans* (A), and detection of unidentified metabolites using GC-ECD (B): diamond-control, square-killed control, triangle-FTOH only, circle- FTOH with additional carbon source (n-octane). As the parent compound disappears, two new peaks (peak 1 and 2) were showed up and increased over time.

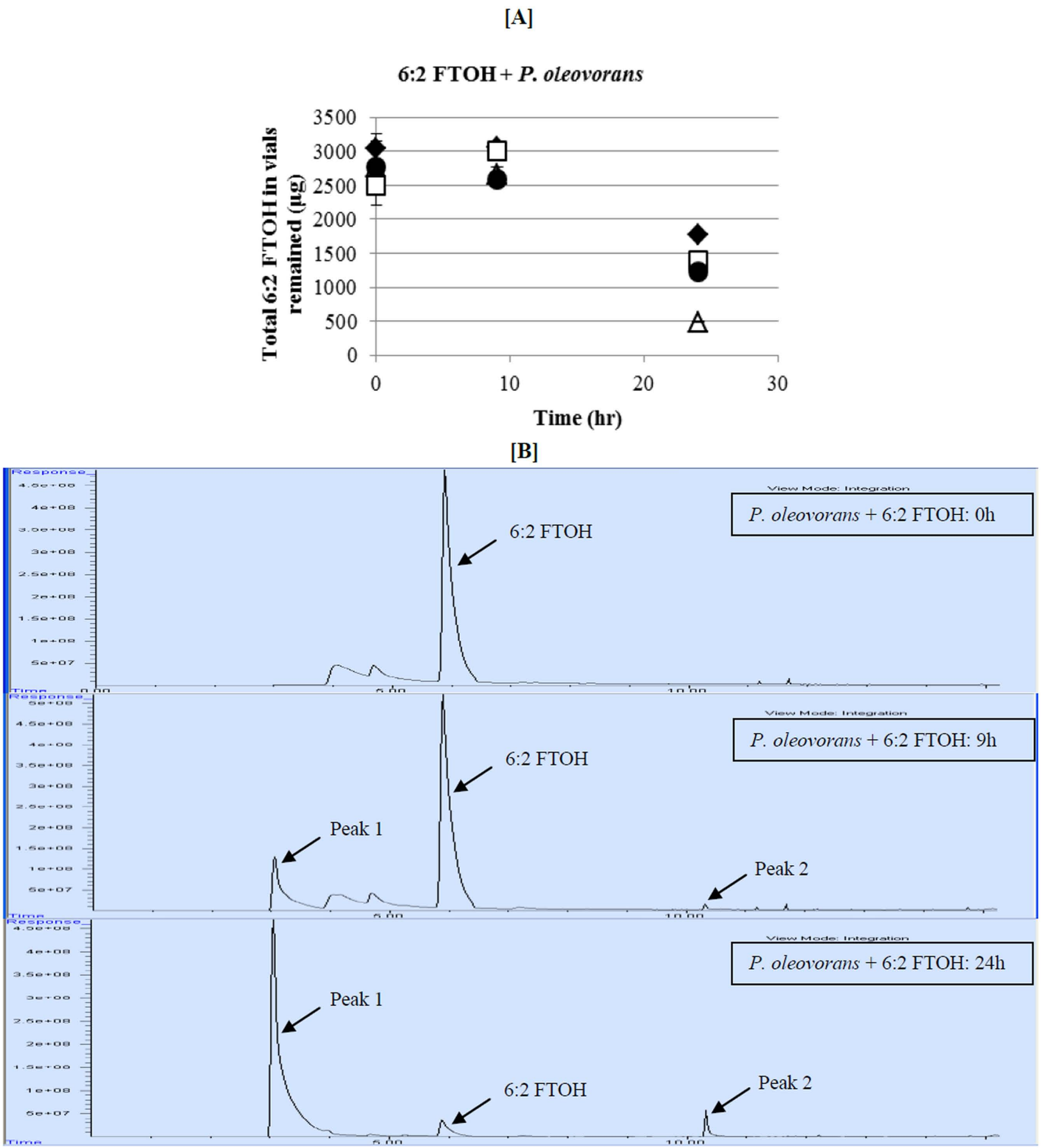

Fig. S3. 6:2 FTOH transformation by *P. oleovorans* (A), and detection of unidentified metabolites using GC-ECD (B): diamond-control, square-killed control, triangle-FTOH only, circle- FTOH with additional carbon source (n-octane). As the parent compound disappears, two new peaks (peak 1 and 2) were showed up and increased over time.

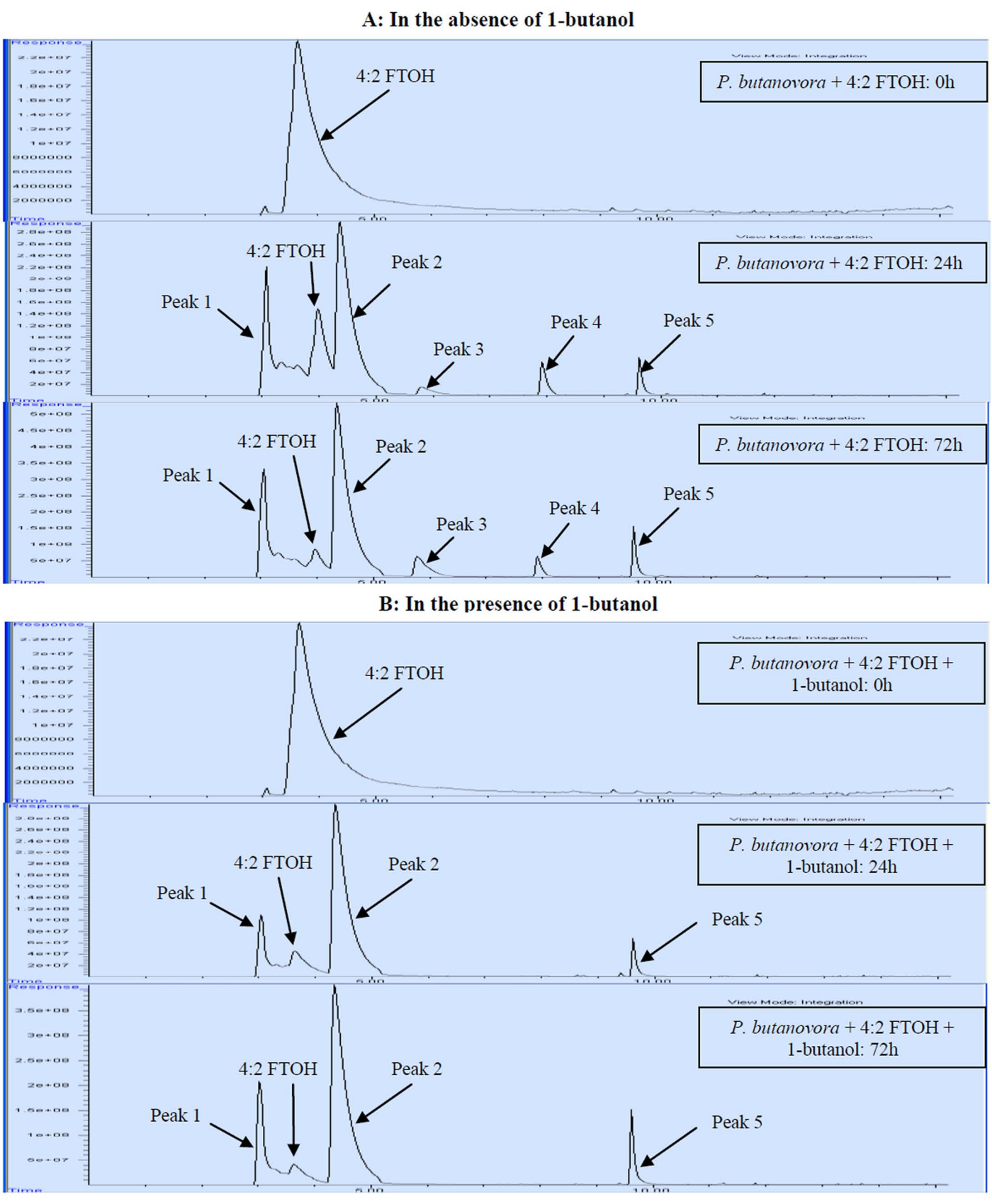

Fig. S2. GC/ECD chromatography for 4:2 FTOH transformation by *P. butanovora* in the absence of 1-butanol (A) and in the presence of 1-butanol (B).

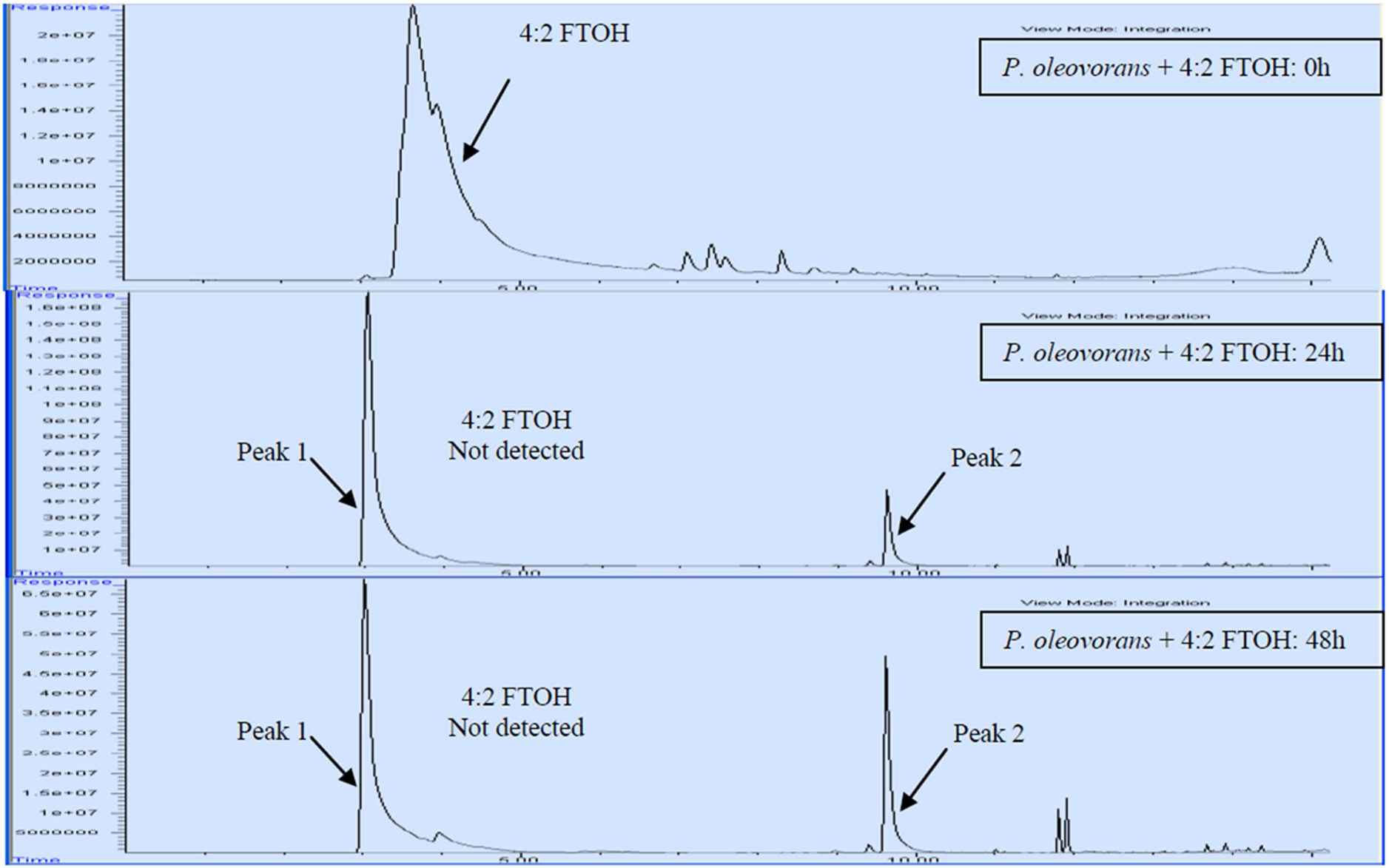

Fig. S1. GC/ECD chromatography for 4:2 FTOH transformation by *P.oleovorans* in the absence of n-octane.