FACTORS AFFECTING BIODEFLUORINATION OF FLUOROTELOMER ALCOHOLS (FTOHS): DEGRADATIVE MICROORGANISMS, TRANSFORMATION METABOLITES AND PATHWAYS, AND EFFECTS OF CO-SUBSTRATES

A Dissertation

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ABSTRACT

Fluorotelomer alcohols (FTOHs, $F(CF_2)_nCH_2CH_2OH$) are emerging contaminants in the environment. Biodegradation of 6:2 and 8:2 FTOHs has been intensively studied using soils and activated sludge. However, little is known about the bacteria responsible for biotransformation of FTOHs. This study deciphered factors affecting biodefluorination of FTOHs and their metabolites, and developed three effective FTOH-degrading consortia.

Two alkane-degrading *Pseudomonas* strains (*P. oleovorans* and *P. butanovora*) can 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. Pathway I led to formation of x:2 ketone (x = n-1), x:2 sFTOH and perfluorinated carboxylic acids (PFCAs). Pathway II resulted in the formation of x:3 polyfluorinated acid and relatively minor shorter-chain PFCAs. Conversely, *P. butanovora* transformed FTOHs by pathway I only.

Mycobacterium vaccae JOB5 (a C_1-C_{22}alkane-degrading bacterium) and *P. fluorescens* DSM 8341 (a fluoroacetate-degrading bacterium) can transform 6:2 FTOH via both pathways I and II with the formation of odd-numbered short-chain PFCAs. In the presence of dicyclopropylketone or formate, *P. oleovorans* transformed 6:2 FTOH six times faster and produced odd-numbered PFCAs. *P. butanovora*, utilized both

pathways I and II in the presence of lactate, and it also produced odd-numbered PFCAs. Unlike *P. oleovorans*, *P. fluorescens* DSM 8341 could slightly convert 5:3 polyfluorinated acid (a key metabolite during 6:2 FTOH degradation, $[F(CF_2)_5CH_2CH_2COOH]$) to 4:3 acid and PFPeA via one-carbon removal pathways.

Three FTOH-degrading consortia transformed FTOHs, with enhanced removal of FTOHs in the presence of n-octane. A higher copy number of *alkB* gene was found to correspond to better removal of FTOHs, suggesting that alkane-degrading bacteria might be the key degraders in the enrichments. The three enrichment cultures showed a similar microbial community structure.

This is the first study reporting that pure strains of alkane- and fluoroacetate-degrading bacteria can bio-transform FTOHs via different or preferred transformation pathways to remove multiple $–CF_2–$ groups from FTOHs to form shorter-chain PFCAs, and to other perfluorinated acids. The results of this study also suggest that enhanced FTOH biodegradation is possible through co-substrate addition and/or using enrichment cultures.

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1. INTRODUCTION AND OBJECTIVES

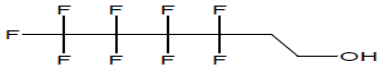
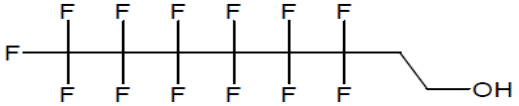
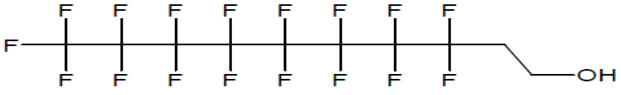
1.1 Introduction

Fluorotelomer alcohols [$F(CF_2)_nCH_2CH_2OH$] (Table 1.1) are a group of fluorinated compounds with even-numbered fluorocarbon chains and an ethanol moiety. They are used as raw materials to manufacture surfactant and polymeric products [1]. FTOH-based compounds have been used in a wide variety of products including paints, adhesives, waxes, polishes, metals, electronics, caulks, agrochemicals, blood substitutes, cookware coatings, refrigerants, fire-fighting foams, and lubricants [1]. From 2000 to 2002, the production of FTOHs was estimated to be 5,000 tons/year in the world [2]. FTOHs have been detected in the troposphere at concentrations ranging from 7 to 196 pg/m^3 [3]or 11 to 165 pg/ m^3 [4], with 6:2 and 8:2 FTOHs in majority.

Abiotic and biotic transformation of FTOHs has been shown as one possible source for perfluorinated carboxylic acids (PFCAs, $[F(CF_2)_mCOOH]$, $2 < m < 8$) [2, 5, 6], which are persistent compounds that have been detected in the environment, wildlife, and humans [7-14]. The structures of perfluorinated compounds are shown in Table A1.1 in Appendix. In 2003, the United States Environmental Protection Agency (U.S. EPA) requested comments on perfluorooctanoic acid (PFOA,$F(CF_2)_7COOH$), a PFCA of concern, due to its potential risks to human health and ecosystem [15]. In 2006, the

U.S. EPA invited eight major manufacturers of fluorinated compounds to participate on a 2010/2015 PFOA stewardship program to eliminate production and emission of these compounds by 2015 [16]. In response to the global PFOA stewardship program, the manufacturers have recently adopted 6:2 FTOH [$F(CF_2)_6CH_2CH_2OH$] as a raw material for the products of FTOH-based polymers [17, 18]. Therefore, new research is particularly needed to better understand the fate and transport of 6:2 FTOH in the environment.

Table 1.1. Chemical structures of FTOHs

Fluorotelomer alcohols (FTOHs)	
4:2 FTOH	
6:2 FTOH	
8:2 FTOH	

The carbon-fluorine bond is one of the strongest bonds in the nature, with up to 130 kcal/mol of bond dissociation energy (BDE) [19]. For example, monofluorinated alkane (CH_3-F) has 115 kcal/mol of BDE which is higher than other carbon-halogen or carbon-hydrogen bonds (104.9 kcal/mol for CH_3-H, 83.7 kcal/mol for CH_3-Cl, 72.1 kcal/mol for CH_3-Br, and, 57.6 kcal/mol for CH_3-I) [20]. Due to the C-F bonds, poly-

and per- fluorinated chemicals such as FTOHs and PFCAs are stable at high temperature (up to 400 ℃ for PFCAs [21]), and resistant to chemical degradation, UV radiation, or weathering [22]. Therefore, FTOHs and the metabolites, PFCAs, are recalcitrant, accumulative and persistent in the environment [22, 23].

However, recent studies have shown that FTOHs, particularly 8:2 FTOH (n=8), are subjected to abiotic degradation in atmosphere and biodegradation in soils and activated sludge [6, 17, 24,25]. Both FTOHs and their metabolites are known to have toxicological effects on laboratory animals and mammalian cell lines. For instance, 6:2 and 8:2 FTOHs exhibited estrogenic activity in the human breast MCF-7 cell line [26]. In mammals, 8:2 FTOH degradation can also result in two transformation products production, PFOA and perfluorononanoic acid (PFNA), which can induce peroxisome proliferation in male mice [27], cause developmental toxicity in mouse neonates [28] and rats [29], and significantly increase mortality in pups [30]. PFNA has not yet been detected as a metabolite during biodegradation of 8:2 FTOH by bacteria [25, 31].

Biodegradation of FTOHs has been observed in activated sludge [32], soils [33, 34], and enrichment cultures with 8:2 FTOH [24] or 1,2-dichloroethane [5]. While biodegradation of FTOHs was once considered only possible on the ethanol moiety of FTOHs, the cleavage of C-F bond in 8:2 FTOH was first reported by Wang et al. [24]. Numerous metabolites, including short-chain perfluorinated carboxylic acids (PFCAs, $[F(CF_2)_mCOOH]$, m = 3, 4, or 5) and x:3 polyfluorinated acids ($[F(CF_2)_xCH_2CH_2COOH]$, x = n-1, n = 6 or 8), have been detected [25]. Possible degradation pathways for 6:2

FTOH and 8:2 FTOH in aerobic soils have been proposed [17, 25]. Most recently, it was reported that 5:3 polyfluorinated acid ([$F(CF_2)_5CH_2CH_2COOH$]) (a metabolite of6:2 FTOH degradation) could be transformed by activated sludge into 4:3 acid, 3:3 acid and short-chain PFCAs [35]. However, all of the previous studies were conducted in mixed cultures, providing little or no information about the microorganisms that are responsible for the FTOH biodegradation. Furthermore, it is unknown which microorganisms can degrade FTOH metabolites, such as the short-chain PFCAs and/or x:3 polyfluorinated acids. Such information is essential for assessing the fate and transport of FTOHs and their metabolites and their environmental impacts.

Many aerobic alkane-degrading bacteria, express enzymes that activate diverse metabolic pathways and these microorganisms can utilize or degrade alkanes and other pollutants of different chain lengths [36-38]. Among these alkane-degraders, *Pseudomonas butanovora* and *Pseudomonas oleovorans* are two well-studied medium-chain (C_5-C_{12}) alkane-degrading bacterial strains. *P. butanovora* was isolated from activated sludge from an oil-refining company [39, 40] and expresses soluble butane monooxygenase (sBMO) that catalyzes the metabolism of C_2-C_9 n-alkanes and a number of primary alcohols [39]. *P. oleovorans* was isolated from soils by co-culturing with n-hexane as a sole carbon source and the isolate can degrade n-octane to octanoic acid [41]. *P. oleovorans* can utilize various substrates including C_6 - C_{12} linear alkanes and alkenes in gas or liquid phase [42]. Another alkane-degrading bacterium, *Mycobacterium vaccae* JOB5 (Gram +), is capable of growing on 2-methylbutane [43] and expresses

diverse monooxygenases that metabolize a wide range of substrates (C_1-C_{22}) [43]. Given that FTOHs are structurally similar to alkanes, it is possible that these alkane degraders may also degrade FTOHs.

Decades of intensive research on dehalogenation, particularly on dechlorination, have identified and characterized numerous dehalogenases capable of breaking C-Cl bonds in aliphatic and/or aromatic compounds [44-55]. Some dehalogenases can defluorinate fluorinated compounds; however, only fluoroacetate dehalogenase is known to hydrolyze C-F bonds in aliphatics [56]. Fluoroacetate dehalogenase was first discovered in a *Pseudomonas* sp. isolated from river mud that is capable of using fluoroacetate as a sole carbon source [57]. *Pseudomonas fluorescens* DSM 8341 and *Burkholderia* sp. FA1 are two of the most studied fluoroacetate-degrading soil isolates. Among seven soil isolates ,*P. fluorescens* DSM 8341 was the most effective fluoroacetate-degrading bacterium [58] and the fluoroacetate dehalogenase expressed by these two strains have a much higher affinity for compounds with C-F bonds compared to those with C-Cl and C-Br bonds [56, 59]. Since PFCAs are structurally similar to fluoroacetate, it is possible that these fluoroacetate-utilizing strains can use fluoroacetate dehalogenase to defluorinate FTOHs.

Enhanced biodegradation can be accomplished by enrichment cultures. Using FTOHs or structurally similar compounds (eg. n-octane) as substrates for enrichment might increase effectiveness of FTOH-degrading microorganisms [5, 33]. Although previous studies have reported biodegradation of FTOH in enrichment cultures with 8:2

FTOH [24]or 1,2-dichloroethane [5], no systematic approach has been attempted to develop an enrichment culture system for enhanced FTOH biodegradation.

Therefore, this study examined the biodegradation potential of FTOHs by several well-studied pure bacterial strains: including bacteria-that degrade alkanes (*P. oleovorans*, *P. butanovora*, and *M. vaccae* JOB5) and a bacterium containing fluoroacetate dehalogenase (*P. fluorescens* DSM 8341). This is the first study attempt to examine the biodegradation of FTOHs (4:2, 6:2 and 8:2 FTOHs) and their known metabolites by these well-studied microorganisms. Since it is expected that 6:2 FTOH will be widely used in the near future, this study also investigated the biodegradation of 6:2 FTOH by the four strains (described above) under different co-substrate conditions. It was expected that addition of co-substrates might enhance the extent of defluorination during 6:2 FTOH degradation and/or result in decreased formation of short-chain PFCAs as end-products. Additional research efforts were focused on development of a mixed culture capable of defluorinating FTOHs to environmental friendly end-products. The performance of these FTOH-degrading enrichment cultures were linked to their unique microbial community structures and the quantity of *alkB* genes, a presumptive catabolic gene for FTOH degradation.

1.2 Goals, objective and hypothesis

The **goal** of this study was to decipher factors affecting biodefluorination of FTOHs and their metabolites, and to develop enhanced biological treatment strategies for removal of FTOHs. To achieve this goal, four specific objectives were identified and accomplished and a description of these objectives, and associated hypotheses and tasks is outlined below.

Objective 1: Examine biodegradation potential of FTOHs by medium-chain alkane-degrading bacteria

Hypothesis: Due to structural similarities between FTOHs and medium-chain alkanes and their broad substrate range, we hypothesize that medium-chain alkane-degrading bacteria can degrade FTOHs.

Task 1a: Examine degradability of FTOHs by two well-studied medium-chain alkane degrading bacteria, *P. oleovorans* and *P. butanovora*

Task 1b: Identify transformation metabolites of FTOHs and possible FTOH degradation pathways by those two strains.

Objective 2: Examine biodegradation potential of 6:2 FTOH by C_1-C_{22} alkane- and fluoroacetate-degrading strains

Hypothesis: We hypothesize that 6:2 FTOH can be degraded by alkane- and fluoroacetate-degrading bacteria.

Task 2a: Examine the biodegradation of 6:2 FTOH by *M. vaccae* JOB5, a C_1-C_{22} alkane-degrading bacterium.

Task 2b: Examine biodegradation of 6:2 FTOH by *P. fluorescens* DSM 8341, a fluoroacetate-degrading bacterium

Objective 3: Examine the effects of co-substrates on biodegradation of 6:2 FTOH

Hypothesis: We hypothesize that the addition of co-substrates will induce expression of different enzymes or provide needed reducing energy to enhance 6:2 FTOH biodegradation. The enhanced degradation can be evaluated with respect to the extent of defluorination or the ability to degrade key metabolites, such as short-chain perfluorinated carboxylic acids (PFCAs) or x:3 polyfluorinated acids, to less concerned products.

Task 3a: Examine the effects of co-substrates on the extent of defluorination of 6:2 FTOH by alkane- and fluoroacetate-degrading bacteria.

Task 3b: Examine biodegradation of short-chain PFCAs by *P. oleovorans* and *P. fluorescens* DSM 8341 in the presence of co-substrates

Task 3c: Examine biodegradation of 5:3 polyfluorinated acid by *P. oleovorans* and *P. fluorescens* DSM 8341 in the presence of co-substrates

Objective 4: Develop and characterize FTOH-degrading microbial consortia

Hypothesis: We hypothesize that alkanes are ideal co-substrates for enriching effective FTOH-degrading consortia.

Task 4a: Develop FTOH-enrichment microbial consortia using activated sludge from a wastewater treatment plant (WWTP) treating perfluorinated-compound-containing wastewater from a fire-fighting training field. Use 8:2 FTOH and/or one of the co-substrates (n-octane or 1-butanol) for enrichment.

Task 4b: Examine the degradation ability of FTOHs by the enrichment cultures. Link the degradation performance to presumptive catabolic gene (*alkB* gene) and associated microbial community structure.

1.3 Dissertation overview

This dissertation consists of six chapters. **Chapter 1** described the problems regarding the occurrence of FTOHs in the environment as well as the goal and objectives of this research. In **Chapter 2**, current literature on FTOHs, potential FTOH-degrading bacteria and molecular tools for microbial community analysis are summarized. The knowledge gaps and research needs in biodegradation of FTOHs are also identified. The results of task 1a and 1b are described in **Chapter 3**. The biodegradation of FTOHs by two pure alkane-degrading bacteria (*P. oleovorans* and *P. butanovora*) was examined. The extent and the amount of FTOH biodefluorination by these strains were identified. The recovery of free fluoride ions at the end of the degradation indicates the occurrence of defluorination of FTOHs. This was the first study attempting to examine biodegradation of FTOHs by these well-studied microorganisms. Materials presented in **Chapter 3** have been published in Biotechnology and Bioengineering [31] and modified to the dissertation format with copyright permission from Wiley Periodicals, Inc., A Wiley Company. Results of the tasks in Objective 2 and 3 are summarized in **Chapter 4**. Specifically, biodegradation of 6:2 FTOH by *M. vaccae* JOB5 (a C_1-C_{22}alkane-degrading bacterium) and *P. fluorescens* DSM 8341 (a fluoroacetate-degrading bacterium) was observed, indicating that bacteria other than the two alkane-degrader strains (*P. oleovorans* and *P. butanovora*) can also degrade FTOH. In addition, the effects of various co-substrates on the types of transformation products and

extent of defluorination of 6:2 FTOH was investigated. Among the tested strains, *P. fluorescens* DSM 8341 was the only strain capable of degrading a selected metabolite, 5:3 perfluorinated acid. In **Chapter 5**, the effects of co-substrates on FTOH degradation by enriched mixed cultures are described. Meanwhile, the microbial community changes in mixed cultures during degradation of FTOHs were analyzed by molecular methods. In **Chapter 6**, the findings of this study are summarized, and the implications of the results of this work and future research directions are also discussed.

2. LITERATURE REVIEW

2.1 Fluorotelomer alcohols (FTOHs)

2.1.1 Chemical and physical properties of FTOHs

FTOHs are produced through a telomerization process with even-numbered fluoroalkyl chains and an ethanol moiety. The telomerization developed by DuPont is defined as a process of reacting a molecule, called telogen, with two or more ethylenically unsaturated molecules, called taxogens [YZ (telogen) + nA (taxogen) → $Y-(A)_n-Z$ (telomere)]. The chemical formula of FTOHs can be expressed as $F(CF_2)_nCH_2CH_2OH$, where n is the number of fluoralkyl chains. Based on the number of the fluoralkyl chains (n) and the non-fluorinated carbons (i.e., 2), FTOHs are commonly referred as n:2 FTOHs. For example, 8:2, 6:2, and 4:2 FTOH represent [$F(CF_2)_8CH_2CH_2OH$], [$F(CF_2)_6CH_2CH_2OH$], and [$F(CF_2)_4CH_2CH_2OH$], respectively. Due to the fluoralkyl chains and ethanol moiety in FTOHs, FTOHs provide unique surface modification properties such as water and oil repellency in polymeric products and wetting and leveling in surfactants [1]. FTOHs are volatile and only slightly soluble in water (140 µg/L for 8:2 FTOH, [60]). The estimated dimensionless Henry's

law constant for 8:2 FTOH is 270 [23]. In water, FTOHs quickly evaporate from the surface [24] and due to the strong C-F bonds, FTOHs are resistant to chemical degradation, UV radiation, or weathering, making them persistent in the environment [22].

2.1.2 Sources and fate of FTOHs in the environments

FTOHs have been detected in wastewater effluent [61] and sludge applied soils [62, 63] and the transformation products such as PFCAs are frequently found in the environment, wildlife, and human [7-14]. However, the origin and environmental fate of FTOHs and their transformation products, particularly PFCAs, are still largely unknown. One potential source could be the residual FTOHs from FTOH-based polymers, and/or the breakage of the covalent bonds of FTOH-based polymers at industrial production facilities, and recent study reported that aqueous solutions of FTOH-based products are released from production facilities into wastewater, and the products may undergo biodegradation in wastewater treatment facilities, landfill or other environments [24]. FTOHs and their metabolites tend to adsorb rapidly into soils and sludge and the sorption is irreversible [24]. Biotic and/or abiotic degradation of polymer and nonpolymeric materials containing FTOHs has been suggested as a source

of atmospheric contamination [2] and this statement is supported by the detection of high concentrations of perfluorinated compounds in the urban troposphere [3, 4].

2.1.3 Toxicity

6:2 and 8:2 FTOHs exhibited estrogenic activity and enhanced proliferation in an *in vitro* assay using MCF-7 human breast cancer cells [26]. Comparing to the reference compound 17β-estradiol (E_2), these FTOHs also induced the expression of estrogen-responsive genes. Exposure of MCF-7 cells to 6:2 and 8:2 FTOH significantly increased the number of cells in the synthesis phase (S-phase) of the cell cycle and stimulated synthesis of new DNA required for cell division (i.e. the increase of cancer cells). Interestingly, other perfluorinated compounds – perfluoro-1-octane sulfonate (PFOS), PFOA and PFNA– did not exhibit estrogenic activity. These results are different from other studies reporting that exposure to PFOA and PFNA (known as 8:2 FTOH metabolites)caused peroxisome proliferation, development toxicity and mortality to animals and mammalian cell lines [27-30].

2.1.4 Abiotic degradation of FTOHs

Ellis et al. [2] showed FTOHs can react with Cl atoms and OH radicals in the air and the reaction rate was not affected by the chain length of FTOHs. However, the fluorinated carbons seemed to deactivate the reaction between $–CH_2-$ groups and Cl atoms or OH radicals, compared to that observed for non-fluorinated alcohols (like ethanol) or glycerol.

Possible pathways for abiotic degradation of FTOHs in the air were proposed by Ellis et al. [6]. During the abiotic degradation, FTOHs first interact with OH radicals and oxygen, followed by a series of reactions to produce PFCAs (Fig. 2.1). The production of OH radicals in the air is associated with the formation of H_2O in molecular clouds. The molecular cloud is a cool dense interstellar region composed primarily of hydrogen and a wide variety of molecules. In the cold environment, the hydronium ion (H_3O^+) is formed from H_2O via proton transfer reactions and is then converted into hydrogen and OH radicals by dissociative recombination ($O + H_3^+ \rightarrow H_2 + OH^+$) [64, 65].

2.1.5 Biotic degradation of FTOHs

It was once considered that only the ethanol moiety, not the C-F bonds, of FTOHs can be biodegraded. In 2005,Wang et al. [24] reported the first evidence for cleavage of C-F bonds in 8:2 FTOH in activated sludge. In a later study, the possible degradation pathways for 8:2 FTOH in aerobic soils was proposed [25]. According to the proposed pathways (Fig. 2.2), 8:2 FTOH is first transformed into 8:2 FTCA (8:2 fluorotelomer acid) which can be catalyzed by alcohol and aldehyde dehydrogenase, and then to 8:2 FTUCA (8:2 fluorotelomer unsaturated acid) via multiple steps, including HF elimination. 8:2 FTUCA is a direct precursor of 7:2 sFTOH, which is then converted to PFOA. It was suggested that the conversion of 7:2 sFTOH to PFOA is catalyzed by monooxygenases. In addition, 7:2 sFTOH can be transformed into the 7:2 ketone and to 2H-PFOA via multiple enzymatic reactions. Through reductive defluorination, 8:2 FTUCA can also be degraded to 7:3 Uacid. 7:3 Uacid is further transformed to α-OH 7:3 acid and to 7:3 acid in which a dehydrogenase might be involved, or to PFHxA (perfluorohexanoic acid) possibly via allylic isomerases and hydrogenases [25]. The enzymes involved in each step of 8:2 FTOH of the proposed degradation pathways have not been confirmed or identified.

With an increasing concern over the large usage of 8:2 FTOH and its transformation products (particularly PFOA), there has been a move to replace 8:2

FTOH with 6:2 FTOH (a shorter FTOH) as a raw manufacture material. Thus, new studies have been initiated to examine the biodegradation potential of 6:2 FTOH in the environment. Similar to 8:2 FTOH biodegradation, 6:2 FTOH was degraded in aerobic soils and mixed bacterial cultures developed from activated sludge previously exposed to fluorinated compounds [17], but with a more rapid rate [17, 25]. Many transformation metabolites were detected during 6:2 FTOH biodegradation by soils and the enrichment culture [17]. These common metabolites reported in this study were 6:2 FTCA, 6:2 FTUCA, 5:2 ketone, 5:2 sFTOH, α-OH 5:3 acid, 5:3 Uacid, PFBA (perfluorobutanoic acid), PFPeA (perfluoropentanoic acid), PFHxA and 5:3 acid. However, 4:3 acid was detected only in aerobic soil, and 5:3 ketone aldehyde [$F(CF_2)_5COCH_2CHO$] and 5:3 Uamide were detected only in the enrichment culture. Interestingly, 5:3 ketone aldehyde and PFPeA were detected during 6:2 FTOH degradation while 7:3 ketone aldehyde and PFHpA (the analogous metabolites) were not detected during 8:2 FTOH degradation, suggesting that (i) 6:2 FTOH is more biodegradable and (ii) new degradation pathways for 6:2 FTOH might be present [17, 25]. 6:2 FTOH degradation pathways were proposed as illustrated in Fig. 2.3 [17]. To further examine the potential for degrading 5:3 polyfluorinated acid in soils and activated sludge, Wang et al. [35] discovered a new degradation pathways, called the "one-carbon removal pathways" [35]. Through these pathways, 5:3 polyfluorinated acid was transformed to α-OH 5:3 acid [$F(CF_2)_5CH_2CH(OH)COOH$] as the dominant metabolite, followed by 5:2 acid [$F(CF_2)_5CH_2COOH$], 5:3 Uacid [$F(CF_2)_5CHCHCOOH$], 5:2 Uacid

[$F(CF_2)_4CFCHCOOH$], and several minor metabolites (PFPeA, PFBA [perfluorobutyric acid, $F(CF_2)_3COOH$], 4:3 acid [$F(CF_2)_4CH_2CH_2COOH$] and 3:3 acid [$F(CF_2)_3CH_2CH_2COOH$])[35]. These degradation pathways result in a production of mixtures of x:3 acid (x<5), α-OH x:3 acid (x = 4 or 5), and short-chain PFCAs (m = 3 and 4). These metabolic pathways are favorable; however, little is known about microorganisms and enzymes responsible for mechanism of these reactions. Identifying pure bacterial strains capable of degrading 5:3 acid via one-carbon removal pathways will provide valuable information for creating favorable environmental and physiological conditions for enhanced degradation of 6:2 FTOH, i.e., beyond 5:3 acid and/or short-chain PFCAs.

2.2 Potential FTOH-degrading bacteria: alkane- and fluoroacetate-degrading bacteria

The chemical structure of FTOHs is very similar to n-alkanes. Since many alkane-degrading bacteria can utilize alkanes with different chain length and also cometabolize a range of aliphatic compounds, it was hypothesized thatalkane-degrading bacteria might be able to degrade FTOHs, either metabolically or cometabolically, into shorter and less-fluorinated metabolites and/or less harmful end-products.

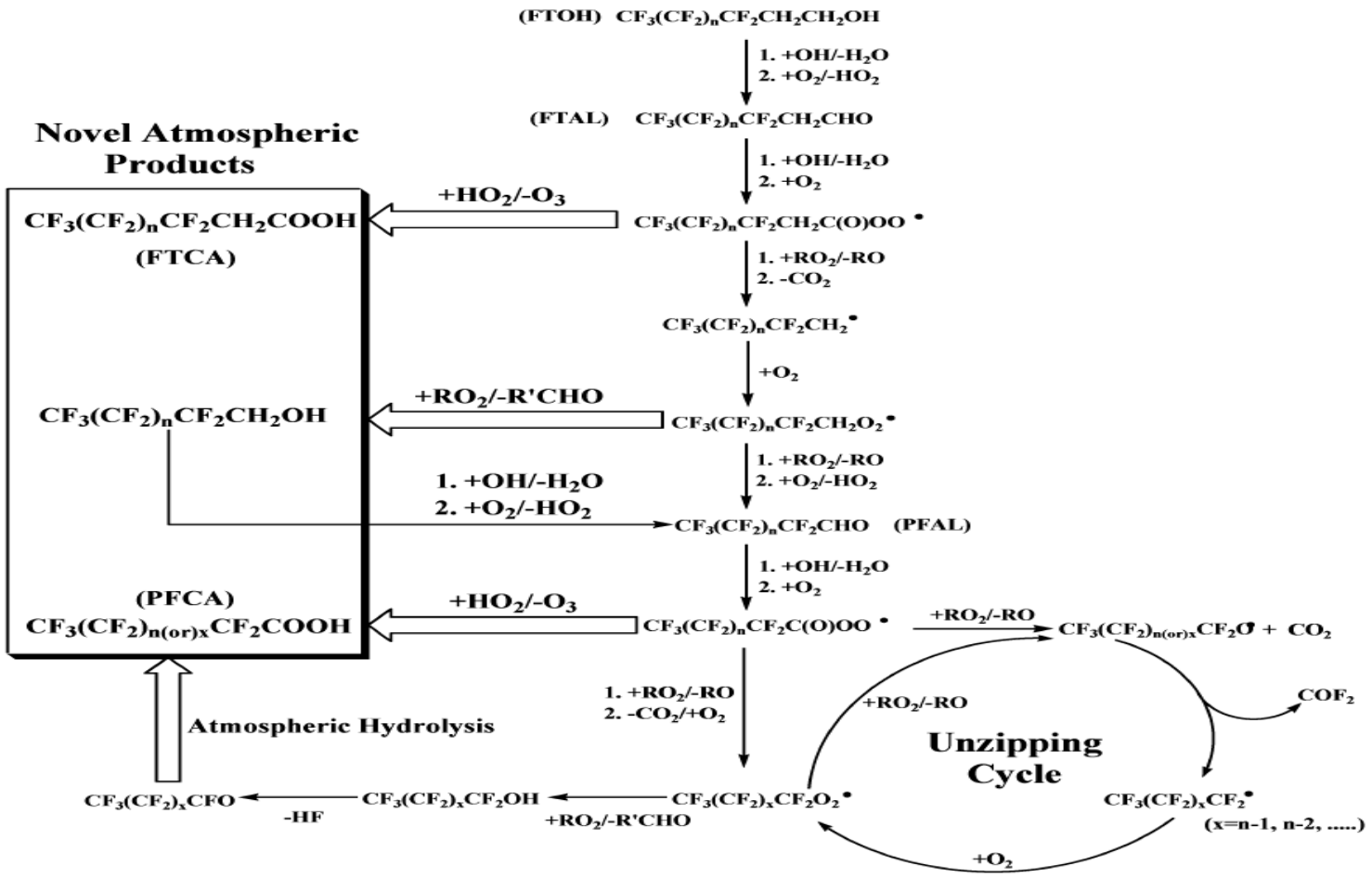


Fig. 2.1.OH-initiated oxidation pathways for telomer alcohols [6]

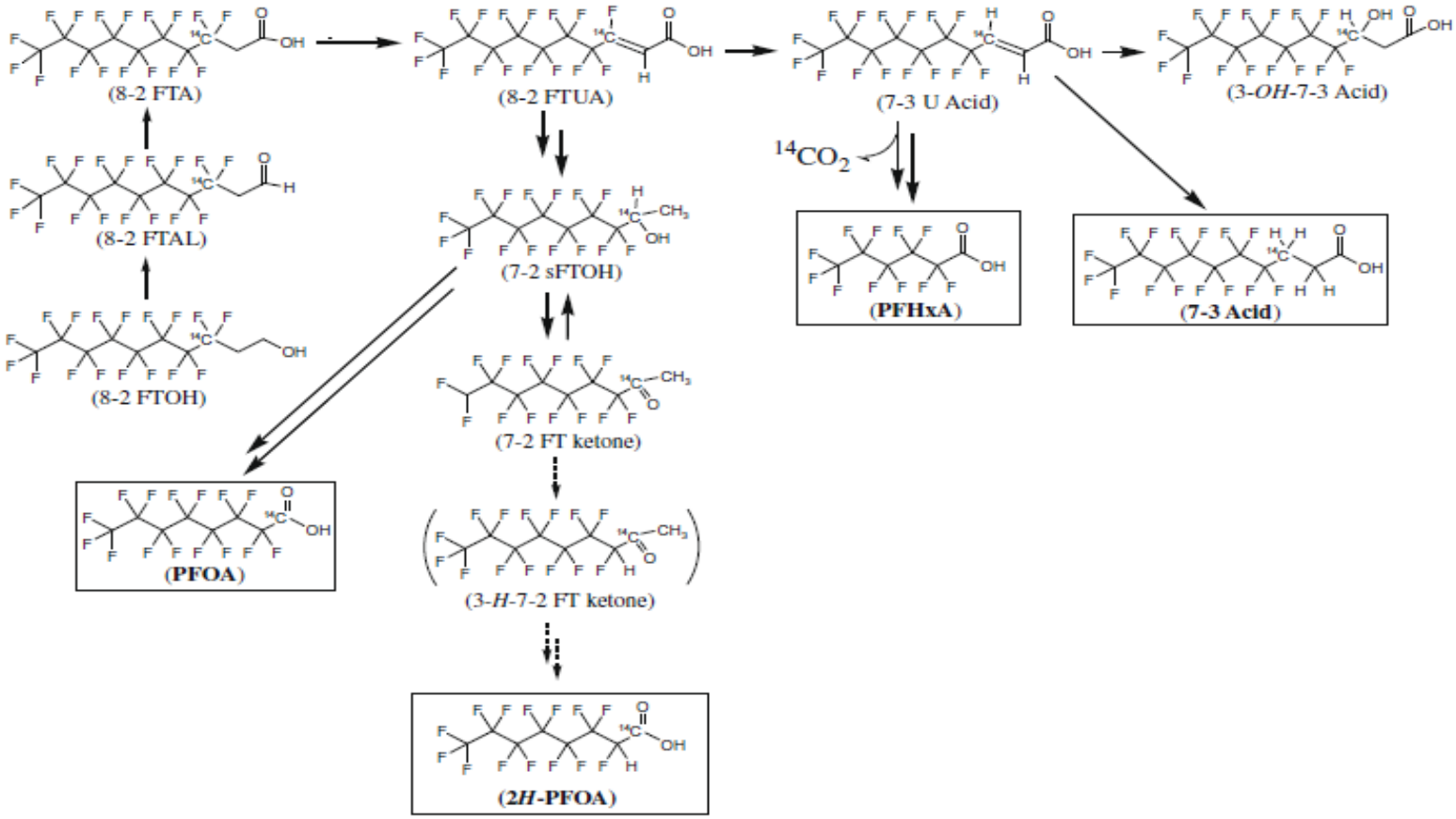


Fig. 2.2. Proposed biodegradation pathways of ^{14}C-labeled 8:2 FTOH [25]

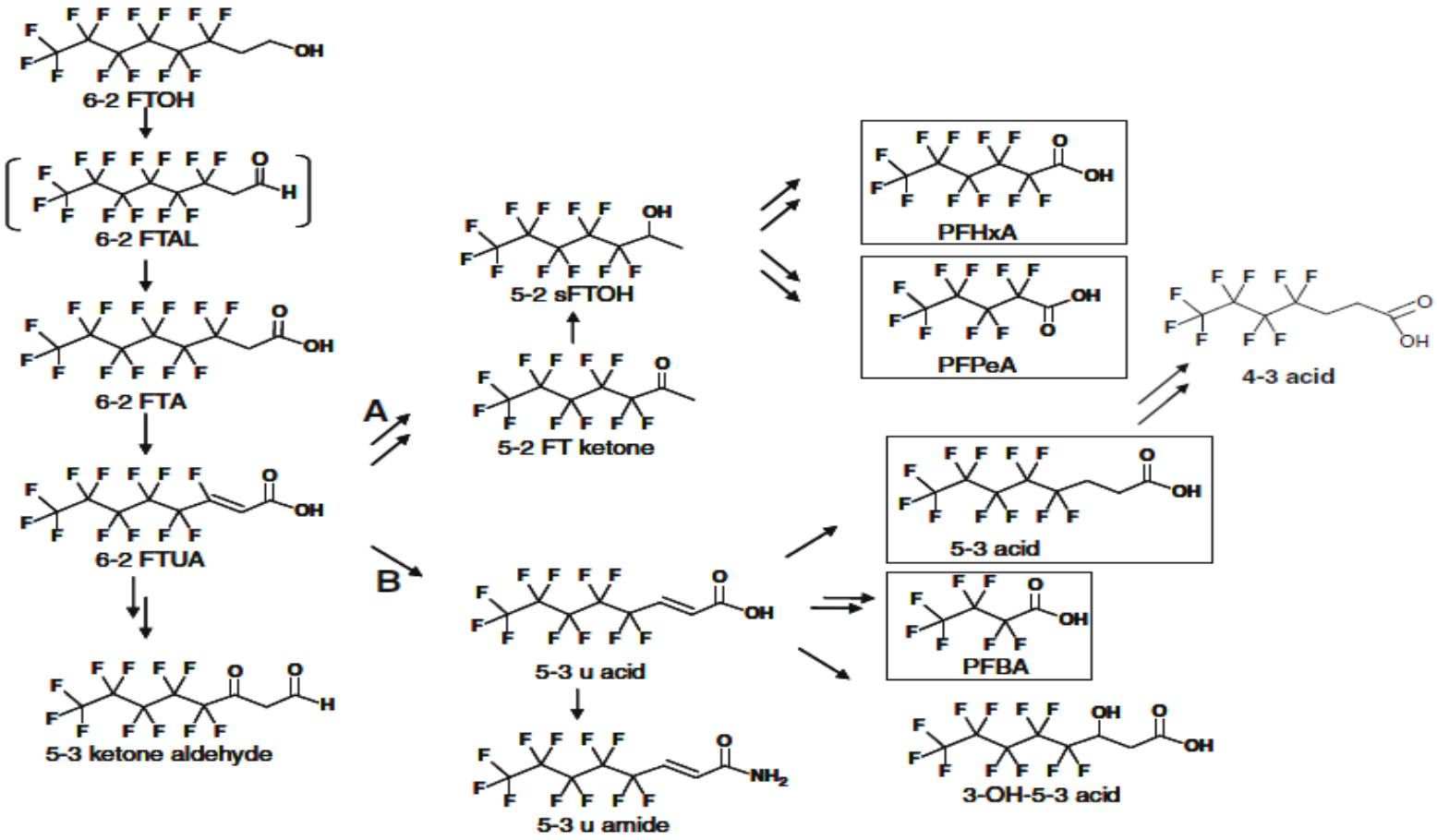


Fig. 2.3. Proposed biodegradation pathways of 6:2 FTOH: The product in brackets is the proposed intermediate and those in boxes are terminal metabolites [17].

To test this hypothesis, three well-studied alkane-degrading bacteria (*P. oleovorans*, *P. butanovora*, and *M. vaccae* JOB5) were selected since these strains are known for their ability to grow on alkanes and to co-metabolize many environmentally persistent pollutants [42, 66-72].

Bacteria, capable of expressing fluoroacetate dehalogenase to cleavage C-F bonds in fluorinated aliphatic compounds, were also selected for this study since fluoroacetate-dehalogenase-expressing bacteria might be able to transform FTOHs into less recalcitrant products. *Pseudomonas fluorescens* DSM 8341 and *Burkholderia* sp. FA1 are two well-studied bacteria expressing fluoroacetate dehalogenases that exhibit a strong affinity toward a C-F bond, but a poor affinity toward chloroacetate and bromoacetate [56, 59]. Below is a brief review on each of these potential FTOH-degrading bacteria.

2.2.1 Alkane-degrading bacteria

Aliphatic hydrocarbons, including n-alkanes, branched alkanes and alkenes, are ubiquitous in the environment [73]. Many alkane-degrading bacteria contain special enzymes, allowing them to utilize or degrade alkanes with different chain lengths and other pollutants [36-38]. Most of microorganisms initiate the oxidation of n-alkanes by hydroxylase (monooxygenase) system [73] and AlkB is one of the key alkane

hydroxylases [38]. In this study, three well-studied alkane-degrading strains, *P. oleovorans*, *P. butanovora* and *M. vaccae* JOB5, were used as model strains to explore their ability to degrade FTOHs and background and information on these strains is given below.

2.2.1.1 *Pseudomonas oleovorans*

P. oleovorans was isolated from soils in 1963 [41] and this strain can grow on n-hexane as a sole carbon source and degrade n-octane to octanoic acid [41]. *P. oleovorans* can oxidize alkanes because it contains an OCT plasmid, encoding a complex of monooxygenases. The OCT plasmid consists of two regions: the *alkBAC* operon (= *alkBFGHJKL* operon) and the *alkR* regulatory region. The *alkBAC* operon encodes alkane hydroxylase (a three component monooxygenase adding one oxygen atom to a substrate), alkanol dehydrogenases and aldehyde dehydrogenases.The expression of *alkB* genes on the OCT plasmid can be gratuitously induced by dicyclopropylketone (DCPK) [74, 75]. Using these enzymes, *P. oleovorans* can also oxidize alkanes to alkanols, alkanals and alkanoic acids. This strain has a broad substrate range, including C_6 to C_{12} linear alkanes, or alkenes in gas or bulk liquid phase[42]. *P. oleovorans*also degrades crude oil [38], chlorinated compounds [76] and methyl tertiary butyl ether (MTBE) in the presence of DCPK [77].

2.2.1.2 *Pseudomonas butanovora*

P. butanovora is a gram-negative, rod-shaped, aerobic bacterium which was originally isolated from activated sludge from an oil-refining company [39, 40]. Soluble butane monooxygeanse (sBMO) expressed by this strain initiates the oxidation of butane to 1-butanol [68]. 1-Butanol is then transformed by two alcohol dehydrogenases to butyraldehyde which is further degraded to butyrate [78]. Both butane and 1-butanol are inducers for sBMO; however, it was reported that 1-butanol was a more efficient inducer of sBMO in this strain [79]. *P. butanovora* can grow on C_2-C_9 n-alkanes and a number of primary alcohols [39] and this strain can also degrade different chlorinated alkanes and aromatics [67, 71, 72].

2.2.1.3 *Mycobacterium vaccae* JOB5

M. vaccae JOB5, isolated from soil, is a gram-positive bacterium. The strain was isolated for its ability to use isopentane (2-methylbutane) as a sole carbon and energy source [43]. The strain can also grow on a wide range of substrates (C_1-C_{22}) [43] and expresses diverse enzymes including butane monooxygenase (BMO) [80], propane monooxygenase (PMO) [81], and AlkB (alkane hydroxylase) [82] in response to its

growth conditions. Propane-grown *M. vaccae* JOB5 are known to degrade trichloroethylene [66], BTEX (benzene, toluene, ethylbenzene, and xylenes) [83], tertiary butyl alcohol (TBA) [82], and 1,4-dioxine [84]. Butane-grown *M. vaccae* JOB5 can degrade chloroform [67], and n-octane-grown *M. vaccae* JOB5 can degrade methyl tertiary butyl ether (MTBE) [85].

2.2.2 Fluoroacetate-degrading bacteria

Fluoroacetate [$F(CH_2)COOH$] is a fluorinated compound that is naturally produced by some plants and a bacterium (*Streptomyces cattleya*). Although fluoroacetate is not toxic to plants and bacteria, it is highly toxic to mammals [86, 87]. Among various known dehalogenases [47, 88-95], fluoroacetate dehalogenase, expressed by fluoroacetate-degrading bacteria, is the only enzyme capable of hydrolyzing the C-F bond of fluoroacetate [56].

Since the first report of bacterial defluorination of fluoroacetate by Goldman[57], several fluoroacetate-degrading microorganisms such as *Moraxella*, *Pseudomonas*, and *Burkholderia*have been isolated [56, 96]. The fluoroacetate dehalogenases expressed by *Moraxella* sp. B and *Burkholderia* sp. FA1 can defluorinate fluoroacetate via a two-step mechanism similar to that catalyzed by haloalkane dehalogenase. In the first step, the aspartate residue at the active site of the enzyme attacks the α-carbon atom

nucleophilically. This attack causes the release of the fluoride ion and formation of the ester intermediate. In the second step, the intermediate is then hydrolyzed by a water molecule to produce glycolate. Figure 2.4 shows the defluorination of fluoroacetate dehalogenase (FAc-DEX H1) from *Moraxella* sp. B [96, 97].

Sodium monofluoroacetate(SFA), consisting of a carboxylic group and a C-F bond, is structurally very similar to a short-chain PFCA. Recent studies [17, 25] have reported that short-chain PFCAs appear to be the accumulative products during degradation of FTOHs. Accordingly, these fluoroacetate-dehalogenase-expressing strains might be able to degrade short-chain PFCAs into diverse transformation metabolites as reported during biodegradation of FTOHs by activated sludge and soils [25]. Thus, this study examined the biodegradation potential of short-chain PFCAs by fluoracetate-degrading bacteria. In addition, the biodegradation potential of x:3 acids by fluoracetate-degrading bacteria were investigated.

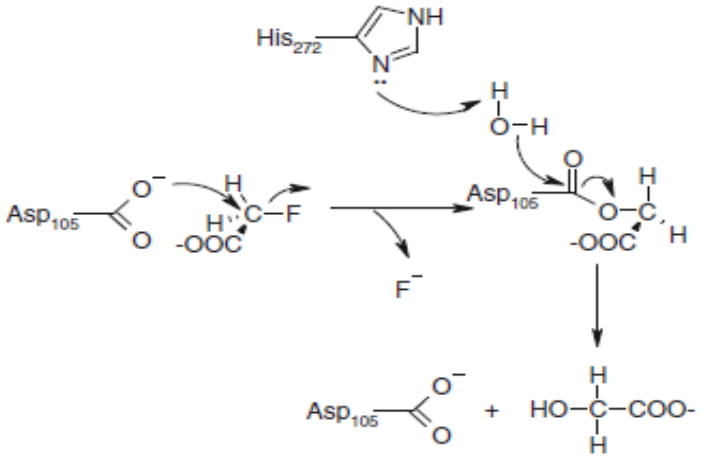


Fig. 2.4. The reaction of defluorination by fluoroacetate dehalogenase, FAc-DEX H1 [59]

2.2.2.1 *Pseudomonas fluorescens* DSM 8341

Pseudomonas fluorescens DSM 8341, one of seven isolates from western Australian soils, is the most efficient strain in defluorinating SFA[58]. The fluoroacetate dehalogenase, expressed by *P. fluorescens*DSM 8341, can also dehaolgenate other halogenated compounds (chloroacetate and bromoacetate) but less efficiently. The enzyme also has a poor substrate specificity toward ethyl fluroacetate and was unable to degrade fluroacetamide, suggesting that the carboxyl group in halogenated compounds might play an important role in substrate affinity. It was also reported that the presence of Mg^{2+} increased the enzyme activity by 20-25% [59].

2.2.2.2 *Burkholderia* sp. FA1

*Burkholderia*sp. FA1 is also a soil fluoroacetate-utilizing bacterium. The dehalogenase expressed by this strain, called FAc-DEX FA1, was specific to haloacetate, particularly fluoroacetate. FAc-DEX FA1 was only induced in the presence of fluoroacetate. The enzymatic structure of FAc-DEX FA1 was similar to that of FAc-DEX H1 from *Moraxella*sp. B [56].

2.3 Molecular techniques used to characterize FTOH-degrading consortia

Most of our knowledge of biodegradation of target compounds has been obtained from laboratory studies of bacterial isolates [98, 99]. However, more than 99% of bacteria are not cultivable in a laboratory setting [100] and results obtained from the isolates are biased and might not be relevant to what occurs in the environment.

In this study, FTOH-degrading consortia were developed and characterized by two specific culture-independent methods. These two methods are real-time terminal restriction fragment length polymorphism (real-time t-RFLP) which has been successfully used to monitor diverse microorganisms in the environment [101-103] and real-time PCR which can be used to quantify the strains and/or catabolic genes [102, 104]. These two methods are briefly described below.

2.3.1 Detection of catabolic genes by real-time PCR

Real-time PCR is a fluorescence-based kinetic PCR method for quantifying the initial number of copies of a target gene [105]. In the first step in real-time PCR, a fluorescence-labeled probe is complementarily bound to the target DNA or nucleic acid stain such as SYBR Green is bound to any existing double stranded DNA in the

sample. During the amplification, the fluorescent label is cleaved and the released fluorescent signals are recorded at the end of each PCR cycle. The detected signals are then compared to a set of standard curves for quantification [106].

AlkB (alkane hydroxylase) is known as one of key enzymes for alkane degradation and the alkB gene has been most commonly used for detection of alkane-degrading bacteria in the environment [107]. From the previous study on degradation of FTOHs by alkane-degrading bacteria [31], we hypothesized alkB gene coding alkane hydroxylase might be one of responsible genes for degradation of FTOHs. Based on former studies for primers design [108-110], forward and reverse primers were chosen to detect microorganisms containing the alkB gene in FTOHs degrading consortia and to investigate the effects of this bacterial group on FTOH degradation in the natural environment.

2.3.2 Characterization of FTOH-degrading microbial community using Real-time terminal restriction fragment length polymorphism (real-time-t-RFLP) assay

A quantitative fingerprinting method, called the real-time terminal restriction fragment length polymorphism (real-time-t-RFLP) assay, has been successfully used to study the microbial community structures in activated sludge, microcosms, and enrichment cultures [101-103]. Real-time-t-RFLP assay, integrating real-time PCR and

t-RFLP, is a quantitative finger printing assay [102]. Briefly, the 16S rRNAs in templates are PCR amplified using fluorescent-labeled primers and probes, and the amplified PCR products are digested with a restriction enzyme. The digested PCR products generate various fluorescence-labeled terminal restriction fragments (T-RF). The microbial community profile is characterized as a series of T-RF peaks, representing different phylotypes in a microbial community. The fluorescence intensity of the peaks reflects the relative abundance of each phylotype [111]. The signals are then compared to a set of standard curves for quantification of the target gene[102, 106]. The gene copy number for a T-RF can be calculated by multiplying the area of the T-RF portion and the total gene copy number in the sample.

3. BIOTRANFORMATION OF FTOHS BY TWO PURE ALKANE-DEGRADING BACTERIA: *P. oleovorans* AND *P. butanovora**

3.1 Introduction

Perfluorinated carboxylic acids (PFCAs) and perfluorinated sulfonic acids are persistent compounds that have been detected in the environment, wildlife, and humans [7-14]. The environmental sources of PFCAs are not fully understood; however, one possible source of PFCAs comes from abiotic and biotic transformation of fluorotelomer alcohols (FTOHs) and their precursors [2, 5,6]

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 [1]. The unique properties of FTOH-based products have led to a wide variety of applications including paints, adhesives, waxes, polishes, metals, electronics, caulks, agrochemicals,

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refrigerants, fire-fighting foams, and lubricants [1]. From 2000 to 2002, the production of FTOH-based products was estimated to be ~6000 tons of fluorotelomer A equivalents/year worldwide [112].

FTOHs are volatile and relatively water insoluble (carbon chain-length dependent) in the absence of a sorbing medium [113], and they have a tendency to be adsorbed strongly to solid matrices such as household dusts [114], soils and activated sludge [24, 113, 115]. Field monitoring studies report detection of FTOHs in the troposphere at concentrations ranging from 7 to 196 pg/m^3 [3] and averaged 87 pg/ m^3 [116] with 6:2 and 8:2 FTOHs being the major components. The major source of environmental FTOHs is postulated to be the residual unreacted FTOH present in commercial products [2]. A recent study suggests that the current practice of applying sludge from WWTPs to soils is a potential pathway to introduce FTOHs and PFCAs to the environment [63].

Similar to the historical aspect of PCB dechlorination [117], microbial removal of $–CF_2–$ groups from FTOHs is considered extremely difficult due to the strength of C-F bonds in the perfluoroalkyl moiety of FTOHs. Early studies report biotransformation of 8:2 FTOH in 1,2-dichloroethane enrichment culture [5], activated sludge [24], and soils [25, 33]. By using ^{14}C-labeled 8:2 FTOH, Wang et al. [24] reported the first evidence of C-F bond cleavage in 8:2 FTOH by activated sludge. In recent years, Liu et al. [17] and Wang et al. [25] reported more FTOH transformation products from

FTOH defluorination and proposed possible transformation pathways for 6:2 FTOH and 8:2 FTOH in aerobic soils. 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 [36-38]. 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 the activated sludge of oil-refining waste [39, 40]. In addition to butane, *P butanovora* can grow on C_2-C_9 n-alkanes and a number of primary alcohols [39]. This strain expresses soluble butane monooxygenase (sBMO) to oxidize butane to 1-butanol [68], which is further converted to butyraldehyde, and then to butyrate [78]. Previous studies also showed that sBMO can degrade a wide range of chlorinated alkanes and aromatics via cometabolic reactions [67, 71,72]. *P. oleovorans* is a soil isolate known for its ability to express alkane monooxygenases to degrade n-octane to octanoic acid [41] and to oxidize various C_6 to C_{12} linear alkanes or alkenes [42]. 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 8:2FTOHs) by the aforementioned two *Pseudomonas* strains. This is the first report to show that individual pure bacterial strains were able to defluorinate FTOHs to form transformation products with shorter chain lengths, resulting in the removal of three $-CF_2-$ groups from FTOHs. Furthermore, this work demonstrated that the two individual microbial strains deployed different pathways to transform FTOHs.

3.2 Materials and methods

3.2.1 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 Japan. Hexane (95 % pure) was obtained from Acros Organics (UK). 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 (Geel, Belgium), and ethanol (95% purse) 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 the 4:2 FTOH transformation test, the pure compound (in liquid form) was used directly. For 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 a 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] PFHxA, the internal standard, was from Wellington Laboratories (Guelph, Ontario, Canada). The rest of the polyfluorinated acid standards used for quantitative analysis were synthesized by DuPont (Wilmington, DE). All standards used have a purity of +97%.

3.2.2 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 [118] with 1% n-octane to an optical density at 660 nm (OD_{660}) of 1.0 before being harvested for experimental use. The cells were centrifuged at 10,000 *g* for 5min, 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 [68] with 2 mM 1-butanol to OD_{600}= 0.5-0.7.

3.2.3 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 *P. oleovorans* (or *P. butanovora*) cell suspension 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 μL 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 to 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 concentrated 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℃ dark room. The vials were sacrificed over different time points: 0, 12 and 24 h for 6:2 and 8:2 FTOH transformation by *P. oleovorans*, 0, 24 and 48 h for 4:2 FTOH transformation by *P. oleovorans* and 0, 1 and 3 d for 4:2, 6:2 and 8:2 FTOH transformation by *P. butanovora*. At each sampling time point, 2 mL of hexane was added to the vials to stop transformation and extract the remaining FTOH. After overnight extraction, a gas tight syringe was used to transfer the hexane layer to clean capped GC amber vials (1.5 mL) which were then stored at -20℃ for later GC/MS analysis. The aqueous layer in the old sample vials (i.e. 22 mL-glass vials) was preserved at 4℃ 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 3300 μg) of 6:2 FTOH was added. For 8:2 FTOH transformation tests, 5 μL of 8:2 FTOH stock solution (40g/L in 95% ethanol) (or 200 μg) was used.

3.2.4 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 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 to the bottles using a 25 μL-gas-tight syringe. Killed controls, containing acid-killed cells (by adding 50 μL of concentrated 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 % 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 from Quantek Instruments, Grafton, MA). The bottles were incubated on a shaker at 150 rpm in a 30 ℃ dark room. At each of following sampling points (0, 0.5, 1, 3, 7, 14, 28 days), two live samples and two killed control bottles were sacrificed for solvent extraction. Twenty milliliters of acetonitrile were added to each of the bottles to extract the remaining FTOH and potential metabolites in

the 10-mL cell suspension. The extraction was carried out for 3 days at 50 ℃ with ~150 rpm shaking. After the extraction, each bottle was resealed with a new septum and preserved in a freezer (-50℃). 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 ℃. 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.

3.2.5 Fluoride measurement

The aqueous samples from the 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 ion concentrations using an Orion 96-09BNWP ion-selective electrode (Thermo Scientific, Beverly, MA) following the manufacturer's protocol. Fluoride standard solutions, ranging from 2~20 mg/L, were prepared by diluting a certified fluoride standard solution (100 mg/L, Thermo Scientific, Beverly, MA) in the corresponding growth media.

3.2.6 GC/MS and GC/ECD analysis

The concentration of 4:2 FTOH was determined using an Agilent Technologies 6890N gas chromatograph (GC) coupled with a 5973N mass selective detector (Agilent Technologies) 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. [34] as follows: 50 ℃ for 2 min, with a first ramp at 20 ℃ min^{-1} to 210 ℃ and a second ramp at 50 ℃ min^{-1} to 280 ℃, and then held at 280 ℃ for 3 min. One microliter hexane extract from each sample was injected into the GC/MS system. The injector temperature was 150 ℃ and the detector temperature was 280 ℃. Helium was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. The 4:2 FTOH used for the transformation test was used to make standard solutions by serial dilution in hexane. A linear calibration curve was obtained for 4:2 FTOH from 0.298 to 2.385 μg (R^2>0.99). The hexane extracts from the transformation tests were also used for screening metabolites potentially 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 (30m × 0.25 mm × 0.25μm film thickness) and an electron capture detector (ECD). The oven temperature program was 45℃ for 2 min, ramping at 10 min^{-1} to 95 ℃ held for 5 min, and ramping at 30 ℃ min^{-1} to 250 ℃. The injector temperature was 150 ℃ and the detector temperature was 300 ℃.

3.2.7 LC/MS/MS analysis

LC/MS/MS analysis was used to identify and quantify metabolites produced during 6:2 and 8:2 FTOH biotransformation. Acetonitrile extracts obtained from the 120-mL bottle samples were analyzed as previously described [18] 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 with 50 μL/mLof sample and an internal standard solution containing 200 ng/mL of [1,2-^{13}C] PFHxA (M+2 PFHxA) and 5000 ng/mL 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, the [1,2-^{13}C] PFHxA internal standard was used for quantification of PFBA, PFPeA, PFHxA, PFHpA, 6:2 FTCA, 6:2 FTUCA, 5:3 acid, 4:3 acid, and 5:3 Uacid. The [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) were spiked into 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. The [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 the previous method [18], 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 Table A3.2 & A3.3 in Appendix.

3.3 Results and discussion

3.3.1 Biotransformation and biodefluorination of 4:2 FTOH by *P. oleovorans*

Biotransformation of 4:2 FTOH was observed by *P. oleovarans* within 48 h in the absence and presence of n-octane (Fig. 3.1 and Table A3.1 in Appendix) along with an increase amounts 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 A3.1). The addition of n-octane inhibited FTOH defluorination by more than two-fold (Table A3.1). This indicates that *P. oleovorans* prefers n-octane as its carbon source over 4:2 FTOH. Table A3.1 in the Appendix presents 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.

3.3.2 Biotransformation and biodefluorination of 4:2 FTOHs by *P. butanovora*

Similarly, *P. butanovora* showed ability to biotransform 4:2 FTOH in the absence and presence of 1-butanol (Fig. 3.1 and Table A3.1) 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. 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 correspond to a defluorination potential of 37.3% in the absence of 1-butanol and 32% in the presence of 1-butanol (Table A3.1).

3.3.3 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 A3.1 in Appendix, 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. A3.2 in Appendix). 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.

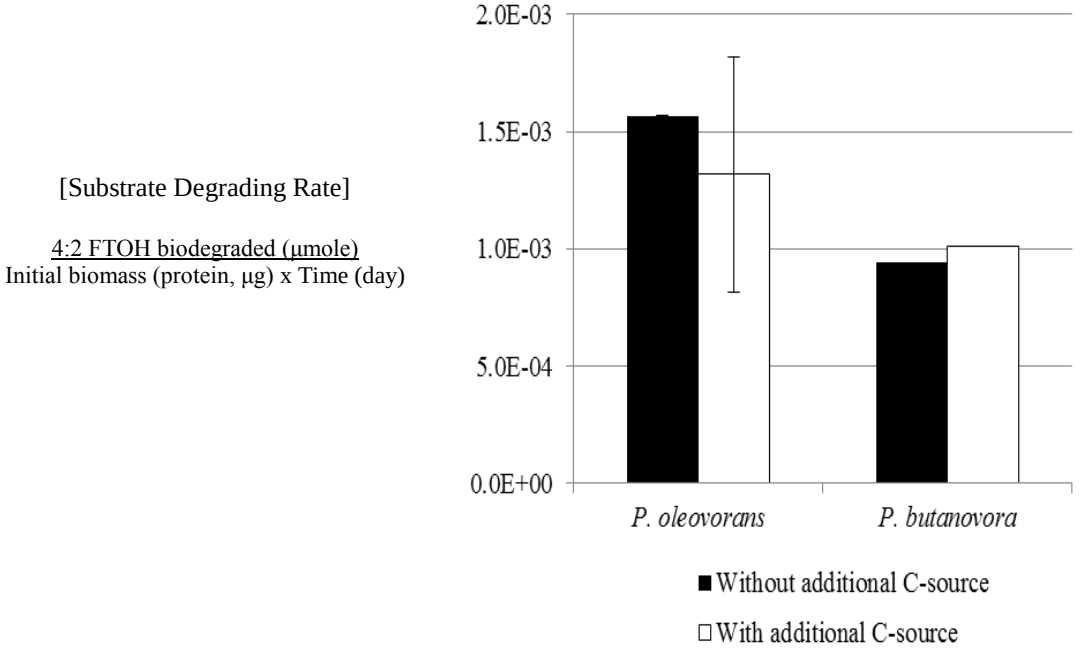


Fig. 3.1. 4:2 FTOH transformation by *P. oleovorans* and *P. butanovora*. *P. oleovorans* and *P. butanovora* were able to transform 4:2 FTOH in the absence and presence of additional carbon sources (n-octane for *P. oleovorans* and 1-butanol for *P. butanovora*). Initial mass of 4:2 FTOH applied is 1590 μg (6.0 μmol) in 5 mL bacterial cell suspension. The bars represent ranges of duplicate samples.

3.3.4 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 short-term transformation tests if the 6:2 and 8:2 FTOH biotransformation by *P. oleovorans* (Figs. A3.3 and A3.4 in Appendix). To identify these metabolites, long-term experiments were conducted and samples were analyzed by LC/MS/MS. Five to eight of 6:2 and 8:2 FTOH transformation products were identified and quantified by LC/MS/MS analysis (Fig. 3.2 for *P. oleovorans* and Fig. 3.3 for *P. butanovora*). Over the course of FTOH biotransformation (28 days), 10 % (v/v) of oxygen in gas phase remained which is a half of the initial oxygen (~ 20 % in gas phase) in live control bottles (Fig. 3.2a and Fig. 3.3a); 103~109 % of 6:2 FTOH initially applied at day 0 and 105~106 % of 8:2 FTOH initially applied at day 0 were still remained in sterile controls (Fig. 3.2b and Fig. 3.3b). These results demonstrate 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 yield 100% of initial FTOH applied. Approximately 40-54% of the initially applied FTOH loss 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 [17, 25]. Second, other potential metabolites formed during FTOH biotransformation were not quantified due to either lack of authentic analytical standards

or unknown identities. Nonetheless, the total mass balance of this study is comparable to that of previous studies on FTOHs with microbial consortia [5, 18,33].

Eight metabolites were detected and quantified during 6:2 and 8:2 FTOH biotransformation by *P. oleovorans*, respectively (Figs. 3.2c-1 and 3.2c-2). According to the quantity of each metabolite detected over time (Fig. 3.2c), 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, to n:2 FTUCA, to x:2 ketone, and then to x:2 sFTOH.

(1) n:2 FTCA(n:2 fluorotelomer saturated carboxylic acid)

$F(CF_2)_nCH_2COOH$, n= 6 or 8

(2) n:2 FTUCA (n:2 fluorotelomer unsaturated carboxylic acid)

$F(CF_2)_{n-1}CF=CHCOOH$, n= 6 or 8

(3) x:2 ketone

$F(CF_2)_xC(O)CH_3$;x= n-1, where n = 6 or 8

(4) x:2 sFTOH (x:2 secondary alcohol)

$F(CF_2)_xCH(OH)CH_3$; x= n-1, where n = 6 or 8

As the 6:2 and 8:2 FTOH concentrations decreased, these four metabolites continued to increase to reach their maximum on day 7 and remained little or no change

until day 14, and then decreased slightly on day 28. Also, on day 3, PFCAs such as PFBA (perfluorobutyric acid) in 6:2 FTOH samples and PFHxA (perfluorohexanoic acid) in 8:2 FTOH samples appeared in trace amounts and increased slightly on day 28, accounting for 0.44 mol% 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. In contrast, PFHxA or PFOA is the major stable transformation product from 6:2 FTOH or 8:2 FTOH biotransformation, accounting for 2.8 mol% 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 until 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 metabolites were detected during 6:2 and 8:2 FTOH biotransformation by *P. butanovora*, respectively (Figs. 3.3c-1 and 3.3c-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. 3.3c). For samples with 6:2 FTOH, the concentrations of 5:2 ketone increased throughout the experiment, whereas in samples with 8:2 FTOH, the

concentrations of 7:2 ketone increased fivefold by 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 until day 28. Also, PFHxA (during 6:2 FTOH transformation) and PFOA (during 8:2 FTOH transformation) were identified on day 1 and 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 (day 28), the observed major intermediates included: n:2 FTUCA, x:2 ketone, and x:2 sFTOH. PFHxA or PFOA is the major stable transformation product from 6:2 FTOH or 8:2 FTOH biotransformation, accounting for 2.9 mol% 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 these study conditions may lack enzymes capable of defluorinating n:2 FTUCA to x:3 Uacid and then of reducing the latter to x:3 acid.

3.3.5 Biotransformation pathways for 6:2 and 8:2 FTOHs

Based on the metabolites detected in this study, two possible pathways for 6:2 and 8:2 FTOH transformation were proposed (Fig. 3.4). Pathway I leads to the

formation of major PFCAs and pathway II leads to x:3 acids and small amounts of shorter-chain PFCAs. These two pathways share the same first three transformation steps before diverging into two different paths (Fig. 3.4). In the first step of the transformation, 6:2 and 8:2 FTOHs were transformed into n:2 FTAL (not analyzed; an assumed intermediate, [24]), which was quickly transformed to n:2 FTCA. Then n:2FTCA was further transformed to n:2 FTUCA, where the first defluorination reaction occurred. This defluorination reaction involved hydrogen fluoride (HF) elimination [18, 25].

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 [$F(CF_2)_{n-1}CF=CHCOOH$, n= 6 or 8] 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 [32]. This view is supported by recent finding that PFBA was formed from 5:3 Uacid in activated sludge through one-carbon removal pathways [35] and not formed from 5:2 sFTOH [17]. 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 [24] hypothesized that PFHxA was from 8:2 FTUCA or from 7:3 Uacid biotransformation. These results indicate 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 [25, 32]. As described earlier, other shorter-chain perfluorinated products could be formed, but were not identified due to the lack of authentic standards. 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.

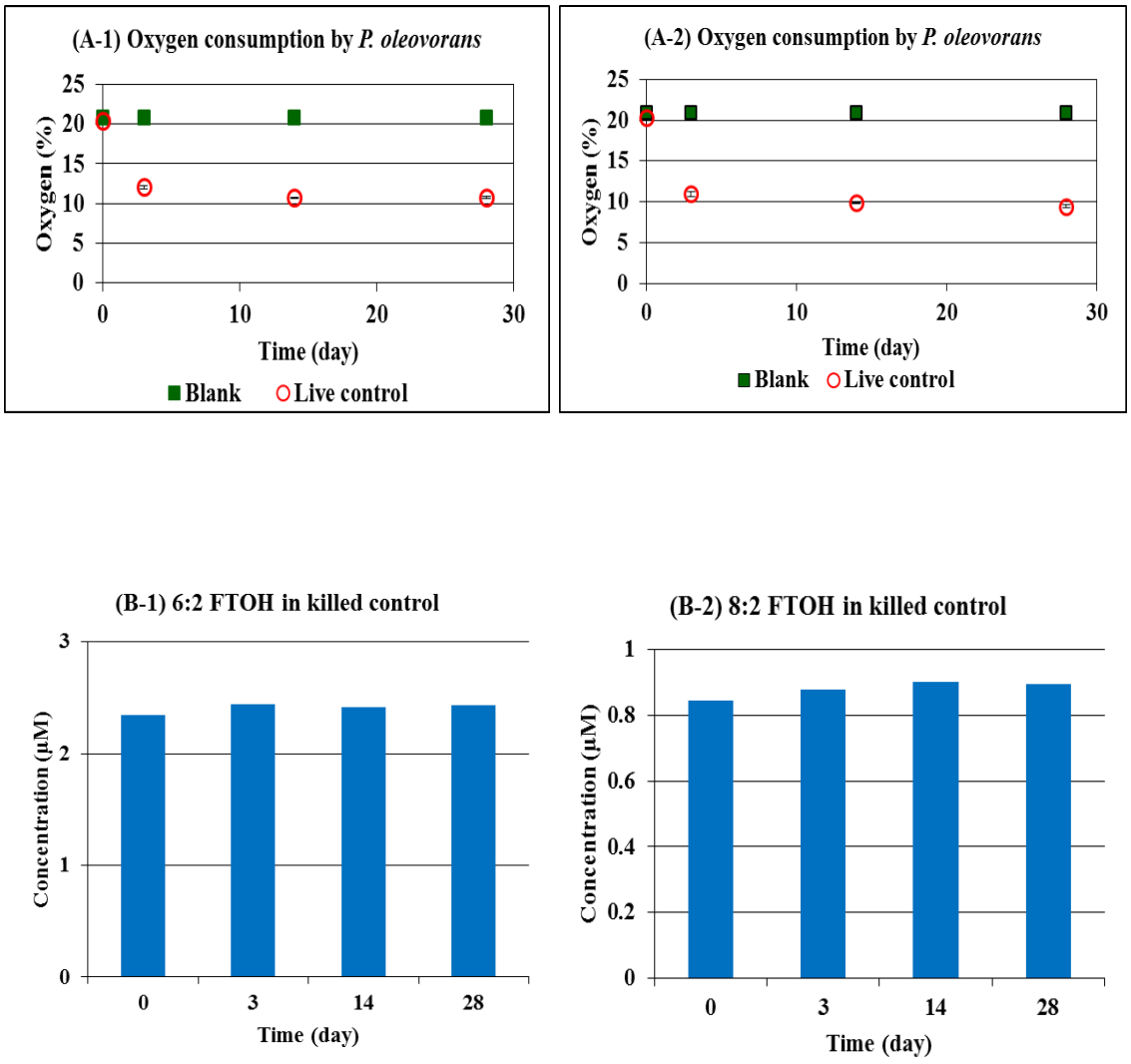


Fig. 3.2. Time trend of metabolite (transformation product) formation during 6:2 and 8:2 FTOH biotransformation by *P. oleovorans*. (a) Changes in oxygen concentrations during FTOH biotransformation; (b) changes of 6:2 and 8:2 FTOH concentration during 28 d in killed (sterile) controls; (c) changes of different metabolite concentrations during 28 d in live samples dosed with 6:2 FTOH or 8:2 FTOH. The transformation products in pathway I and II are grouped together in (c).

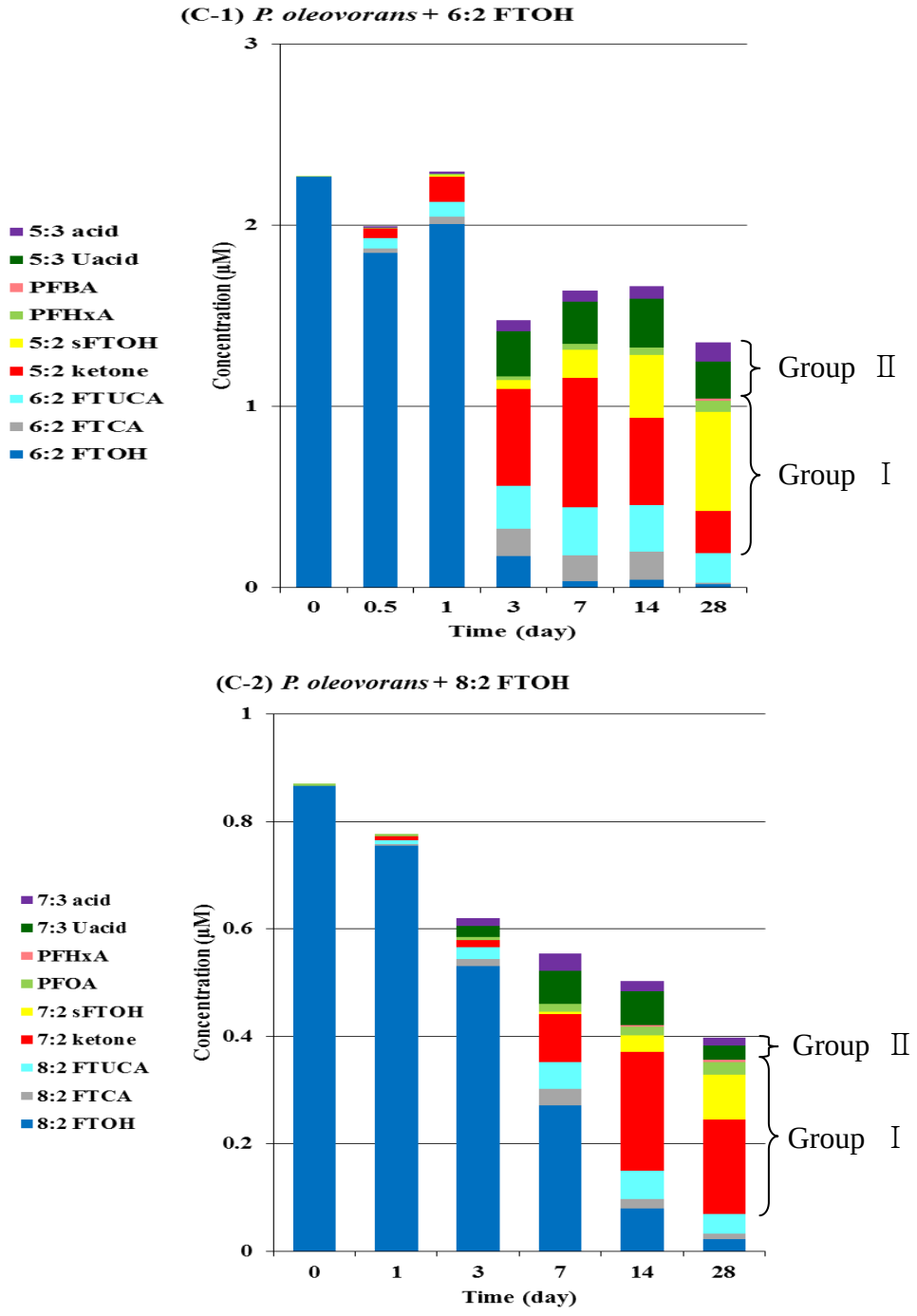


Fig. 3.2. Continued

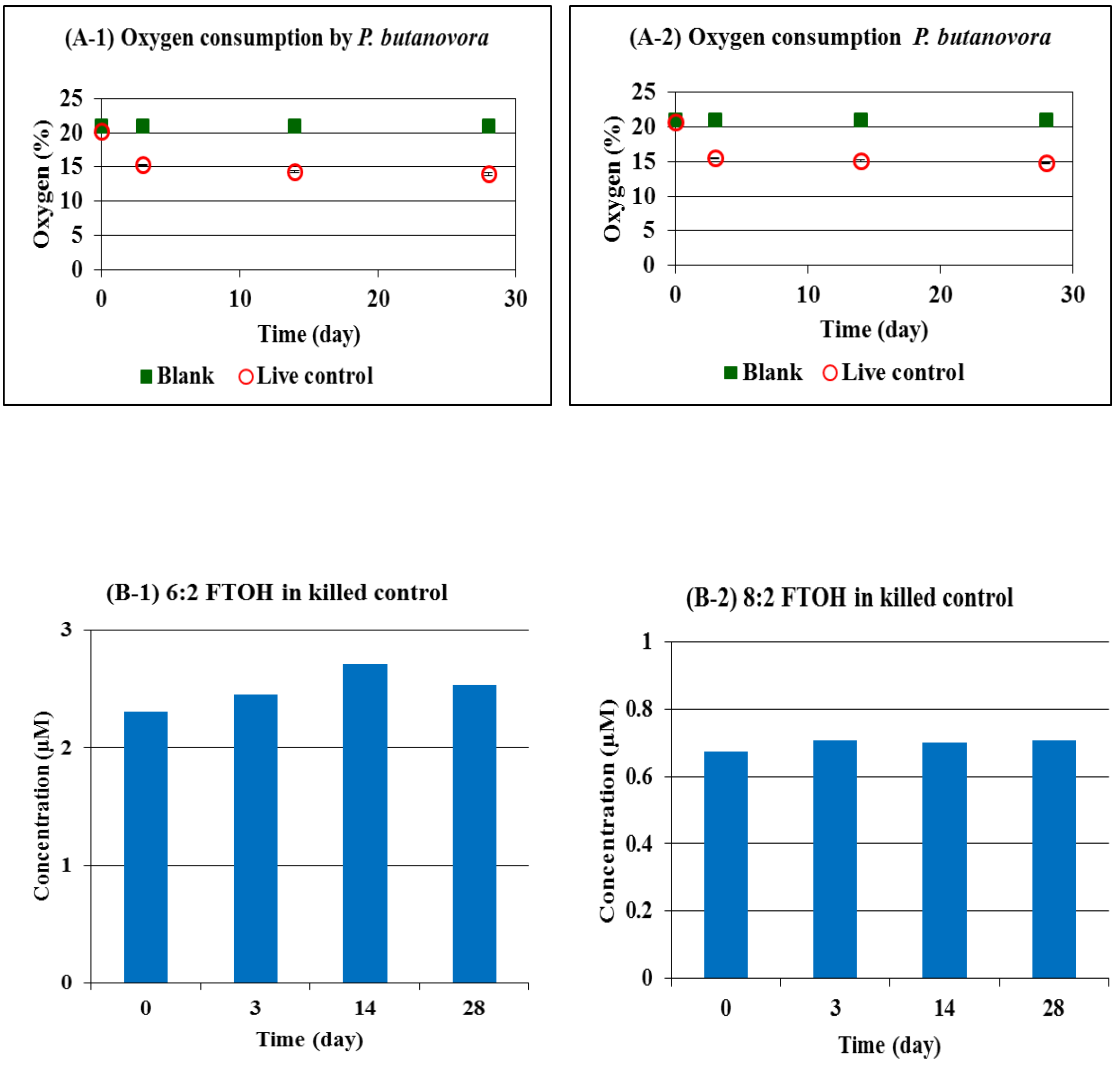


Fig. 3.3. Time trend of metabolite formation during 6:2 and 8:2 FTOH biotransformation by *P. butanovora*. (a) Changes in oxygen concentrations during FTOH biotransformation; (b) changes of 6:2 and 8:2 FTOH concentrations during 28 d in killed controls; (c) changes of different metabolite concentrations during 28 d in live samples dosed with 6:2 FTOH or 8:2 FTOH. The transformation products in pathway I and II are grouped together in (c).

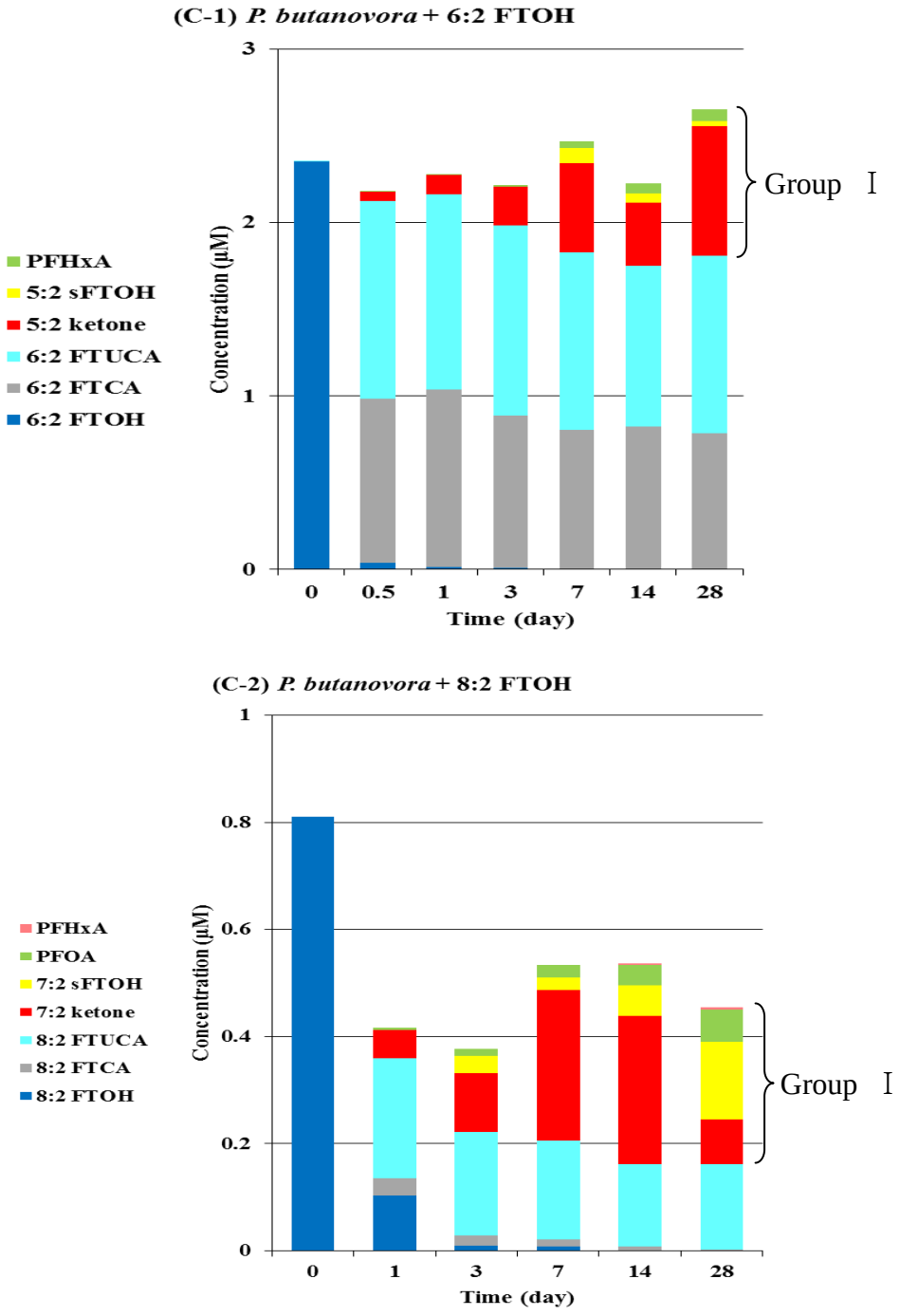


Fig. 3.3. Continued

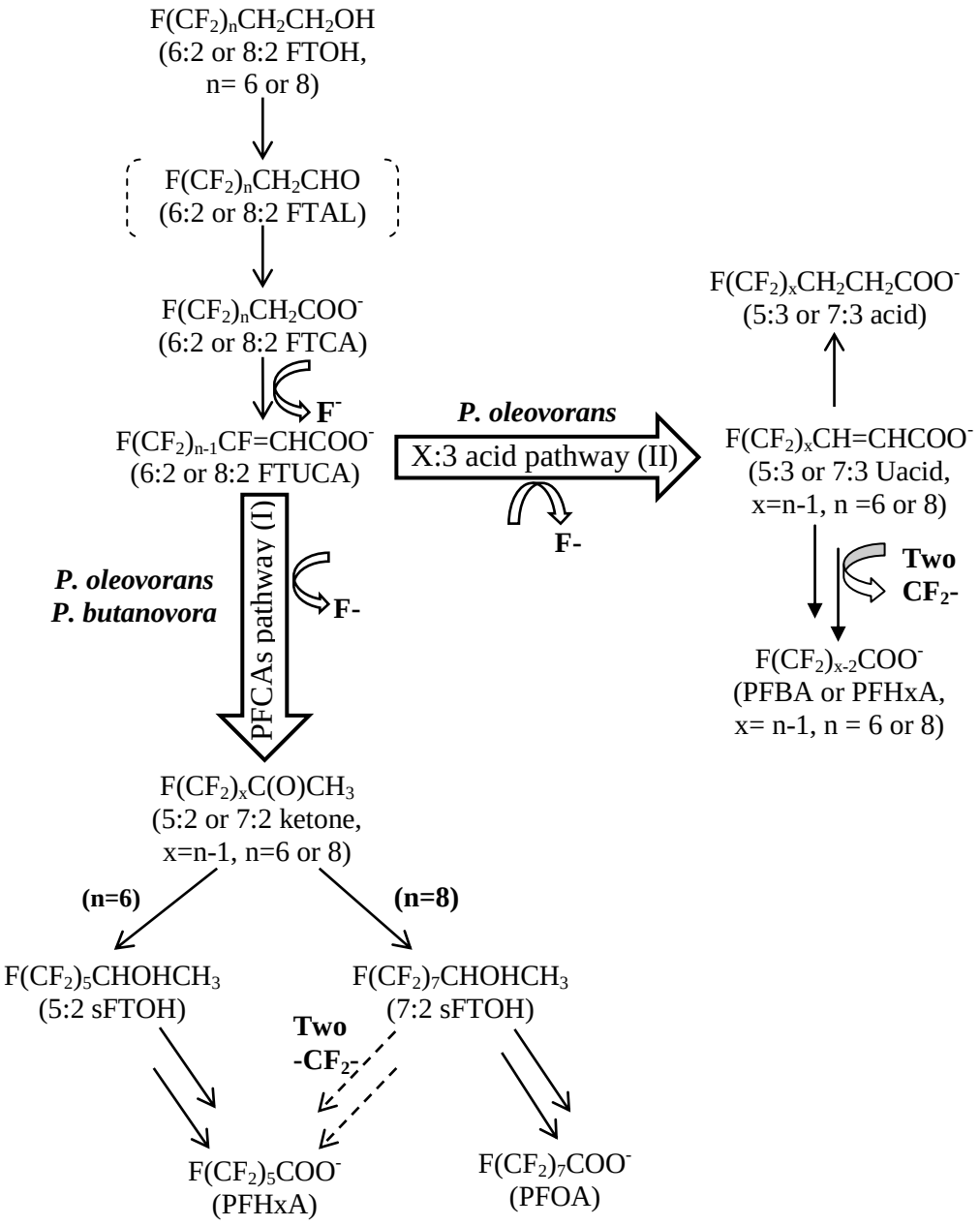


Fig. 3.4. 6:2 FTOH (n = 6) and 8:2 FTOH (n = 8) biotransformation pathways by *P. oleovorans* and *P. butanovora.* Pathway I leads to major PFCAs (perfluorinated carboxylic acids) whereas pathway II leads to 5:3 acid or 7:3 acid and small amounts of shorter-chain PFCAs. *P. oleovorans* utilized both 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.

4. AEROBIC BIOTRANSFORMATION OF 6:2 FLUOROTELOMER ALCOHOL BY ALKANE- AND FLUOROACETATE-DEGRADING BACTERIA

4.1 Introduction

The worldwide detection of perfluorocarboxylic acids [PFCAs, $F(CF_2)_mCOOH$, $2 < m < 8$][14, 119-124], particularly perfluorooctanoic acid (PFOA, m=7), has prompted intensive research efforts to better understand their sources [112], fate, and transport [125, 126] in the environment. Under high experimental dosage, PFOA is known to induce peroxisome proliferation in male mice [27], cause development toxicity in mouse neonates [28] and rats [29], and significantly increase mortality in pups [30]. Early studies reported that 8:2 fluorotelomer alcohol [8:2 FTOH, $F(CF_2)_8CH_2CH_2OH$] is a raw material for the manufacture of fluorinated surfactants and fluorinated polymers and attributed to be a precursor of PFOA [127]. Accordingly, in an effort to eliminate the global production of PFOA and its precursors [16], the manufacturers recently adopted 6:2 FTOH [$F(CF_2)_6CH_2CH_2OH$] as a raw material to manufacture FTOH-based products [17, 18]. Therefore, a better understanding of 6:2 FTOH biodegradation potential is essential for assessing its fate and transport in the environment.

Previous research efforts reported biodegradation pathways and novel metabolites (transformation products) of 8:2 FTOH in various environmental matrices

[24, 25, 31,32]. Recently, a few studies observed biodegradation of 6:2 FTOH in soils, activated sludge, and sediment [17, 18, 128], and by pure cultures of two alkane-degrading *Pseudomonas* strains [31]. Compared with 8:2 FTOH biodegradation observed in soils, activated sludge, and sediment, a faster 6:2 FTOH degradation and more extensive breakdown products were observed [17, 18, 128]. PFPeA [perfluoropentanoic acid, $F(CF_2)_4COOH$], PFHxA [perfluorohexanoic acid, $F(CF_2)_5COOH$], and 5:3 polyfluorinated acid [5:3 acid, $F(CF_2)_5CH_2CH_2COOH$] were major metabolites formed during 6:2 FTOH degradation in aerobic soils. A later 90-day study using activated sludge suggested that 5:3 acid can be further defluorinated and decarboxylated via novel "one-carbon removal pathways" to form metabolites with shorter carbon-chain length. For example, through these novel pathways, 5:3 acid can be degraded into 4:3 acid as a result of removing one CF_2 group from 5:3 acid. The reactions for one $–CF_2–$ removal can continue for 4:3 acid to form 3:3 acid. 5:3 acid was first converted to 5:3 Uacid [$F(CF_2)_5CHCHCOOH$], then α-OH 5:3 acid [$F(CF_2)_5CH_2CH(OH)COOH$] (as the dominant intermediate metabolite), 5:2 acid [$F(CF_2)_5CH_2COOH$], 5:2 Uacid [$F(CF_2)_4CFCHCOOH$], and then to several poly- and per-fluorinated carboxylates (PFPeA, PFBA [perfluorobutyric acid, $F(CF_2)_3COOH$], 4:3 acid [$F(CF_2)_4CH_2CH_2COOH$] and 3:3 acid [$F(CF_2)_3CH_2CH_2COOH$])[18, 25, 35]. These results suggest that more extensive defluorination of 6:2 FTOH is possible through the one-carbon removal pathways and other yet to be identified novel microbial defluorination mechanisms.

Our previous study [31] demonstrated that two pure alkane-degrading bacteria, *P. oleovorans* and *P. butanovora* defluorinated 6:2 FTOH via one or two degradation pathways, called PFCA pathway and x:3 acid pathway. In 28-day experiments, *P. oleovorans* used both pathways to degrade 6:2 FTOH to produce a wide range of metabolites: 5:2 ketone [dominant metabolite, $F(CF_2)_5C(O)CH_3$], 5:2 sFTOH [$F(CF_2)_5CH(OH)CH_3$], PFHxA, PFBA and 5:3 acid. In contrast, 6:2 FTOH degradation by *P. butanovora*, produced PFCAs but not 5:3 acid, suggesting that only PFCA pathway was involved in producing 5:2 ketone, 5:2 sFTOH, and PFHxA. The two pure strains did not generate short-chain odd-numbered PFCAs (e.g., PFPeA) or 4:3 acid as observed in 6:2 FTOH degradation by mixed bacterial cultures [17], implicating that other microorganisms might be able to degrade 6:2 FTOH via different pathways. As 5:3 acid degradation requires longer incubation time (up to 90 days) [35], it was unclear if 5:3 acid (the key the metabolite from 6:2 FTOH degradation viax:3 pathway) could be further transformed by *P. oleovorans* or other candidate microorganisms.

*Mycobacterium vaccae*JOB5 (Gram-positive alkane-degrading bacteria) and *P. fluorescens* DSM 8341 (fluoroacetate-degrading bacteria) were tested to determine the possibility of 6:2 FTOH degradation by these pure strains other than the *P. oleovorans* or *P. butanovora*. *M. vaccae* JOB5 utilizes a wide range of linear alkanes (C_1-C_{22}) as growth substrates [43]. *M. vaccae* JOB5 is known to degrade trichloroethylene[66], BTEX (benzene, toluene, ethylbenzene, and xylenes) [83], tertiary butyl alcohol (TBA) [82], or 1,4-dioxane [84] when grown on propane; chloroform [67] after grown on

butane; and methyl tertiary butyl ether (MTBE) [85] after grown on n-octane. *M. vaccae* JOB5 was selected to determine if C_1-C_{22} alkane-degrading bacterium can also degrade and defluorinate 6:2 FTOH to different extents. *P. fluorescens* DSM 8341 can grow on fluoroacetate [$F(CH_2)COO$-]as a sole carbon source and express fluoroacetate-dehalogenase to cleave off the C-F bond of fluoroacetate [59]. Among various dehalogenases studied [47, 88-95], fluoroacetate-dehalogenase is the only enzyme that specifically hydrolyzes the C-F bond [56]. Given that *P. fluorescens* DSM 8341can express fluoroacetate-dehalogenase and that the structure of fluoroacetate is similar to fluorinated acids (like 5:3 acid or short-chain PFCAs), experiments were conducted to determine if this fluoroacetate-degrading bacterium can degrade 6:2 FTOH.

This study also examined if the extent of 6:2 FTOH biodegradation can be improved by providing different co-substrates. It is hypothesized that the addition of different co-substrates can induce needed degradative enzymes and/or provide needed reducing power if the degradation of 6:2 FTOH is reducing energy dependent. Three different co-substrates, dicycloproplyketone (DCPK), formate, and lactate were used. DCPK, a gratuitous inducer of *alk* genes on OCT plasmid in *P. oleovorans* [74, 75], was provided to induce the expression of alkane monooxygenases. Formate was added as an external reducing energy source. Lactate, an odd-numbered carbon source, is known to induce enzymes in *P. butanovora* to produce pyruvate and odd-number fatty acids [71]. In *P. butanovora,* lactate serves as an effective exogenous reductant for the soluble butane monooygenase (sBMO) during co-metabolism [72, 80] and pyruvate, an

antioxidant, might protect the cells from damages caused by cooxidation [71]. Thus, lactate was selected and added to the degradation experiments containing *P. butanovora.* Additional experiments were conducted to study the degradation potential of 5:3 polyfluorinated acid (a key metabolite during 6:2 FTOH degradation) by *P. oleovorans* (alkane-degrading bacteria) and *P. fluorescens* DSM 8341 (fluoroacetate-degrading bacteria).

4.2 Materials and methods

4.2.1 Chemicals

6:2 FTOH (1H, 1H, 2H, 2H-perfluorooctanol, CAS # 647-42-7, 97 % pure) was obtained from Alfa Aesar (Lancashire, UK). 5:3 Acid ($C_5F_{11}CH_2CH_2CO_2H$, > 96% purity) was synthesized by DuPont Inc.,Wilmington, DE. The 5:3 acid contains approximately 4% of non-fluorinated impurities, which has no potential to produce transformation products that were observed during 5:3 acid degradation. 1-Butanol (99.4 % pure) was purchased from Fisher Scientific (Fair Lawn, NJ) and n-octane (97 % pure) from Acros Organics (Geel, Belgium). Sodium fluoroacetate (SFA) was purchased from Pfaltz & Bauer, Inc (Waterbury, CT) and sodium chloroacetate (SCA)

from Sigma-Aldrich (Louis, MO). DCPK was obtained from Oakwood Products, Inc (West Columbia, SC),sodium formate from MP Biomedicals, Inc (Solon, Ohio), sodium lactate from Alfa Aesar (Ward Hill, MA), and sodium citrate from Mallinckrodt Chemicals (Phillipsburg, NJ). Acetonitrile (> 99% pure) was purchased from Acros Organics (Geel, Belgium), ethanol (95% pure) from IBI scientific (Peosta, IA), and sodium hydroxide from EM Science (Gibbstown, NJ). 6:2 FTOH and 5:3 acid was 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 (PFBA, PFPeA, PFHxA, and PFHpA) 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 ketone was from TCI America (Portland, OR). The 4:3 acid ($C_4F_9CH_2CH_2CO_2H$; 96% purity with $C_4F_9CH_2CH_2COOCH_2CH_2CH_2C_4F_9$, as the major impurity) and α-OH 5:3 acid [$C_5F_{11}CH_2CH(OH)COOH$, 98%] were synthesized by DuPont. The [M+5] 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 standards have a purity of + 97 %.

4.2.2 Bacterial strains and growth conditions

P. oleovorans was purchased from America Type Culture Collection (ATCC number # 29347). *P. oleovorans* was grown in P1 medium [118] 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 for experimental use. *P. butanovora* was generously provided by Dr. Daniel J. Arp, Oregon State University. The cell suspension was prepared similarly to *P. oleovorans*. Briefly, *P. butanovora* was pregrown in ATCC1581 medium [68] with 2 mM 1-butanol to OD_{600}= 0.5-0.7 before harvested for experimental use. *M. vaccae*JOB5 was generously provided by Dr. Robert Steffan, Shaw Environmental, Inc.(Lawrenceville, New Jersy). Similarly, *M. vaccae* JOB5 was pregrown in NMS medium with 2 mM 1-butanol and 0.01 % yeast extract to OD_{600}= 0.5-0.7 before use. *P. fluorescens* (DSM 8341) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). *P. fluorescens*DSM 8341 was grown in Luria Broth (LB) medium to obtain high cell density (over OD_{600} = 1.0). The cells were centrifuged at 10,000 *g* for 5min, washed with 0.1 M phosphate buffer (pH = 7), and then re-grown in 457 mineral medium (Brunner) with 0.1% SFA and 0.05 % yeast extract to induce the expression of fluoroacetate dehalogenase. The expression of fluoroacetate-dehalogenase was confirmed by the detection of fluoride in the growth medium. The fluoride ion was

determined as described by Kim et al. [31] with anion-selective electrode (Orion 96-09BNWP).Then, the fluoroacetate-degrading cells were centrifuged (10,000 *g* for 5min), washed (0.1 M phosphate buffer, pH = 7), and the pellets were resuspended in NMS medium to reach OD_{600} = 0.6 ~ 0.7for experimental use.

4.2.3 6:2 FTOH biotransformation test

The experiments for 6:2 FTOH biotransformation by each of the selected strains were performed in a series of 120-mL glass bottles containing cell suspension (10 mL), 6:2 FTOH (4.125 g/L in 50 % ethanol), and one co-substrate (formate, lactate, or DCPK). Table 4.1lists the experimental labels and summary of each condition. Experiments using *P. oleovorans* were supplied with 0.05 % n-octane and formate (1 mM) or DCPK (1 mM). Experiments using *P. butanovora* were supplied with 2 mM 1-butanol with lactate (5 mM). Experiments using *M. vaccae* JOB5 were supplied with 2mM 1-butanol with or without formate (1 mM). For experiments using *P. fluorescens* DSM 8341, 1 g/L SCA and 0.05 % yeast extract with or without formate (1 mM) were used.

Sample preparations including live and sterile controls, incubation, extraction, and instrumental analysis were the same as described in Chapter 3.

4.2.4 5:3 Polyfluorinatedacid degradation test

The experiments for 5:3 acid biotransformation were conducted as described for the 6:2 FTOH degradation tests, except that 5:3 acid was used. Only *P. oleovorans* and *P. fluorescens* DSM 8341were used in these experiments. The chemicals added to each condition are summarized in Table 4.2.

At each sampling point (0, 2, 7, 14, 28, 56 and 90 days), two active samples and two sterile control bottles were sacrificed for a two-step solvent extraction. For the first extraction, twenty milliliters of acetonitrile was added to each bottle to extract the remaining 5:3 acid and potential metabolites in the 10-mL cell suspension. The suspensions were extracted for 3 days at 50 ℃ with ~150 rpm shaking. After extraction, the bottles were centrifuged (× 300 g, 20 min) and the extracts passed through 0.22 µm-pore sized filter paper, and the filtered extracts were stored at –50 ℃ for further analysis. The used filter paper and spent septum were added to each bottle containing the pellet, 5 mL of acetonitrile and 0.15 mL of 1M NaOH for the second extraction. The extraction was carried out at the same condition as the first extraction. After the second extraction, the extracts were filtered through a 0.45-µm pore size filter disk and preserved at –50 ℃ separately. Each extract from the first and second extraction was analyzed by LC/MS/MS individually.

4.2.5 Fluoride measurement and LC/MS/MS analysis

The degradation of sodium fluoroacetate by *P. fluorescens* DSM 8341 was determined by measuring free fluoride ion concentration in the liquid medium. The fluoride measurement and LC/MS/MS analysis were conducted as described in the previous work [31].

4.3 Results and discussion

4.3.1 Biodegradation of 6:2 FTOH by 1-butanol grown *M. vaccae* JOB5

A Gram positive alkane-degrading bacterium, *M. vaccae* JOB5, biodegraded 6:2 FTOH into at least nine metabolites in the presence of 1-butanol (and formate) (Fig. 4.1). Almost 100 mol% of 6:2 FTOH applied at day 0 was recovered in the sterile controls, suggesting the integrity of the experimental systems and the suitability of the extraction method. The average recovery was 76 mol% for active culture based on the sum of remaining 6:2 FTOH and quantifiable metabolites on day28 (Fig. 4.1). The mass balance is comparable to that observed in previous FTOH degradation studies with microbial consortia [5, 18,33]. The missing portions could be attributed to two possible

factors: (i) non-extractable or non-quantifiable bound-residue formed between fluorinated compounds and biological organic components, and (ii) unknown metabolites that were not quantifiable due to lack of authentic analytical standards [17, 25].

Except for the formation of PFPeA, *M. vaccae* JOB5 produced the same transformation products as those observed in the degradation of 6:2 FTOH by *P. oleovorans* [31]. It was not surprising because *M. vaccae* JOB5 can use a wide range of substrates (C_1-$C_{22}$of n-alkane) [43] and *alkB* gene purified from this strain was similar to that of *P. oleovorans* (currently renamed as *P. putida* GPo1) [82]. However, the degradation of 6:2 FTOH by *M. vaccae* JOB5 was slower than those by *P. oleovorans* and *P. butanovora* [31]. Most of 6:2 FTOH was still remaining on day 3 (75.5 mol% of 6:2 FTOH remaining) and was not completely transformed on day 28 (4.6 mol% of 6:2 FTOH remaining) (Fig. 4.1). Formation of 6:2 FTCA **[**$F(CF_2)_6CH_2COOH$] and 6:2 FTUCA **[**$F(CF_2)_5CF=CHCOOH$] started on day 3 and reached 13.1 mol% and 32.2 mol% on day 28, respectively. 5:2 ketone, 5:2 sFTOH and PFHxA emerged on day 7 and increased to 14.8 mol%, 4.9 mol% and 0.89 mol% on day 28, respectively. Interestingly, an odd-numbered PFCA (PFPeA) was detected at a trace amount (0.26 mol%) on day 28, which was not detected with *P. oleovorans*. Additionally, lower levels of 5:3 acid (3.4 mol%), 5:3 Uacid (1.1 mol%) and PFBA (0.37 mol%) accumulated from day 3until day 28.

Table 4.1. Characteristics of four pure strains and experimental conditions for 6:2 FTOH biotransformation.

Type of strain	Strain name	Enzyme induced based on the experimental conditions	Co-substrate added during degradation tests	Sample Label
Alkane-degrading bacteria	*P. oleovorans*	Alkane monooxygenase	n-octane	A1
			n-octane + formate	A2
			n-octane + DCPK	A3
	P. butanovora	Soluble butane monooxygenase	1-butanol	B1
			1-butnaol + lactate	B2
	M. vaccaeJOB5	Monooxygenases	1-butanol	C1
			1-butanol + formate	C2
Fluoroacetate-degrading bacteria	*P. fluorescens* DSM 8341	Fluoroacetate-dehalogenase	SCA+ YE	D1
			SCA + YE + formate	D2

Table 4.2. The experimental conditions for 5:3 acid biotransformation.

Strain	Target compound	Co-substrateadded during degradation tests
P. oleovorans	5:3 acid dissolved in ethanol	n-octane
		DCPK
		DCPK + (sodium) citrate*
P. fluorescens DSM 8341		SFA+ YE
		SFA + YE + formate

*DCPK induced alkane hydroxylase efficiently in the presence of citrate (non-alkane carbon source) [129]

The presence of formate (reducing energy) did not affect the degradation pattern, rate or the metabolites produced(Fig 4.1b). Unlike *P. butanovoa*, it was unclear if 1-butanol can effectively induce butane monooxygenases (BMO) in the strain JOB5. As the strain JOB5 was pregrown and also supplemented with 1-butanol for 6:2 FTOH degradation tests, it was possible that BMO and/or alkane monooxygenase were not expressed or present at low levels which would explain why the strain JOB5 degraded 6:2 FTOH at slower pace that *P. butanovora* [31]. Additional 6:2 FTOH experiments using butane-grown JOB5 with the addition of butane are needed.

4.3.2 Biodegradation of 6:2 FTOH by *P. fluorescens* DSM 8341

P. fluorescens DSM 8341, a fluoroacetate-degrading bacterium, transformed 6:2 FTOH into at least nine metabolites (Figs. 4.2a and 4.2b). Compared to the results of 6:2 FTOH degradation experiments using *P. oleovorans* and *P. butanovora* [31], degradation of 6:2 FTOH was slower by *P. fluorescens*DSM8341. The transformation products were the same as those observed for *P. oleovorans*, except for two odd-numbered PFCAs: PFPeA and PFHpA.

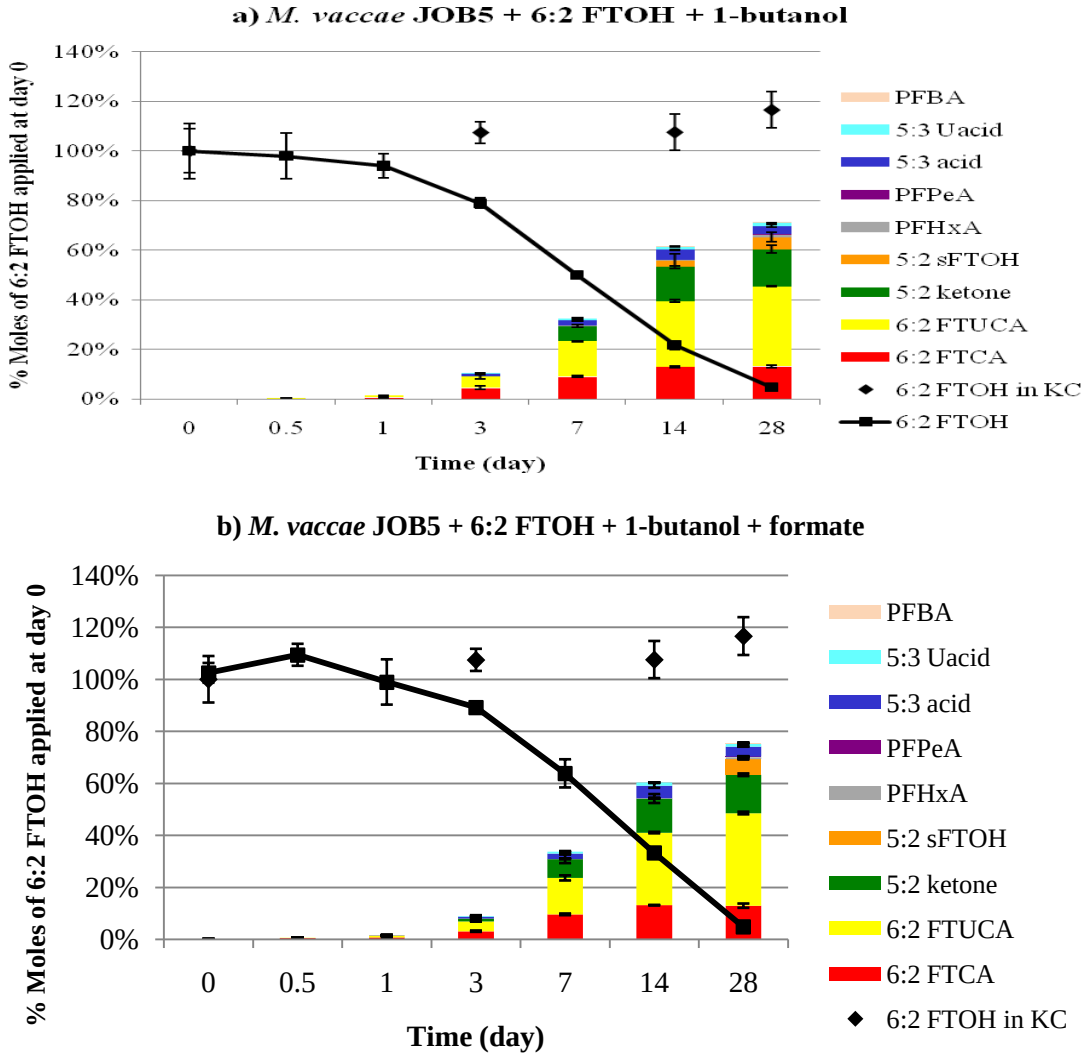


Fig. 4.1. Biotransformation of 6:2 FTOH by *M. vaccae* JOB5 in the presence of a) 1-butanol and b) 1-butanol and formate. The solid square shows the decrease of 6:2 FTOH in samples and the solid diamond represents 6:2 FTOH in sterile controls (killed controls, KC) taken intermittently on days 0, 3, 14 and 28. The portion of metabolites in samples over time is described with different colors in mole %of 6:2 FTOH applied at day 0.

A similar degradation pattern was observed in the presence and absence of formate (Fig. 4.2a and 4.2b). The degradation of 6:2 FTOH did not occur until day 1; however, the amount of 6:2 FTOH decreased dramatically to 38.6 mol% on day 3, to 2.1 mol% on day 7,then undetectable on day 28. As 6:2 FTOH quickly decreased after day 3, 6:2 FTCA (53.8 mol% to 67.4 mol%) and 6:2 FTUCA (9.7 mol% to 14.9 mol%) increased significantly from day 3 to day 14. On day 28, 6:2 FTCA decreased to 38.2 mol%, but 6:2 FTUCA was still increasing (15.6 mol%), suggesting that the defluorination step from 6:2 FTUCA to 5:3 U acid was inefficient compared with other bacterial strains studied so far. Meanwhile, other metabolites, 5:2 ketone, 5:2 sFTOH, PFHxA and PFPeA, also appeared on day 3. The amounts of these metabolites continued to increase after day 3:20.7 mol% for 5:2 ketone, 27.4 mol% for 5:2 sFTOH, 1.5 mol% for PFHxA, and 0.57 mol% for PFPeA on day 28. 5:3 Uacid and 5:3 acid accounted for 0.25 mol% and 4.7 mol% on day 28, respectively.

PFHpA (0.03 mol%) was detected for the first time in 6:2 FTOH degradation by bacterial strain. This trace level of PFHpA could come from impurities, rather than 6:2 FTOH aerobic biotransformation. If PFHpA indeed came from 6:2 FTOH, a possible mechanism for PFHpA formation is the α-oxidation of 6:2 FTCA. However, a previous study examining FTOH metabolic products in rat hepatocytes suggested that α-oxidation of 6:2 FTCA is not possible or unfavorable [130]. It was believed that HF elimination of 6:2 FTCA to produce 6:2 FTUCA is a more favorable reaction than α-oxidation of 6:2 FTCA, which requires α-hydroxylation of 6:2 FTCA [130]. In the

presence of formate, PFBA(a shorter-chainPFCA) was detected on day 28 (Fig. 4.2b), suggesting that formate facilitated *P. fluorescens* DSM 8341 to remove multiple –CF_2– groups from 6:2 FTOH. The production of PFBA could come from 5:3 acid defluorination via the one-carbon removal pathway, or from 5:2 sFTOH degradation through unknown degradation pathways. More research is needed to elucidate the degradation pathways leading to PFBA.

4.3.3 Effects of co-substrate on 6:2 FTOH biotransformation by *P. oleovorans*

Based on our previous work [31], these experiments were conducted to determine if addition of co-substrates can enhance 6:2 FTOH degradation by *P. oleovorans*. Experiments were conducted with the addition of formate (as additional substrate and/or reducing energy source) or DCPK (as an alkane hydroxylase inducer).

As displayed in Figure 4.3a, *P. oleovorans* was able to degrade > 90 % of the available 6:2 FTOH within 3 days (7.7 mol% of 6:2 FTOH applied on day 0remained) and produced at least 8 metabolites in the presence of n-octane. In the presence of formate or DCPK, six times faster degradation rate for 6:2 FTOH was observed (Figs. 4.3b and 3c). *P. oleovorans* transformed > 90% of 6:2 FTOH within 12 hours when formate or DCPK was present along with n-octane, 9.1 mol% and 5.1 mol% of 6:2 FTOH remained on day 0.5, respectively.

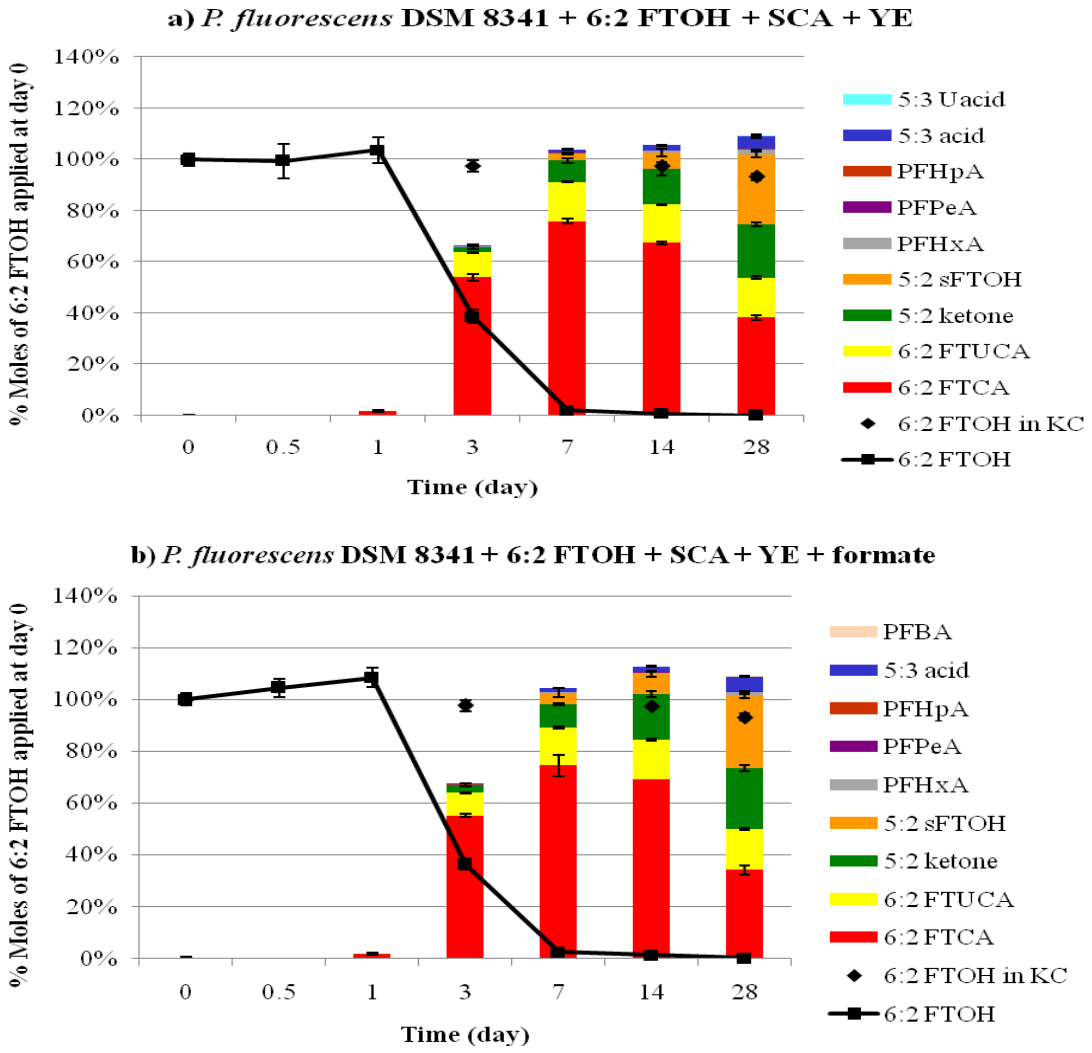


Fig. 4.2. Biotransformation of 6:2 FTOH by *P. fluorescens* DSM 8341 in the presence of a) SCA and YE, and b) SCA, YE and formate. The solid square shows the decrease of 6:2 FTOH in samples and the solid diamond represents 6:2 FTOH in sterile controls (killed control, KC) taken intermittently on days 0, 3, 14 and 28. The portion of metabolites in samples over time is described with different colors in mole% of 6:2 FTOH applied at day 0.

In the presence of DCPK or formate, as 6:2 FTOH transformed quickly, the amount of 6:2 FTCA increased to ~ 7 mol% on day 0.5 and decreased to ~ 1 mol% on 28 days. About 27 mol% of 6:2 FTUCA was produced on day 0.5, accumulated to ~ 29 mol% and decreased to ~ 26 mol% on day 28 (Fig. 4.3b and 3c). The maximum observed quantity of 6:2 FTUCA (~ 29 mol%) was three times higher than that observed in sample with n-octane only (Fig. 4.3a). Regardless of the presence of DCPK or formate, the quantities of metabolites (35-40%) produced via the PFCA pathway (i.e. summation of 5:2 sFTOH, 5:2 ketone and PFHxA) were similar to those observed in the presence of n-octane only (40%) (Fig 4.3a). These results suggest that rapid transformation of 6:2 FTOH into 6:2 FTUCA is possible by adding DCPK to enhance the expression of enzymes involved in the first steps of 6:2 FTOH degradation or by adding formate to supplement needed reducing energy source to defluorinate 6:2 FTCA more efficiently .

Two odd-numbered PFCAs (about 0.2 mol% of PFPeA and 0.02 ~ 0.07 mol% of PFHpA) were observed when DCPK or formate was provided. In addition, 5:3 acid (1.7 ~ 2.1 mol%) and 5:3 Uacid (6.1 ~ 7.2 mol%) were produced in samples with DCPK or formate, while 4.7 mol% of 5:3 acid and 9.1 mol% of 5:3 Uacid were formed in sample with n-octane only). The results support our previous finding that *P. oleovorans* has both pathway I and II (or PFCA pathway and x:3 pathway, respectively). This finding also suggests the presence of alternative pathways for producing odd-numbered PFCAs.

4.3.4 Effects of co-substrate on 6:2 FTOH biotransformation by *P. butanovora*

Previously, *P. butanovora* transformed 6:2 FTOH into at least 5 metabolites in the presence of 1-butanol, utilizing the pathway I (PFCA pathway) [31]. *P. butanovora* could transform > 95 % of 6:2 FTOH (1.7 mol% of 6:2 FTOH applied at day 0remained) within 12 hours and 6:2 FTOH was not detected after 7 days (Fig. 4.4a) [31]. Additional co-substrate lactate was added to examine if it provides needed reducing energy and the effects of odd-numbered carbon source on 6:2 FTOH biodegradation. Lactate was selected because it induces enzymes to produce odd-numbered fatty acids and serves as exogenous reductants for sBMO. Pyruvate, the first product of lactate degradation, is an antioxidant that can protect cells from oxidative damages [71, 72,80]

Unexpectedly, in the presence of lactate, the transformation of 6:2 FTOH was slower than that observed in samples with only 1-butanol. Approximately 80 mol% of 6:2 FTOH remained on day 0.5, followed by 0.77 mol% remained on day 7, and was undetectable on days 14 ~ 28 (Fig. 4.4b). This seven times slower transformation is attributed to *P. butanovora* preference for lactate over 6:2 FTOH. However, transformation of 6:2 FTOH in the presence of lactate was much more diverse in terms of the number of metabolites detected.

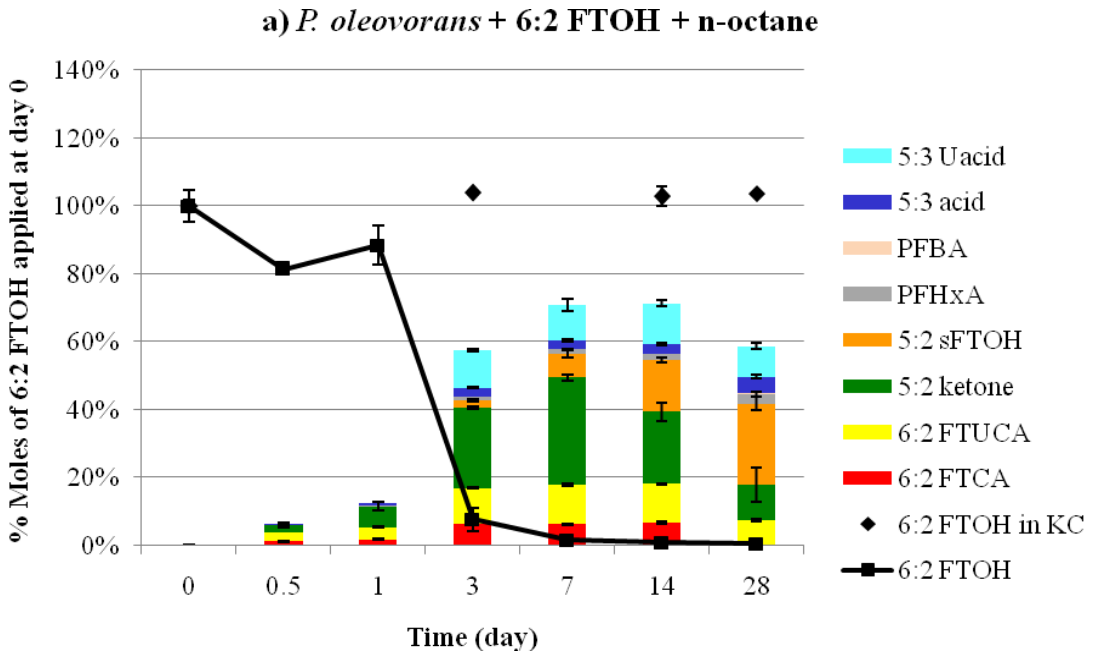
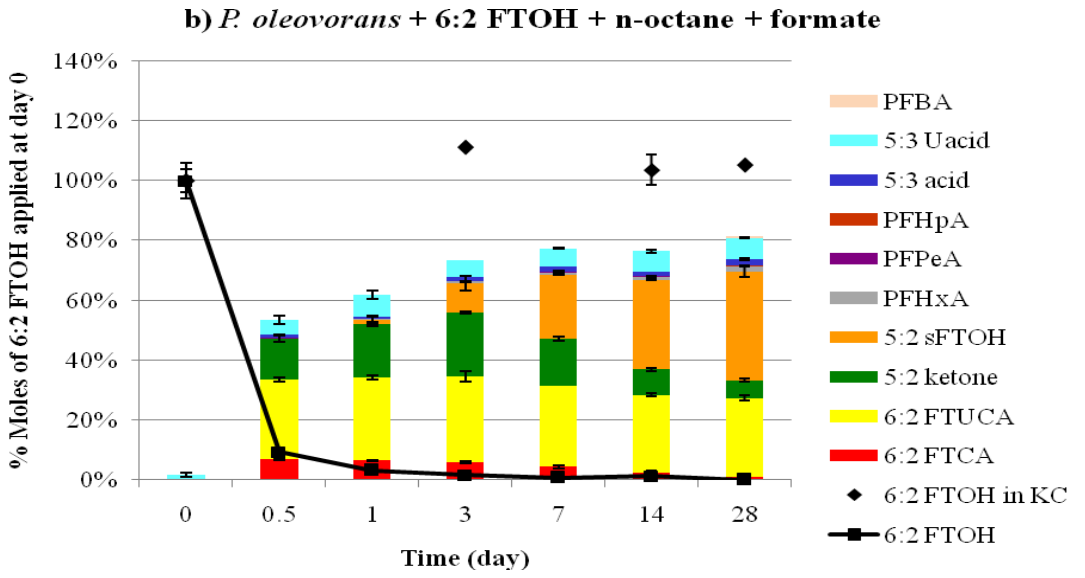


Fig. 4.3. Biotransformation of 6:2 FTOH by *P. oleovorans* in the presence of a) n-octane (revised from [31]), b) n-octane and formate, and c) n-octane and DCPK. The solid square shows the decrease of 6:2 FTOH in samples and the solid diamond represents 6:2 FTOH in sterile controls (killed control, KC) taken intermittently on day 0, 3, 14 and 28. The portion of metabolites in samples over time is described with different colors in mol% of 6:2 FTOH applied at day 0.



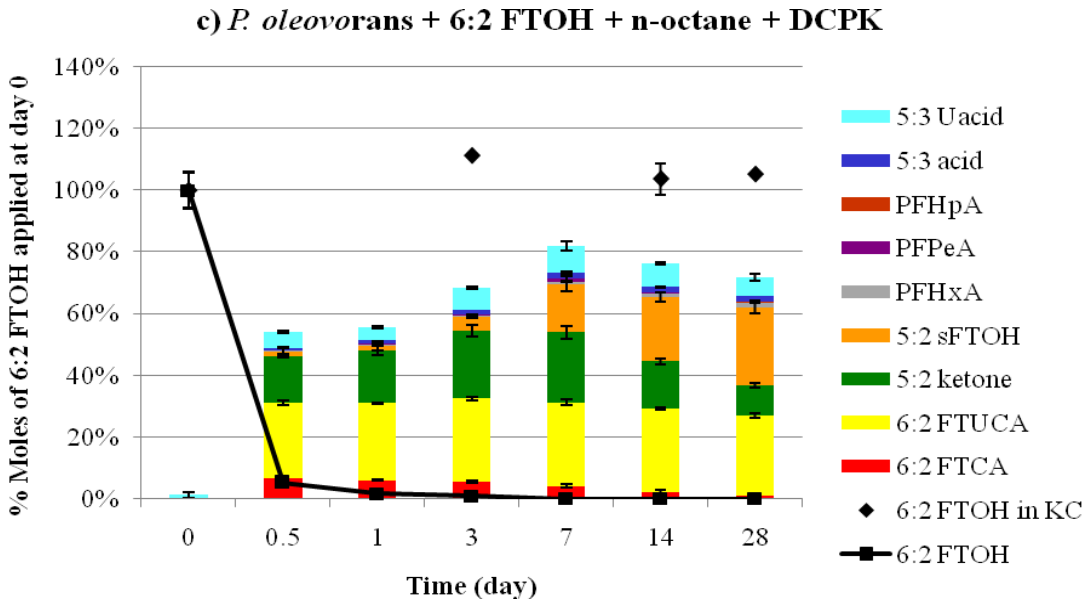


Fig. 4.3. Continued

The most interesting finding of the experiments using *P. butanovora* and lactate was the detection of metabolites: PFPeA, PFHpA, 5:3 acid and 5:3 Uacid, which were not observed in previous study with *P. butanovora* with 1-butanoladded [31]. As lactate is an odd-numbered compound with a carboxylic acid at the terminal, *P. butanovora* expressed more diverse enzymes to utilize lactate than for 1-butanol. Both 5:3 acid and 5:3 Uacid (a product from 6:2 FTUCA defluorination or 5:3 acid degradation via one-carbon removal pathway) also have molecular structures with a carboxylic acid at the terminal. *P. butanovora* produced more 5:3 Uacid (38 mol% of 6:2 FTOH applied at day 0) than *P. oleovorans* did (9.1 mol%) [35]. PFPeA accounted for 0.24 mol% on day 28; a trace amount of PFHpA (0.0004 mol%) was detected on day 0.5 but rapidly disappeared. These results strongly suggest that the extent of 6:2 FTOH biodegradation by *P. butanovora* is substrate-dependent and an odd-numbered carbon substrate like lactate can promote 6:2 FTUCA defluorination to 5:3 Uacid and decarboxylation to PFPeA, respectively.

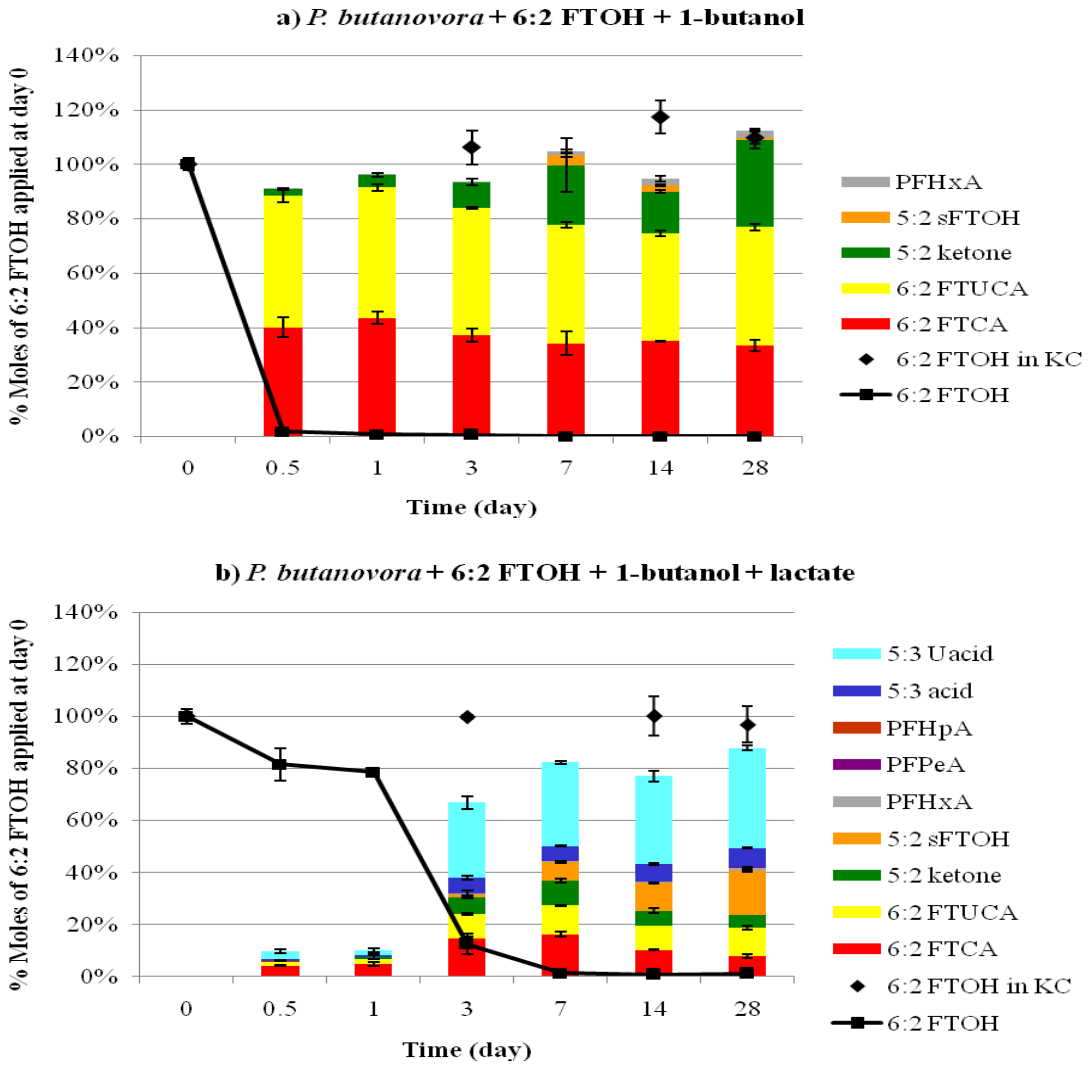


Fig. 4.4. Biotransformation of 6:2 FTOH by *P. butanovora* in the presence of a) 1-butanol (revised from [31]) and b) 1-butanol and lactate. The solid square shows the decrease of 6:2 FTOH in samples and the solid diamond represents 6:2 FTOH in sterile controls (killed control, KC) taken intermittently on days 0, 3, 14 and 28. The portion of metabolites in samples over time is described with different colors in mol% of 6:2 FTOH applied at day 0.

4.3.5 Degradation of 5:3 acid, a selected transformation product

Another research effort was placed on determining if 5:3 acid can be further degraded by alkane- and fluoroacetate-degrading bacteria. Since 5:3 acid is the major and the end product in x:3 degradation pathway (pathway II) during 6:2 FTOH biodegradation, it is expected that the production of PFCAs via PFCA pathway (pathway I) can be minimized if x:3 acid degradation pathway can be promoted. Thus, two strains, *P. oleovorans* and *P. fluorescens* DSM 8341 were used and the 5:3 acid degradation experiments were conducted in the presence of different co-substrates.

P. oleovorans was unable to show any significant degradation of 5:3 acid in the presence or absence of DCPK (data not shown). However, in the presence of sodium fluoroacetate and yeast extract, *P. fluorescens* DSM 8341 could slightly transform 5:3 acid into at least 6 metabolites (Fig. 4.5).

During the first 14 days, 5:3 acid was converted to 5:3Uacid (up to 3.0 mol%). The reactions between 5:3 acid and 5:3 Uacid were believed to be reversible [17, 35]. On day 28, 5:2 Uacid was detected (0.31 mol%) and then decreased over time. Production of 4:3 acid was first observed on day 14 (0.37 mol%), increased to 1.54 mol% on day 56, and then declined on day 90.From day 56 to day 90, PFPeA was detected and its amount increased to 0.36 mol%. The molar yields of 4:3 acid and PFPeA are 9 and

15 time lower, respectively, than that in activated sludge [35], and 4:3 acid molar yield is comparable to that in soil dosed with 5:3 acid [18].

A similar degradation pattern and metabolites were observed when formate was added (data not shown), suggesting that formate did not enhance 5:3 acid transformation by *P. fluorescens* DSM 8341.

Based on the metabolites detected, these results suggest that *P. fluorescens* DSM 8341could transform 5:3 acid via one-carbon removal pathways [35], although to a less extent compared with that in activated sludge. The ability of *P. fluorescens* DSM 8341 to degrade 5:3 acid, if enhanced, could lead to a more complete defluorination of6:2 FTOH by promoting synergy among various FTOH-degrading microorganisms in a mixed culture with proper co-substrates to enhance defluorination and decarboxylation (mineralization) of 6:2 FTOH.

4.3.6 Modification of 6:2 FTOH biotransformation pathways

Based on the results from this study, Figure 4.6 shows a revised biotransformation pathway for 6:2 FTOH [31]. The steps shared by all four strains under tested conditions are marked in red arrows: 6:2 FTOH is first converted into 6:2 FTAL (not analyzed; an assumed intermediate [24]), 6:2 FTCA, and then to 6:2 FTUCA.

The first defluorination occurs via hydrogen fluoride (HF) elimination when 6:2 FTCA is converted into 6:2 FTUCA [18, 25]. PFHpA was detected in several samples containing *P. oleovorans* with n-octane and DCPK (or formate), *P. fluorescens* DSM 8341 with SCA and YE (and formate) and *P. butanovora* with 1-butanol and lactate. However, whether PFHpA is originated from 6:2 FTCA is not clear since α-oxidation of 6:2 FTCA is not favorable [130].

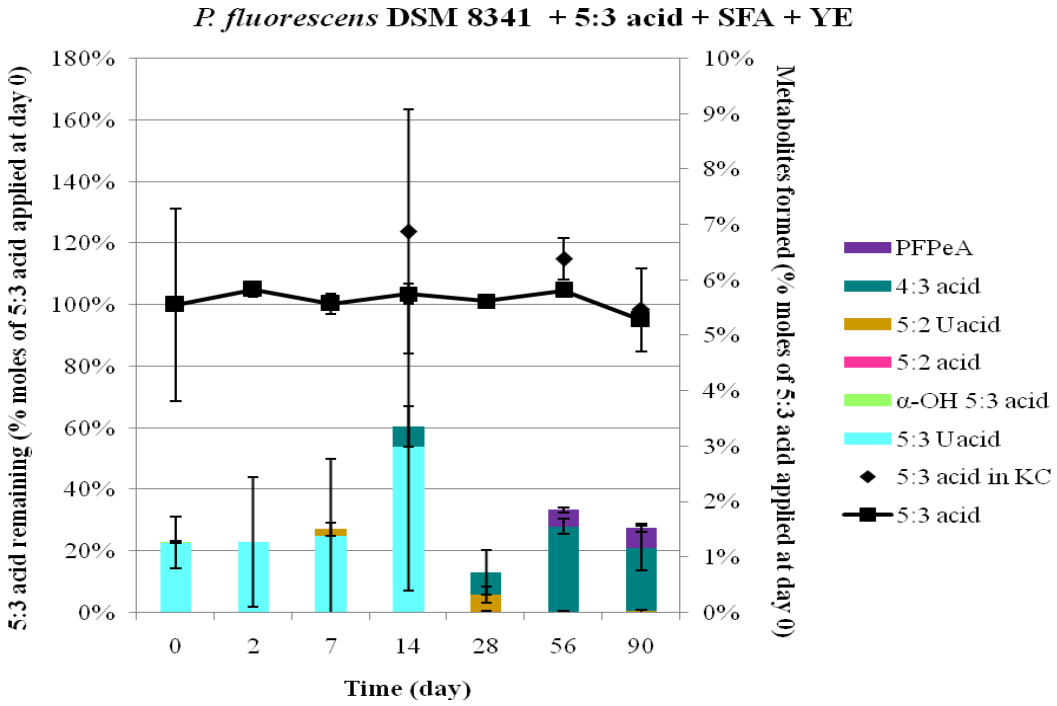


Fig. 4.5. 5:3 acid biotransformation by *P. fluorescens* DSM 8341 in the presence of sodium fluoroacetate and yeast extract. The solid square shows the decrease of 5:3 acid in the sample and the solid diamond represents 5:3 acid in sterile control (killed control, KC) taken intermittently on day 0, 14, 56 and 90. The portion of metabolites in the sample over time is described in bar charts with different colors in mol% of 5:3 acid applied at day 0.

In the pathway I (PFCA pathway), 6:2 FTUCA is converted to 5:2 ketone in which multiple enzymatic steps might be involved; the mechanisms for 8:2 FTUCA to 7:2 ketone were proposed in previous study [131]. 5:2 ketone is then converted to 5:2 sFTOH, and finally to short PFCAs. In the pathway II (x:3 acid pathway), 6:2 FTUCA is transformed into 5:3U acid, 5:3 acid, and PFPeA.

Through pathway I (PFCA pathway), 5:2 sFTOH can be converted into three possible products: PFHxA, PFPeA, and PFBA. The conversion of 5:2 sFTOH to PFHxA was proposed earlier in previous studies [17, 31] and confirmed again in this study. The production of PFHxA was only observed in aerobic soil when incubated with 5:2 sFTOH, not with 5:3 acid [17]. This view was validated by the results of our previous study - PFHxA was detected while no 5:3 acid was produced during the 6:2 FTOH degradation by *P. butanovora* with 1-butanol [31]. In this study, PFHxA was also not detected during the transformation of 5:3 acid by *P. fluorescens* DSM 8341.

The possibility of production of PFPeA from 5:2 sFTOH was proposed based on the following findings: (i) PFPeA was detected in all samples of strains employing both pathway I and II (except *P. oleovorans* with n-octane only) in this study and (ii) PFPeA was produced when 5:2 sFTOH was incubated in aerobic soils [17]. However, it is still not clear if PFPeA can be produced in both pathways because 5:3 acid was degraded into PFPeA via one-carbon removal pathways in Wang et al. [35] and by *P. fluorescens* DSM 8341 in this study.

The third possibility is that PFBA was converted from 5:2sFTOH via pathway I, based on the following observations: (i)*P. oleovorans*, *M vaccae* JOB5 and *P. fluorescens* DSM 8341 utilized both pathway I and II, and produced PFBA, and (ii) *P. oleovorans* and *P. fluorescens* DSM 8341 did not produce PFBA during 5:3 acid transformation (i.e. in pathway II). *P. butanovora* did not produce PFBA either when taking pathway II only (1-butanol only) or when utilizing both pathways (in the presence of 1-butanol and lactate), indicating *P. butanovora* might not have ability to form PFBA. However, it is hard to conclude because *M. vaccae* JOB5 was not tested for 5:3 acid transformation and Wang et al. [35] monitored the formation of PFBA during 5:3 acid degradation in soils. Transformation of longer-chain PFCAs to shorter ones (for example, from PFHxA to PFPeA or PFBA) was not observed with the strains tested in any condition (data not shown).Experiments using 5:2 sFTOH and these strains are needed to confirm this degradation pathway to PFBA.

Since we did not observe any degradation of PFCAs to a shorter-chain PFCA or other transformation products with the strains used in this study under any conditions (data not shown), it was not considered that shorter PFCAs were originated from the longer PFCAs.

Through x:3 acid pathway (pathway II), 6:2 FTUCA can be defluorinated to 5:3 Uacid first, and then to 5:3 acid via a reduction step facilitated by the reducing power, NAD(P)H [17]. 5:3 acid can be further degraded via one-carbon removal pathways if the reversible dehydrogenation of 5:3 acid to 5:3 Uacid is not rate-limiting. This view was

supported by the experimental results of this study using *P. oleovorans*, *M. vaccae* JOB5 and *P. fluorescens* DSM 8341 under all the tested conditions, and *P. butanovora* with lactate. If 5:3 acid was degraded similar to a fatty acid via β-oxidation, the expected metabolite would be PFHxA [35]. However, no PFHxA was detected during the 5:3 acid transformation with *P. fluorescens* DSM 8341. Instead, 5:3 Uacid, 5:2 Uacid, 4:3 acid and PFPeA were detected, strongly supporting the occurrence of one-carbon removal pathways in *P. fluorescens* DSM 8341. To employ β-oxidation for 5:3 acid transformation, 5:3 acid has to be activated by an acyl-Coenzyme A synthetase for further multiple defluorination and such activation was not observed in activated sludge [35]. Similarly, 5:3 acid also may not be activated by acyl-Coenzyme A synthetase in *P. fluorescens* DSM 8341, which instead utilized the one-carbon removal pathways to degrade 5:3 acid.

In Figure 4.6, PFPeA was proposed to be converted from 5:2 sFTOH in pathway I (major route) [17] and from 4:2 sFTOH in pathway II (minor route via one-carbon removal pathways) [35]. To clarify the origin of PFPeA and other PFCAs, it can be hypothesized that certain enzymes can deploy the same degradation pathways by keep going through a cycle from polyfluorinated acid, polyfluorinated unsaturated acid, ketone, secondary alcohol, and then PFCAs with decreased carbon-chain lengths, respectively (Fig. 4.7).

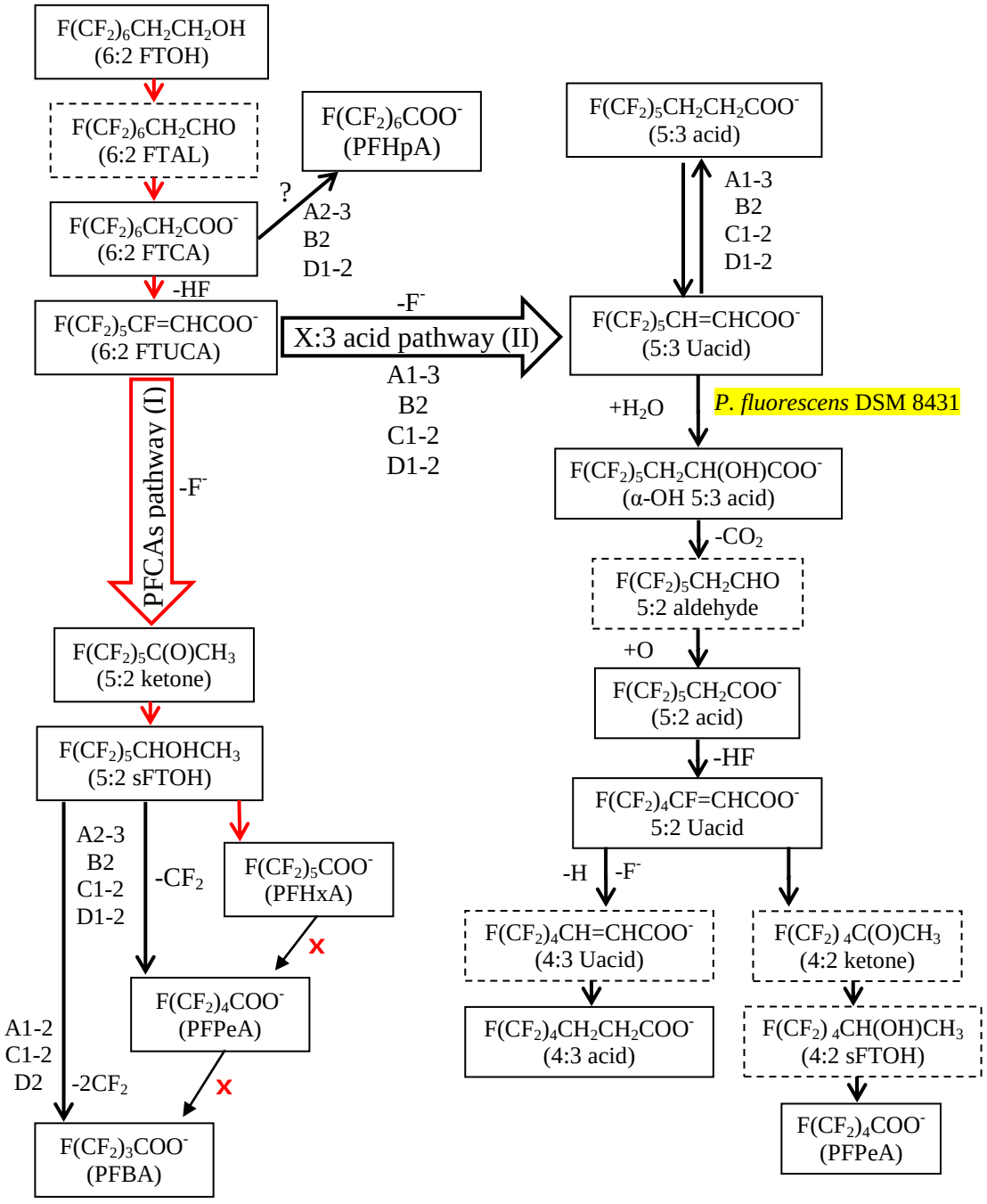


Fig. 4.6. Proposed 6:2 FTOH degradation pathways by alkane-degrading bacteria and fluoroacetate-degrading bacteria. The pathways taken commonly by all the bacteria are presented in red arrows. Pathways taken by different strains are marked with the labels (A1-D2 from Table 4.1). Compounds in boxes with dotted line are assumed metabolites, not analyzed.

In the proposed metabolic/degradation cycle, 6:2 FTOH is converted into 5:2 sFTOH as described above. It was proposed 5:2 sFTOH can produce two types of diols depending on the location of oxygen inserted: 5:2 diol Ⅰ (oxygen inserted at the terminal) and 5:2 diol Ⅱ (oxygen inserted in the middle of the compound) in cycle 1. From 5:2 diol Ⅰ, 5:1 aldehyde is produced with losing two hydrogens which is oxidized to an even-numbered PFCA, PFHxA. 5:2 diol Ⅱ is converted to 4:1 aldehyde with defluorination and loss of two hydrogens and 4:1 aldehyde is further transformed to an odd-numbered PFCA, PFPeA. This supports the formation of PFHxA and PFPeA via PFCA pathway. Assuming the same mechanism can be applied to 6:2 FTOH, it was proposed that PFHpA could be converted from 6:2 FTOH by producing a diol and then 6:1 aldehyde, which could be converted to PFHpA.

6:2 FTUCA can be converted to 5:3 Uacid, α-OH 5:3 acid, and then 5:2 aldehyde via the one-carbon removal pathways. 5:2 aldehyde is oxidized to 5:2 acid which starts a new cycle and 4:2 sFTOH is transformed to PFPeA and even-numbered PFBA through the formation of corresponding diols and aldehydes. This explains that PFPeA may also be produced via x:3 acid pathway. We did not observe PFBA during 5:3 acid degradation by *P. oleovorans or P. fluorescens* DSM 8341; however, Wang et al. [35] observed the formation of PFBA during 5:3 acid degradation by activated sludge. From 4:2 acid, formed from the “Cycle 2” of Fig. 4.7, the third cycle starts to produce PFBA and possibly a shorter-chained PFCA as the same manner in “Cycle 2”. We did

not observe any degradation of PFCAs to shorter PFCAs or other products and thus PFCAs are the terminal metabolites during microbial degradation of 6:2 FTOH and other polyfluorinated chemicals such as 5:3 acid. More studies are warranted, especially the degradation experiments using selected metabolites such as x:2 acid or x:2 sFTOH which might provide additional information to confirm the hypothesis of diol formation during 6:2 FTOH biodegradation that could lead to shorter-chain and odd numbered PFCAs. A set of kinetic models was developed to describe 6:2 FTOH degradation and formation of its metabolites. The detail of developed model was included in Table A4.1 in Appendix. Results of 6:2 FTOH degradation was used to validate these models (Figure A4.1 in Appendix).

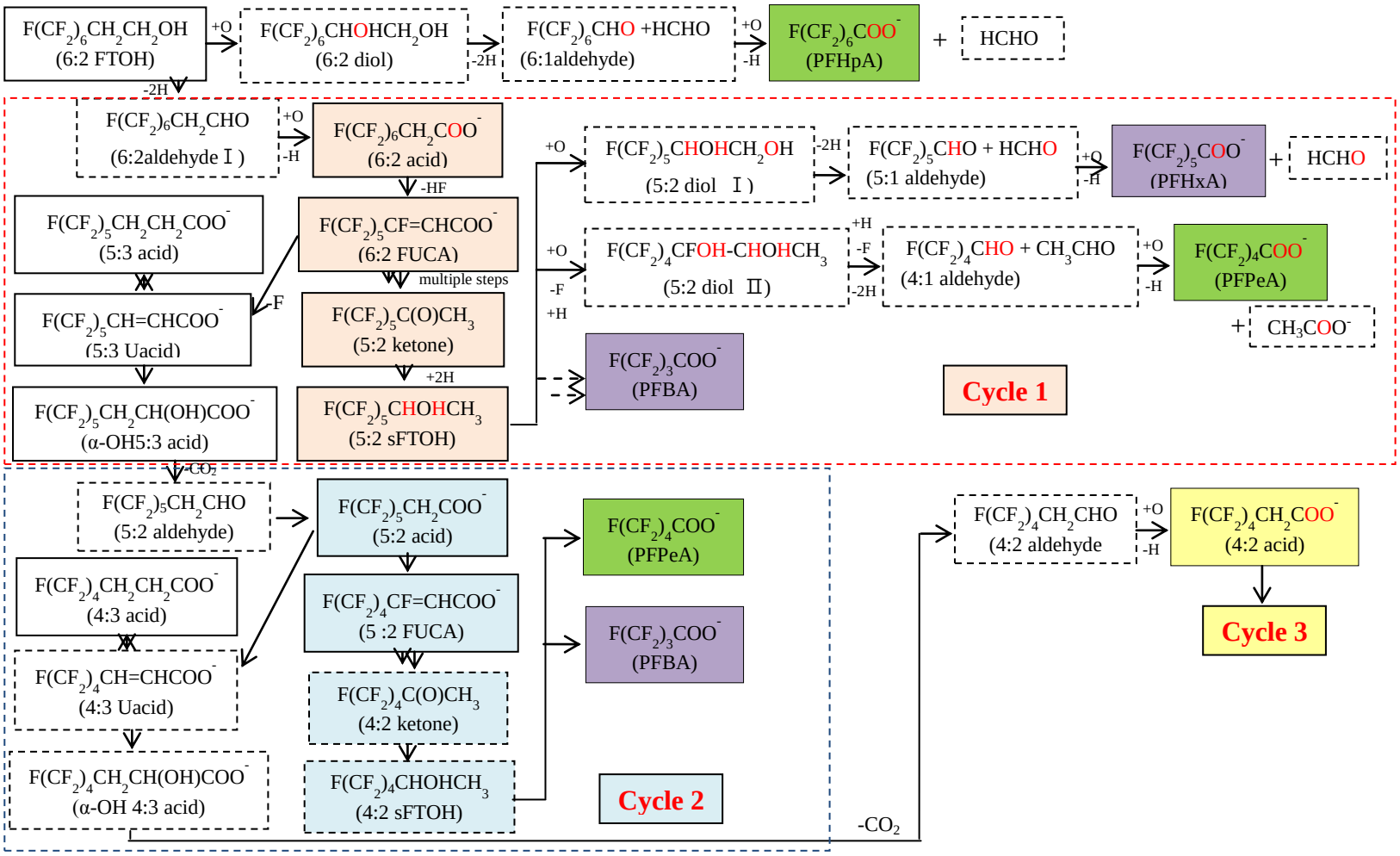


Fig. 4.7. Proposed enzymatic metabolic/degradation cycles to degrade 6:2 FTOH in bacteria. Even-numbered (in purple) and odd-numbered (in green) PFCAs are terminal products of each cycle. The dashed arrows are possible unknown reactions.

5. DEVELOPMENT AND CHARACTERIZATION OF FTOH-DEGRADING MICROBIAL CONSORTIA

5.1 Introduction

FTOHs [$F(CF_2)_nCH_2CH_2OH$] are emerging environmental contaminants. They are used to manufacture a number of products due to its water- and oil- repelling properties [1]. 6:2 and 8:2 FTOH showed estrogen potential on MCF-7 human breast cancer cells [26]. Also, 8:2 FTOH transformation products, especially perfluorooctanoic acid (PFOA), are known to cause many toxicological effects on lab animals [27-30].

Many studies have demonstrated that FTOHs are biodegradable in mixed culture or pure strains alkane-degrading bacteria[17, 18, 24, 25, 31,32]. Our previous study has also shown that FTOHs were degraded by n-octane- and butane-degrading bacteria [31], suggesting that enhanced biodegradation of FTOH might be feasible through enriching these alkane-degrading cultures. Enrichment an inoculum on FTOHs or structurally similar compounds (such as n-octane or 1-butanol) might increase the chance to obtain FTOH-degrading bacteria [33, 132]. Enhanced biodegradation of FTOHs can be accomplished by enrichment cultures containing FTOH-degrading consortia.

The objective of this study was to develop effective FTOH-degrading bacterial consortia and examine their ability to degrade FTOHs to environmental friendly end products. It was hypothesized that enhanced FTOH degradation by the enrichment cultures are attributed to the possession of the enzymes actively involved in the degradation. In our previous study, it was shown that alkane-degrading bacteria could defluorinate FTOHs efficiently [31]. Therefore, AlkB (alkane hydroxylase), a key enzyme for alkane degradation [107], was selected to detect alkane-degrading bacteria in the enrichment cultures to investigate the relation between the amount of degradative enzyme and FTOH degradation efficiency. The detection of degradative enzymes is more efficient than 16S rRNA to capture the target microorganisms because the catabolic enzymes are evolved much faster [99].

As different co-substrates used to develop FTOH-degrading consortia might lead to different patterns of microbial communities, the characteristics of the FTOH-degrading consortia were also investigated using molecular tools. Molecular methods based on DNAs directly from the bacteria in the environment provide more accurate and abundant information about the microorganisms than the traditional culture-dependent methods [99, 133]. Two molecular tools, real-time PCR for *alk* gene quantification[99] and real-time terminal restriction fragment length polymorphism (real-time-t-RFLP) assay for microbial community structure analysis [102], were used in this study.

5.2 Materials and methods

5.2.1 Chemicals

1H, 1H, 2H, 2H-heptadecafluoro-n-decanol (8:2 FTOH,CAS # 678-39-7, >96% pure), 1H, 1H, 2H, 2Hperfluorooctanol(6:2 FTOH, CAS # 647-42-7, 97% pure), and 1H, 1H, 2H,2H-perfluorohexan-1-ol (4:2 FTOH, CAS # 2043-47-2) were purchased from TCIAmerica (manufactured in Tokyo, Japan), Alfa Aesar (Lancashire, UK), and Aldrich (Shinagawa-Ku, Japan), respectively.Hexane (95% pure) was obtained from Acros Organics(Morris Plains, NJ). 1-Butanol (99.4% pure) was purchasedfrom Fisher Scientific (Fair Lawn, NJ) and n-octane(97% pure) was from Acros Organics (Geel, Belgium). Ethanol (95% pure) was from IBI scientific (Peosta, IA).

5.2.2 Enrichment of FTOH-degrading microbial consortia

FTOH-degrading microbial consortia were developed in 250 mL-glass bottles using activated sludge as inocula. The sludge was collected from a WWTP receiving wastewater from a fire-fighting training field at Texas A&M University. The activated sludge was first diluted in NMS medium to reach VSS ≒ 500 mg/L before use. The

diluted activated sludge (100 mL) was then distributed into a 250- mL-autoclaved glass bottle and 1 % n-octane, 5 mM 1-butanol or no growth substrate was provided. After the bottles were sealed with PTFE/SIL septa, 100 µL of 8:2 FTOH (40 g/L stock solution dissolved in ethanol) was added using a gas-tight syringe. The enrichment cultures were maintained by transferring into the same fresh medium as described above, biweekly for about six months. The enrichment cultures were named as FEO (enriched on 8:2 **F**TOH in **e**thanol and n-**o**ctane), FEB (8:2 **F**TOH in **e**thanol and 1-**b**utanol) and FE (8:2 **F**TOH in **e**thanol). The ability of the enrichment cultures to degrade 8:2 FTOH was supported by the fluoride ion measurement as described previously [31].

5.2.3 FTOH degradation test

The experiments for biodegradation of 4:2, 6:2 and 8:2 FTOHs by enrichment cultures were performed in a series of 22-mL glass vials containing cell suspension (1 mL), one of FTOHs, and one of co-substrates (1-butanol or n-octane). The enrichment cultures (1 mL) were transferred into vials containing fresh NMS medium (total volume = 5 mL). One percent of n-octane, 5 mM of 1-butanol or no substrate was added and the vials were sealed with butyl rubber septa and aluminum caps before FTOH addition. Five micro-liters of 40 g/L 8:2 FTOH (dissolved in ethanol), 1 µL of 6:2 FTOH or 4:2 FTOH (pure chemical, liquid) was injected into vials using a 25 µL-gas-tight syringe.

Killed controls, containing acid-killed cells (by adding 50 μL of concentrate sulfuric acid), each additional carbon source and FTOH, were also prepared to assess parent compound recovery during the degradation. The vials were placed on a shaker at 150 rpm in a 30 ℃ dark room. At each of following sampling points (0, 2 and 4 days), two live samples and two killed controls were sacrificed for solvent extraction. Two milliliters of hexane was added into the vials using a gas-tight syringe and the remaining FTOH was extracted overnight. The hexane layer was withdrawn using a gas-tight syringe and transferred into a new capped GC amber vial (1.5 mL). The samples were stored at – 20 ℃ until GC/MS analysis. The aqueous layer in the old sample vials were preserved at 4 ℃ and later used for fluoride ion measurement. GC analysis and fluoride measurement were conducted as described in our previous work [31].

5.2.4 DNA extraction from enrichment cultures

Twenty milliliters of cell suspensions were taken from each enrichment culture after about six months of transfer. The cell suspensions were centrifuged (10,000 *g* for 5min) and the pellets were used for DNA extraction. The whole genomic DNAs of the enrichment cultures were extracted using the FAST DNA Spin Kit for Soil(MP, Solon, OH) and the DNAs were preserved at –20℃ before used for *alkB* gene detection and real-time-t-RFLP analysis.

5.2.5 Real-time PCR for quantification of *alkB* gene and real-time-t-RFLP analysis for characterization of microbial community structure

The extracted DNA was used as templates in real-time PCR assay for *alkB* gene quantification. Amplification and detection were carried out in 96-well plates with SYBR-Greens PCR 2X Master Mix. The *alkB* gene was amplified using the forward primer (5'-AAYACNGCNCAYGARCTNGGNCAYAA-3') and reverse primer (5'-AAYACNGCNCAYGARCTNGGNCAYAA-3') [108-110]. Each reaction was run in a final volume of 25 μL with 1X final concentration of SYBR-Greens PCR 2X Master Mix, 4 mM of $MgCl_2$, 5 pmol final concentration of each primer, 10 μg/μL of BSA, and 2 μL of each DNA sample. Amplifications were carried out using the following ramping profile: After 2 min at 50 ℃, 15 min at 95 ℃, followed by 1min at 94 ℃, 1 min at 55 ℃, and 1 min at 72 ℃ for 39 cycles. The iCycler iQ™ 5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) was used to quantify the amount of *alkB* gene in samples. The plasmid containing *alkB* gene from *P. oleovorans* was constructed using TOPO TA cloning Kit (Invitrogen) and used for standard curves (range of 1.42×10^3 to 1.42×10^{10} copies of *alkB* gene).

Real-time-t-RFLP analysis was carried as described by Yu et al. [102]. Briefly, a region of 16S rRNA was amplified using fluorescence labeled forward primer 16SHex1055f (5'-hexachlorofluorescein-ATGGCTGTCGTCAGCT-3'), reverse primer

16S1392r (ACGGGCGGTGTGTAC-3'),and TaqMan probe 16STaq1115f (5'-[6-carboxyfluorescein]-CAACGAGCGCAACCC-[6-carboxytetramethylrhodamine]-3').

The PCR mixture was prepared and 16S rRNA was amplified as described in Yu et al.[102], except using a plasmid carrying *E. coli* 16S rRNA gene for preparing standard curves (2.28×10^1 to 2.28×10^8 copies of 16S rRNA gene). The PCR products were separated on a 1.5% agarose gel in 1X Tris-acetate-EDTA buffer, and the DNA bands were excised for gel extraction (QIA quick Gel Extraction Kit, Maryland).The purified PCR products were then digested with restriction enzyme MspI and the digested PCR products were analyzed by AMI 3100 sequencer at the Gene Technology Lab, Texas A&M University.

5.3 Results and discussion

5.3.1 FTOH biodegradation by three enrichment cultures

The mixed cultures enriched with n-octane, 1-butanolor no substrate along with 8:2 FTOH (dissolved in ethanol) could degrade and defluorinate FTOHs (Fig. 5.1).FEO could degrade FTOHs most efficiently among the three consortia with 25 mol% removal of 8:2 FTOH applied at day 0, 59 mol% of 6:2 FTOH, and 68 mol% of 4:2 FTOH,

respectively. This indicates that n-octane might be the most efficient co-substrate among tested carbon sources to induce the enzymes involved in FTOH degradation. Unlike 8:2 FTOH degradation test that 8:2 FTOH was supplied through 8:2 FTOH stock solution (i.e., containing some ethanol), during degradation tests for 6:2 and 4:2 FTOHs, they were amended as pure liquid compounds. The amended 6:2 and 4:2 FTOH were degraded by FEO, suggesting that these two FTOHs were degraded by the enzymes that were already present in FEO. These results support that a short-chained FTOH was degraded better than a long-chained FTOH by FEO (4:2 FTOH> 6:2 FTOH> 8:2 FTOH). Interestingly, the chain length of FTOH did not have significant effects on the extent of defluorination (4 % for 8:2 and 6:2 FTOHs, 3 % for 4:2 FTOHs).

By enriching with 1-butanol, FEB was the second most effective FTOH-degrading consortia with 20 mol% removal of 8:2 FTOH, 14 mol% of 6:2 FTOH and 68 mol% of 4:2 FTOH. The removal of 4:2 and 6:2 FTOH supports that the enzymes induced by 1-butanol is responsible for the degradation. Different from those observed for FEO and FE, FEB defluorinated 3 % of 8:2 FTOH and 1 % of 6:2 FTOH, but showed very high defluorination with 4:2 FTOH (9 %). In fact, this high defluorination of 4:2 FTOH was consistent with our previous study that *P. butanovora*, 1-butanol grown bacteria, defluorinated 4:2 FTOH in much higher degree than *P. oleovorans*, n-octane grown bacteria did [31].

FE could degrade a slight amount of 8:2 FTOH (15 mol%) with 3 % of defluorination. The poor degradation was also observed with 4:2 FTOH (8 mol%) and

6:2 FTOH (3 mol%) which applied as pure liquid compounds (not dissolved in ethanol). This result indicates that FE degrades FTOHs co-metabolically using enzymes induced by ethanol.

Due to the structure similarity between n-alkanes and FTOHs, it was hypothesized that bacteria containing alkane-degrading enzymes might be responsible for FTOH degradation. The copy number of *alkB* gene, a biomarker to detect alkane-degrading bacteria, was quantified from each of FTOH-degrading consortium. As shown in Figure 5.2a, FEO contained the highest number of *alkB* gene, followed by FEB and FE. It seemed n-octane was a better co-substrate than 1-butanol or ethanol to induce alkane-degrading enzymes, and the more expression of AlkB brings better degradation of FTOHs.

Figure 5.2b shows the portion of (detected) alkane-degrading bacteria in the total microorganisms in each FTOH-degrading consortium. The amount of total microorganisms (i.e. total copy number of 16S rRNA gene) was the highest in FEO, followed by FEB and FE (data now shown). Although FE contained the highest portion of alkane-degrading bacteria in total microbial community, least removal of FTOHs were observed for FE. These results indicate that the enzymes expressed by alkane-degrading bacteria in FE may be not actively involved in FTOH degradation. While FEO had the highest number of *alkB* gene, a relatively lower population of alkane-degrading bacteria was observed in the community. The better FTOH degradation observed in FEO could be explained by three hypotheses. First, the FTOH

degradation is more dependent on the absolute number of the degradative bacteria. Second, FEO might contain many diverse alkane-degrading bacteria; however, they were not detected by the primers used in this study. The primers used in this study could detect *alk*B genes from *P. oleovorans* grown on n-octane and *P. butanovora* grown on 1-butanol or hexane (data not shown). However, it still remains unclear if the primers miss some alkane-degrading bacteria which are actively involved in FTOH degradation. Third, different substrates induce different types of alkane monooxygenases with different affinity toward FTOHs. For example, alkane monooxygenases expressed for medium-chain alkanes would not be effective for degrading short-chain or long-chain alkanes [110, 134,135]. It was possible that the n-octane supplied to FEO simply promoted microorganisms with certain types of alkane monooxygenases suitable for degrading FTOHs.

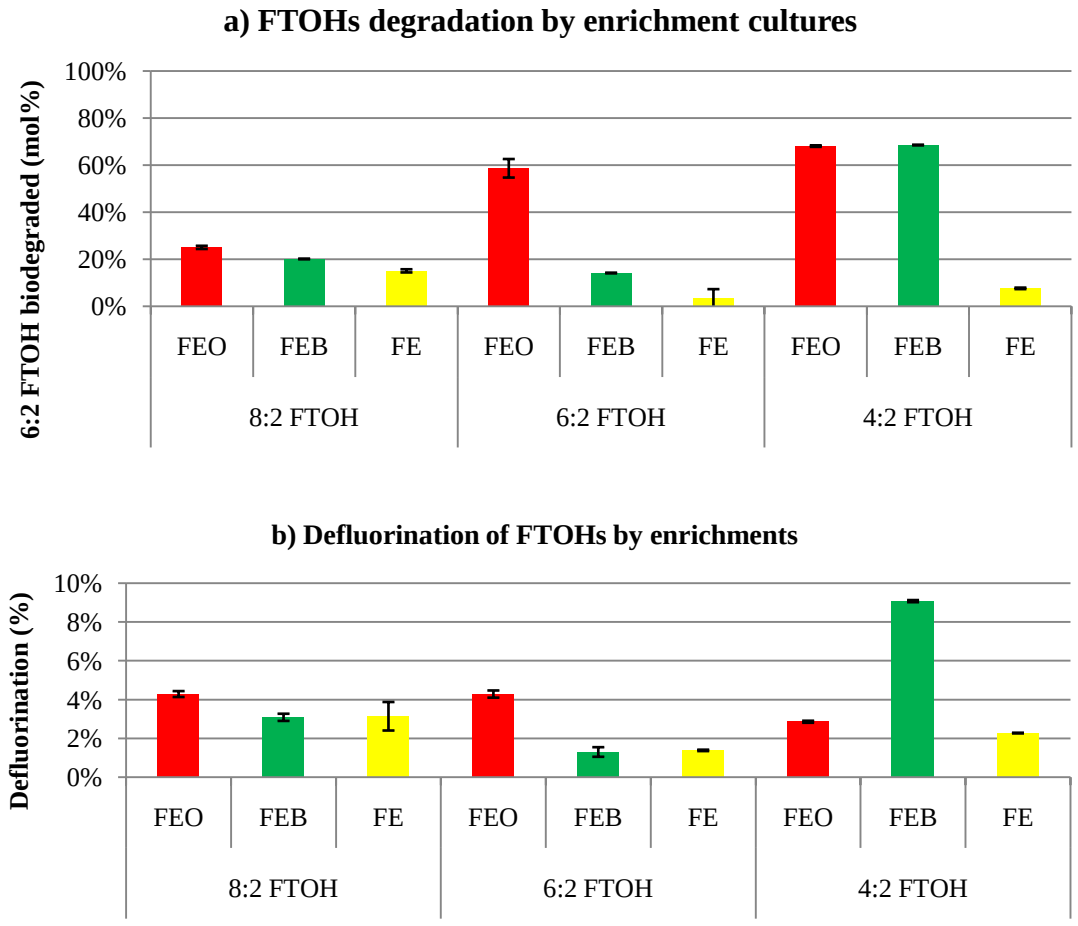


Fig. 5.1. FTOH degradation (a) and defluorination (b) by each enrichment. FEO: enrichment grown on 8:2 **F**TOH dissolved in **e**thanol and n-**o**ctane, FEB: enrichment grown on 8:2 **F**TOH dissolved in **e**thanol and 1-**b**utanol, and FE: enrichment grown on 8:2 **F**TOH dissolved in **e**thanol. The degradation is presented in mol% of FTOH biodegraded of FTOH applied at day 0.The defluorination (b) was calculated like the following description. When 1 µM of FTOH (464.12 µg/L of 8:2 FTOH, 350 µg/L of 6:2 FTOH, and 264 µg/L of 4:2 FTOH) is completely defluorinated 17 µM of fluoride for 8:2 FTOH, 13 µM for 6:2 FTOH, and 9 µM for 4:2 FTOH is released. Therefore, based on the concentration of FTOH biodegraded, the defluorination can be expressed as {measured fluoride concentration (µM) / ideal fluoride concentration (µM) x 100}, where ideal fluoride concentration is {(17 µM of fluoride) / (concentration of 8:2 FTOH biodegraded, µM)} in a case of 8:2 FTOH.

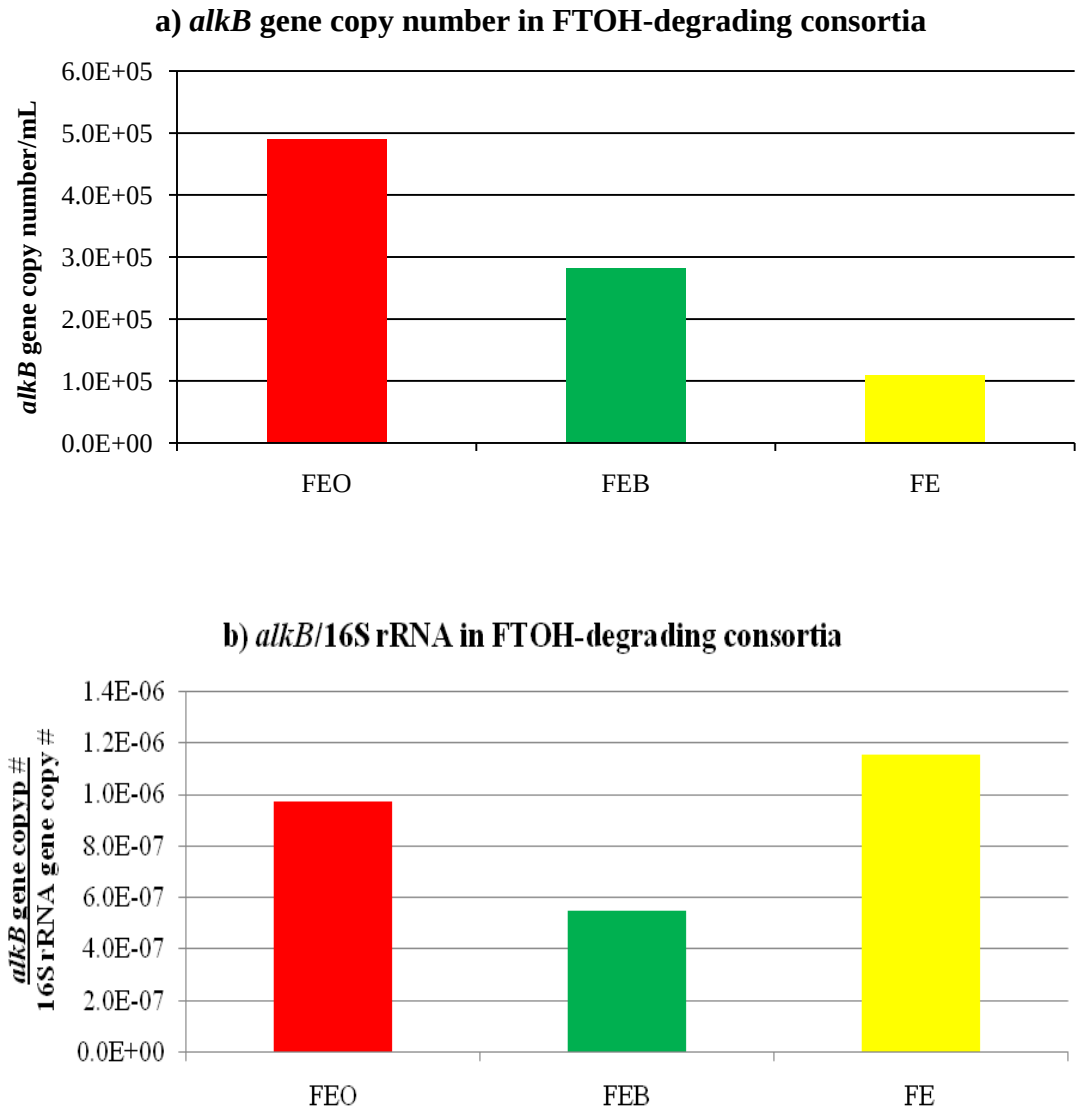


Fig. 5.2. The copy number of *alkB* gene in each FTOH-degrading consortium (a) and ratio of alkane-degrading bacteria (detected) in total bacteria in *alkB*/16S rRNA (b).FEO: enrichment grown on 8:2 **F**TOH dissolved in **e**thanol and n-**o**ctane, FEB: enrichment grown on 8:2 **F**TOH dissolved in **e**thanol and 1-**b**utanol, and FE: enrichment grown on 8:2 **F**TOH dissolved in **e**thanol.

5.3.2 Microbial community structures of FTOH-degrading consortia

On the third day of fresh transfer, when the enrichment cultures showed the highest growth (OD_{600}nm $\fallingdotseq$ 0.5 ~ 0.7), the samples were taken for real-time-t-RFLP analysis. T-RFs shorter than 35 bp or longer than 350 bp in length, or those with less than 1 % of peak area were ignored. All three enrichment cultures showed similar patterns of T-RF profiles (Fig. 5.3). However, the quantity of microbial community was different depending on which additional carbon source was provided. FEO consisted of microorganisms with 103, 104, 106, 115 and 327 bp of T-RF, and had the highest quantity of the communities. FEB and FE had same T-RF patterns (99, 104, 105, 327 and 350 bp), but FE had much less amount of microorganisms in the community. When 16 rRNA sequences of *P. oleovorans* and *P. butanovora* (FTOH-degraders [31]) were ideally digested with the same restriction enzyme, 108 bp and 106 bp of T-RFs would be produced, respectively. It indicates that all three enrichment cultures might have similar strains to *P. oleovorans* or *P. butanovora* in the community.

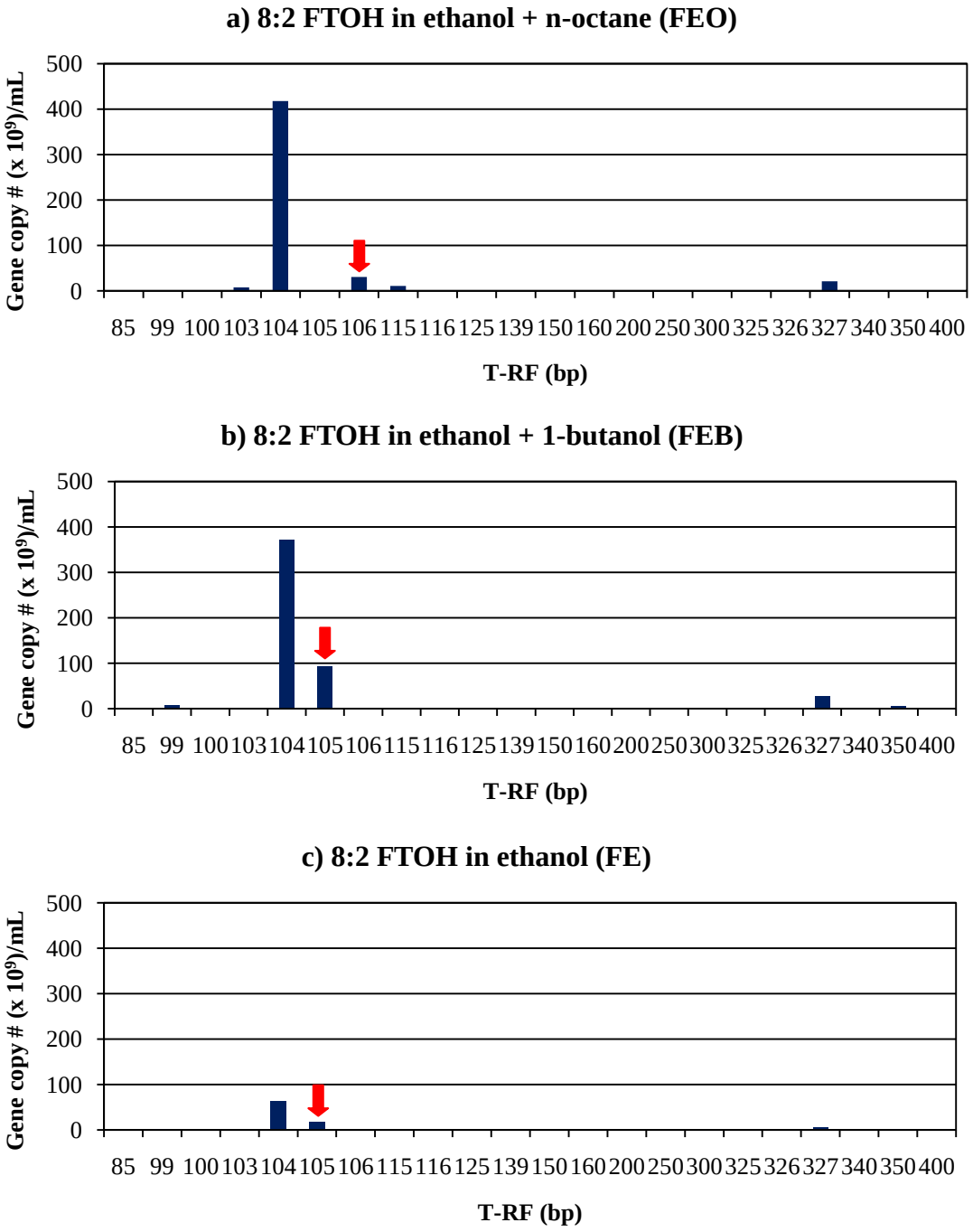


Fig. 5.3. T-RF profiles and 16S rRNA gene copy number in three enrichment cultures. The red arrow presents a possible T-RF for *P. oleovorans* or *P. butanovora.*

6. SUMMARY, CONCLUSIONS AND FUTURE STUDIES

6.1 Summary and conclusions

This is the first study reporting that pure alkane- and fluoroacetate-degrading bacteria could defluorinate FTOHs. Three effective FTOH-degrading enrichment cultures were also developed with different co-substrates, and their microbial community structures were molecularly characterized. The overall hypothesis of this work was that "Bacteria containing degradative enzymes for structurally similar substrates are responsible for FTOH biotransformation in the natural environment". The hypothesis was supported by the results listed below.

1) Medium-chain alkane-degrading bacteria, *P. oleovorans* and *P. butanovora* could defluorinate 4:2, 6:2 and 8:2 FTOHs, with a higher degree of defluorination for 4:2 FTOH. Based on the transformation products detected, two possible pathways were proposed for 6:2 and 8:2 FTOH degradation by *P. oleovorans* and *P. butanovora*. The PFCA pathway (pathway I) led to the production of x:2 ketone, x:2 sFTOH, PFCAs (PFHxA for 6:2 FTOH and PFOA for 8:2 FTOH). The x:3 acid pathway (pathway II) resulted in the formation of

x:3 polyfluorinated acid (x:3 acid) and relatively minor shorter-chain PFCAs (PFBA for 6:2 FTOH and PFHxA for 8:2 FTOH). *P. oleovorans* transformed 6:2 and 8:2 FTOH via both pathways I and II, while *P. butanovora* only utilized pathway I. The formation of shorter-chain PFCAs suggests that a complete enzyme system does exist in pure bacterial strain to remove multiple $–CF_2$-groups from FTOHs.

2) Gram-positive alkane-degrading bacteria, *M. vaccae* JOB5, and fluoroacetate-degrading bacteria, *P. fluorescens* DSM 8341, also could transform 6:2 FTOH into at least nine metabolites via both PFCAs and x:3 acid pathways (pathways I and II). These two strains degraded 6:2 FTOH much slower than *P. oleovorans* or *P. butanovora*, but produced newly detected odd-numbered short-chain PFCAs such as PFPeA or PFHpA. This result shows the possibility of more diverse bacteria in the environment which can transform 6:2 FTOH possibly in more various mechanisms.

3) The presence of co-substrates affected on the degradation of 6:2 FTOH by the strains tested. The presence of DCPK (alkane hydroxylase enhancer) caused much faster transformation of 6:2 FTOH in *P. oleovorans* and the formation of odd-numbered PFCAs (PFPeA and PFHpA). In the presence of lactate (an odd-numbered carbon and reductant), *P. butanovora* was able to utilize not only the PFCA pathway but also the x:3 acid pathway to transform 6:2 FTOH and produce odd-numbered PFCAs (PFPeA and PFHpA).

4) No observation for PFCAs (PFOA, PFHxA and PFBA) degradation by any strain tested. However, *P. fluorescens* DSM 8341 slightly transformed 5:3 acid into 5:3 Uacid, α-OH 5:3 acid, 5:2 acid, 5:2 Uacid and then 4:3 acid and PFPeA via one-carbon removal pathways. This result indicates that complete mineralization of 6:2 FTOH might be possible by synergy of diverse enzymes expressed by various FTOH-degrading bacteria in mixed cultures.

5) Based on the transformation products in PFCA pathway, x:3 acid pathway and one-carbon removal pathway, a possible generalized cycle in 6:2 FTOH was proposed. In this cycle, it was proposed that x:2 perfluorinated acid might be converted to x:2 unsaturated perfluorinated acid, x:2 ketone, and then x:2 sFTOH. x:2 sFTOH might be then transformed to corresponding short-chain PFCA, and (x-1):2 perfluorinated acid which goes through the cycle repeatedly.

6) FTOH-degrading consortia were developed by enriching on n-octane (FEO), 1-butanol (FEB), or no substrate (FE) along with 8:2 FTOH dissolved in ethanol. FTOHs were degraded co-metabolically by the three consortia and n-octane seemed to enhance the degradation greater than other co-substrates. The amount of *alkB* gene (a biomarker for alkane-degrading bacteria) was in order of FEO > FEB > FE and the higher gene copy number of *alkB* led to the better degradation of FTOHs. Real-time-t-RFLP showed that the three enrichment cultures had similar patterns of T-RF profiles, but different quantities of microbial community depending on the carbon source (n-octane > 1-butanol

>ethanol). All of the three FTOH-degrading consortia contained microbial groups having similar T-RF to *P. oleovorans* and *P. butanovora*, FTOH-degraders.

6.2 Future studies

This study provided information that several bacteria are possibly responsible for FTOH degradation in the environment. The microbial community of FTOH-degrading consortia were also characterized and discussed. However, the results of this study brought many questions and drew attentions to several future studies. Below are suggestions for future studies that might help answer the unanswered questions in this study.

1) The specific enzymes responsible for each step for FTOH degradation were not investigated in this study and remain unknown. Purification and identification of enzymes expressed during the degradation will provide better understanding of FTOH biotransformation.

2) Even though the biotransformation pathways for FTOHs were proposed, some parts such as the origin of PFPeA are still unclear. More degradation

studies of selected metabolites such as x:2 ketone, x:2 sFTOH or (x-1):2 acid are necessary to elucidate FTOH pathways to deeper levels.

3) Based on the results of this study, it might be possible to construct artificial microbial community or enrichment cultures for enhanced FTOH degradation. For example, *P. oleovorans* transforms FTOHs very fast (especially, in the presence of DCPK or formate), but cannot degrade further than 5:3 Uacid. *P. fluorescens* DSM 8341 is relatively slow in FTOH degradation, but able to transform 5:3 acid to more down-streamed metabolites. Therefore, the mixture of these two strains can used to investigate the possibility of FTOHs mineralization. The substrate competition and toxicity of each substrate need to be studied first before constructing the combined cultures.

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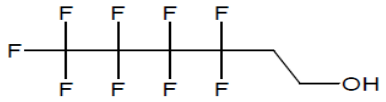
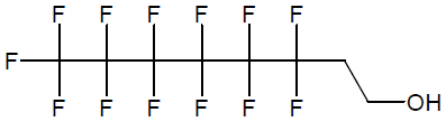
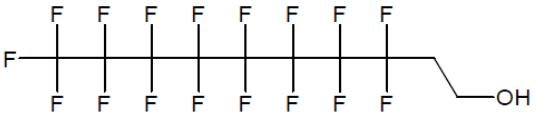
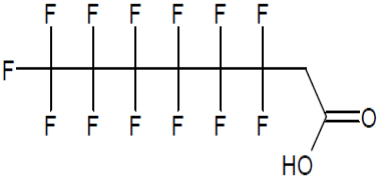
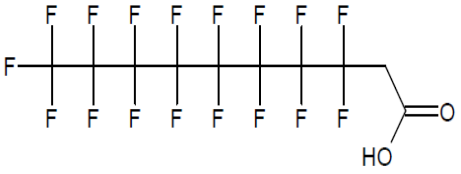
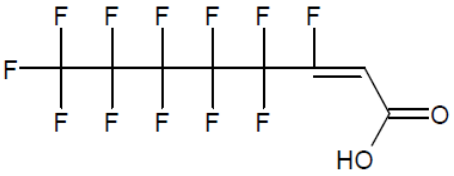
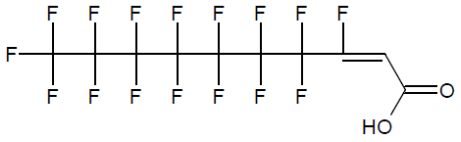
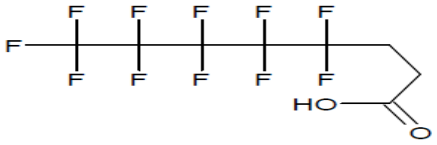
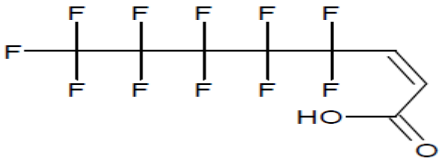
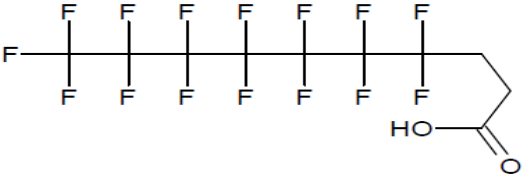
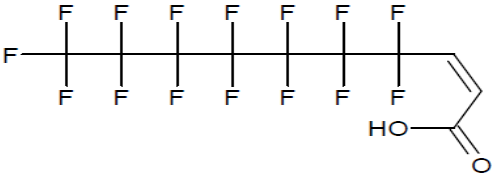
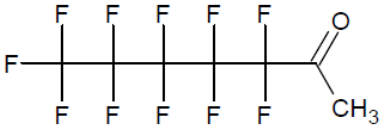
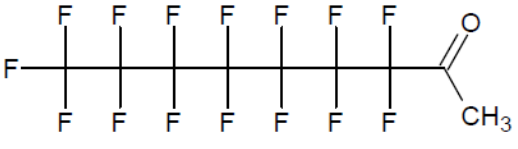
APPENDIX

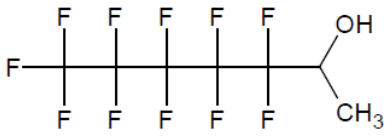
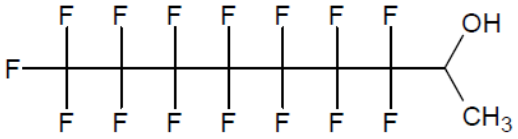
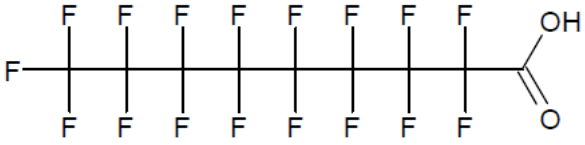
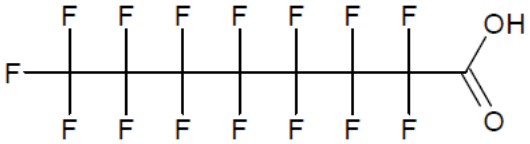
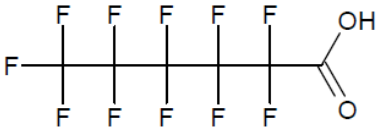
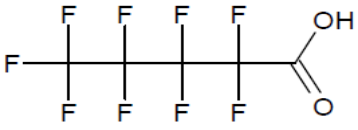
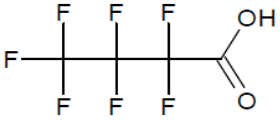
FIGURE or TABLE Page

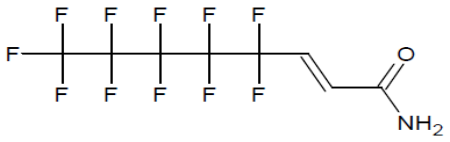
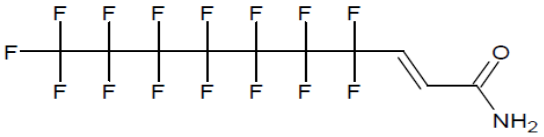
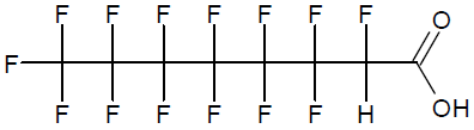
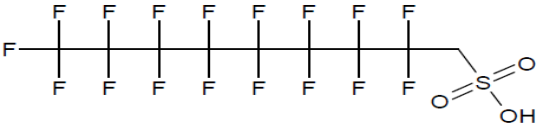
Page

Table A1.1. Structures of perfluorinated compounds

Compound	Structure
Fluorotelomer alcohols (FTOHs)	
4:2 FTOH	
6:2 FTOH	
8:2 FTOH	
Fluorotelomer (unsaturated) acid	
6:2 FTCA	
8:2 FTCA	
6:2 FTUCA	

8:2 FTUCA	
Polyfluorinated acid	
5:3 acid	
5:3 U acid	
7:3 acid	
7:3 U acid	
ketone	
5:2 ketone	
7:2 ketone	

Secondary fluorotelomer alcohols	
5:2 sFTOH	
7:2 sFTOH	
Perfluorinated carboxylic acids (PFCAs)	
Perfluorononanoic acid (PFNA)	
Perfluorooctanoic acid (PFOA)	
Perfluorohexanoic acid (PFHxA)	
Perfluoropentanoic acid (PFPeA)	
Perfluorobutanoic acid (PFBA)	

Other perfluorinated compounds	
5:3 u amid	
7:3 u amid	
2H PFOA	
perfluoro-1-octane sulfonate (PFOS)	

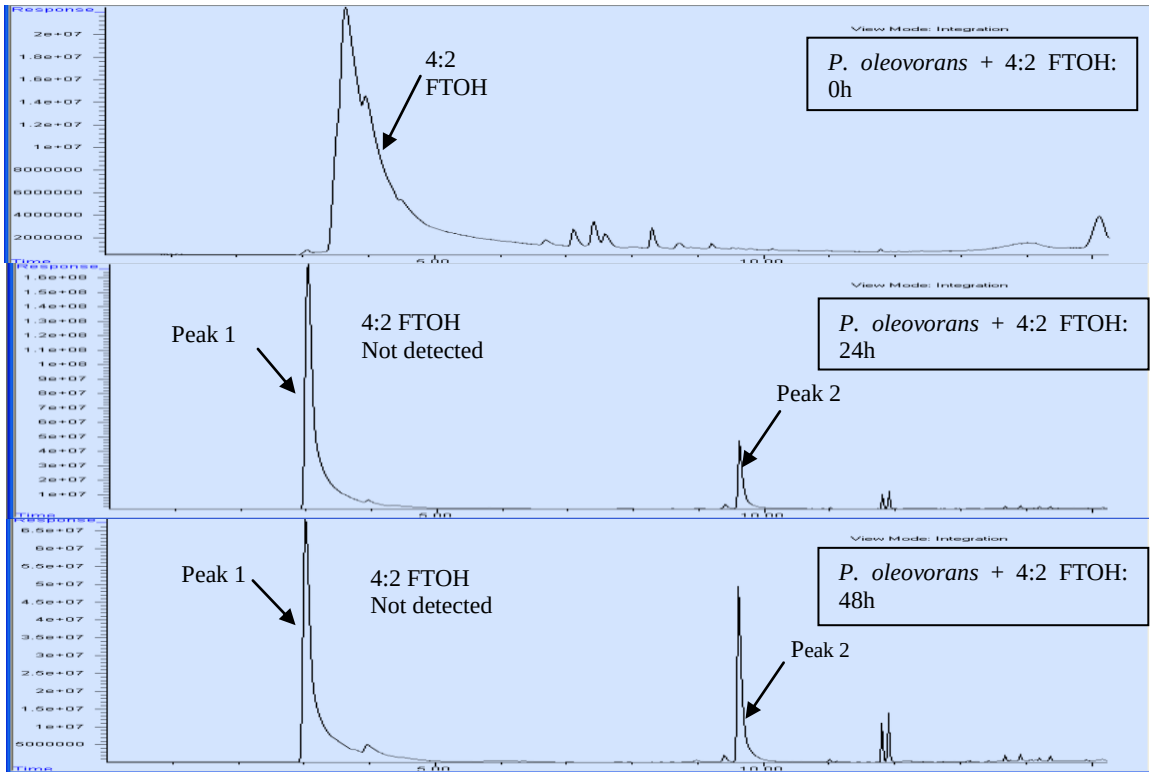


Fig. A3.1. GC/ECD chromatography for 4:2 FTOH transformation by *P.oleovorans* in the absence of n-octane.

A: In the absence of 1-butanol

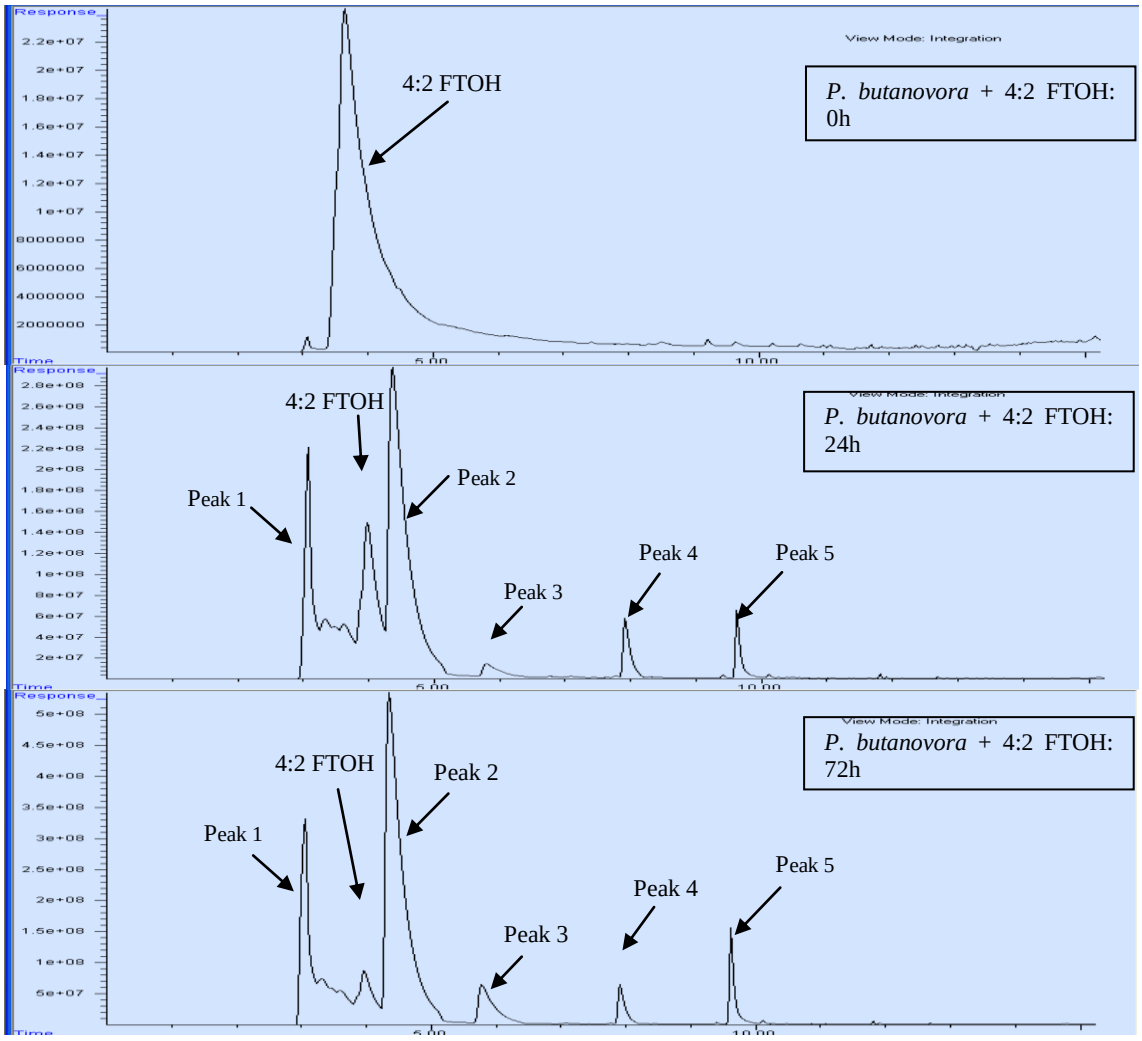


Fig. A3.2. GC/ECD chromatography for 4:2 FTOH transformation by *P. butanovora* in the absence of 1-butanol (A) and in the presence of 1-butanol (B).

B: In the presence of 1-butanol

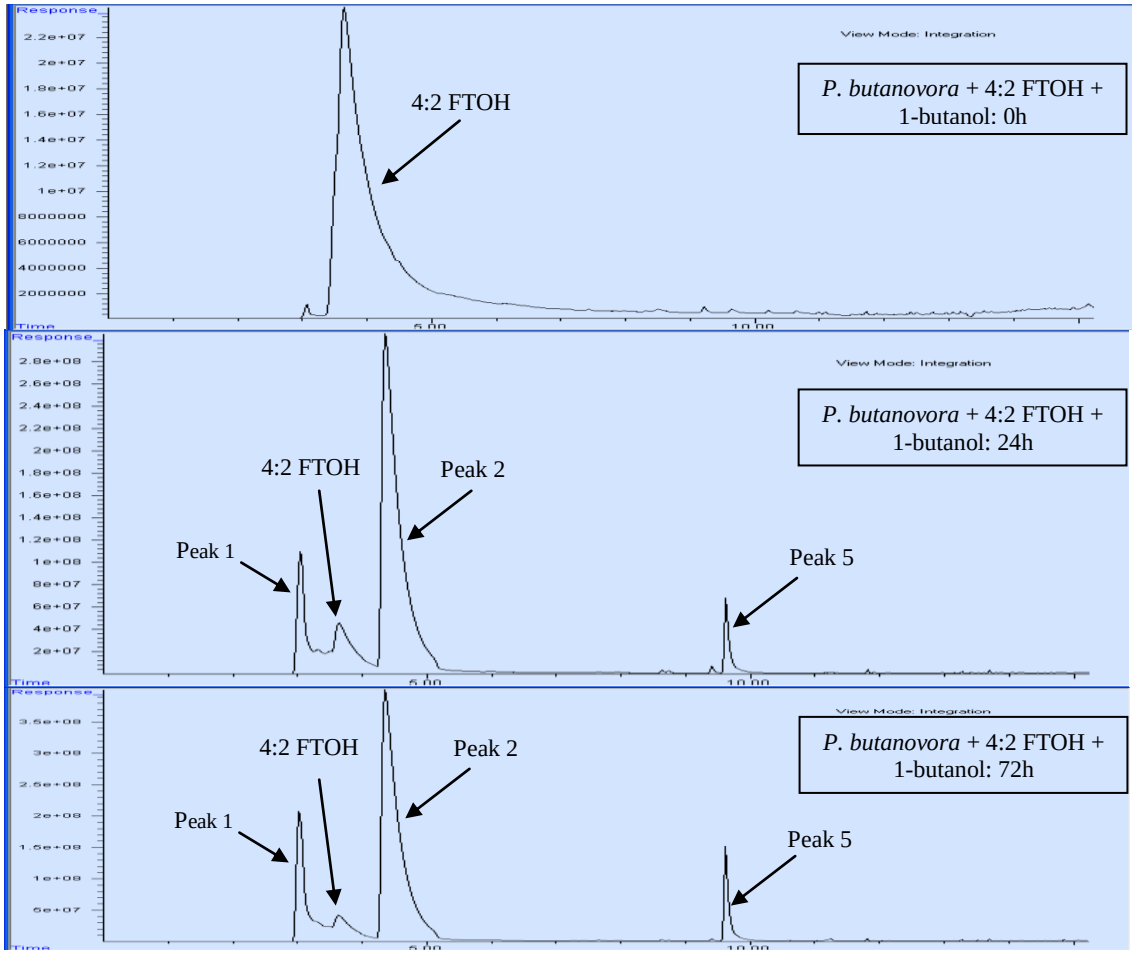
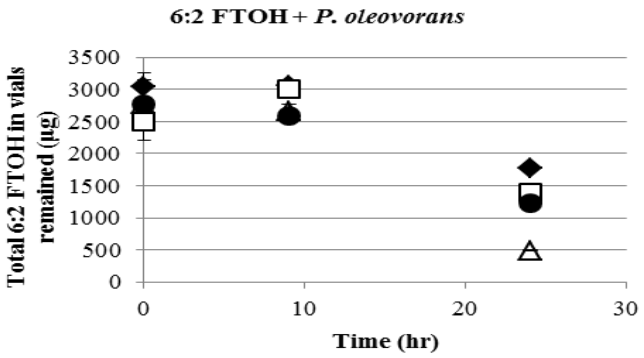


Fig. A3.2. Continued



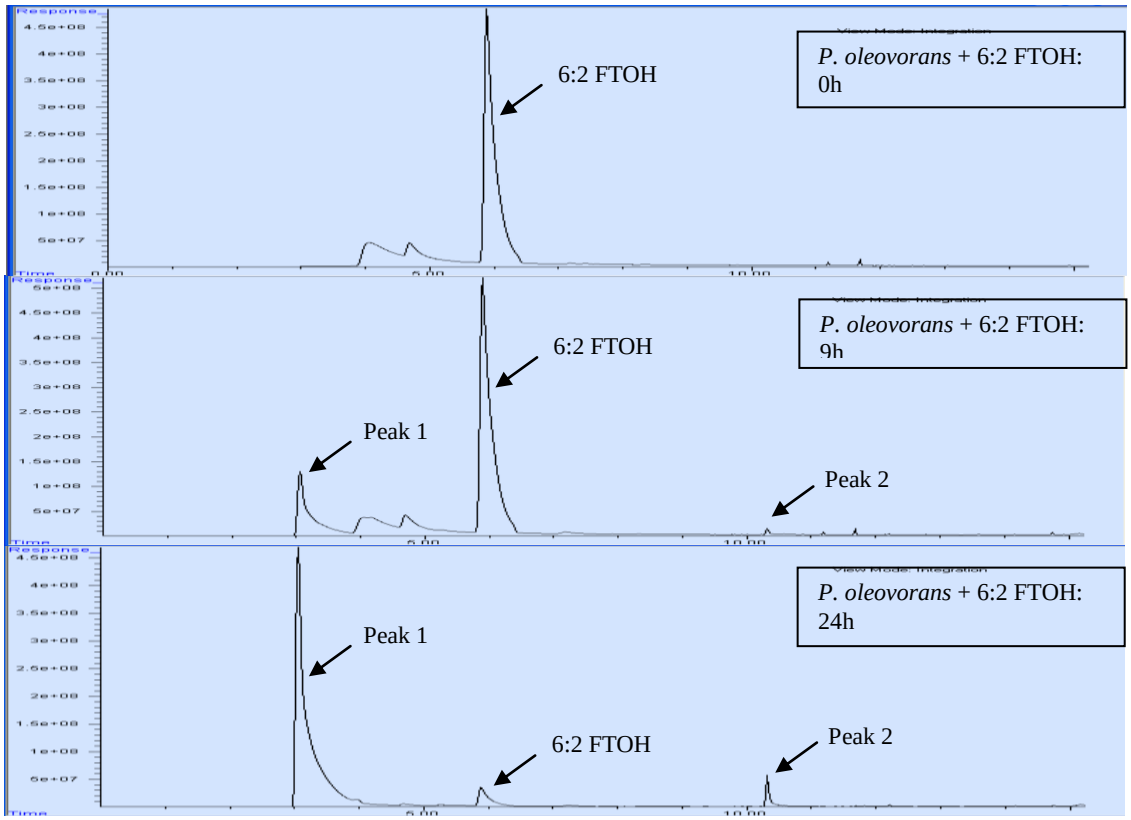


Fig. A3.3. 6:2 FTOH transformation by *P. oleovorans* (A), and detection of unidentified metabolites using GC-ECD (B): diamond-control, square-killed control, triangle-FTOH only, circle- FTOH with additional carbon source (n-octane). As the parent compound disappears, two new peaks (peak 1 and 2) were showed up and increased over time.

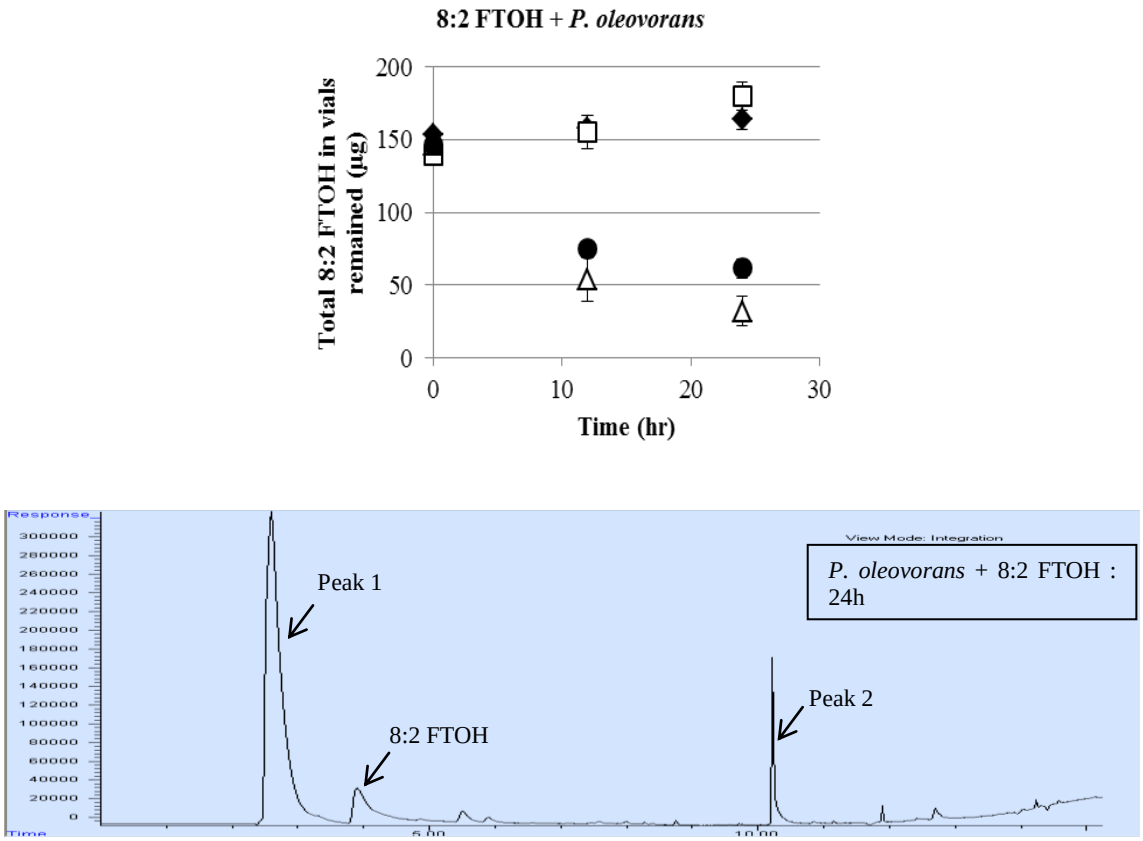


Fig.A3.4**.** 8:2 FTOH transformation by *P. oleovorans* (A), and detection of unidentified metabolites using GC-ECD (B): diamond-control, square-killed control, triangle-FTOH only, circle- FTOH with additional carbon source (n-octane). As the parent compound disappears, two new peaks (peak 1 and 2) were showed up and increased over time.

Table A3.1. 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 in 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 (μM)		4:2 FTOH biodegraded[a] (μM)		Fluoride measured (μM)		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
	1205	1205	751	894	400	826	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
	1205	1205	405	467	1174	1574	32	37.3

*: When 1 μM of 4:2 FTOH (264 μg/L) is completely defluorinated, 9 uM of fluoride is released. Therefore, the complete defluorination of 4:2 FTOH biodegraded (a) will result in (9 × a) μM of fluoride release (production) ideally.

§: {400 μM of measured fluoride /(9 μM of fluoride × 751 μM of 4:2 FTOH biodegraded)} × 100 % = 5.9 %

Table A3.2.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℃
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 FTCA (2.1) / 377 > 293 / 16 / 16 6-2 FTUCA (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 Extractor (V) = 0 RF Lens (V) = 0 Source Temperature (℃) = 120 Desolvation Temperature (℃) = 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 A3.3**.**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℃
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 FTCA (2.1) / 477 > 393 / 16 / 16 8-2 FTUCA (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 (℃) = 120 Desolvation Temperature (℃) = 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 A3.4. FTOHs remaining and transformation products formed per initially applied FTOHs on 28 day (%)

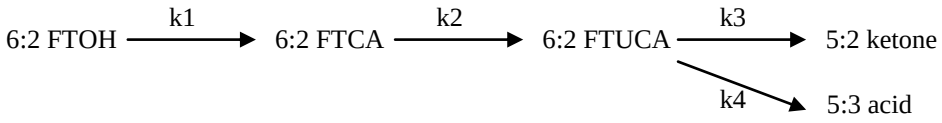
	P. oleovorans	*P. butanovora*		*P. oleovorans*	*P. butanovora*
6:2 FTOH remaining [a]	0.85	ND	8:2 FTOH remaining [a]	2.51	ND
Transformation products formed [b]			Transformation products formed [b]		
6:2 FTCA	0.23	33.4	8:2 FTCA	1.21	0.28
6:2 FTUCA	7.26	43.5	8:2 FTUCA	4.3	19.58
5:2 ketone	10.3	31.8	7:2 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

Table A4.1. Kinetic models of 6:2 FTOH degradation and metabolites formation

<table>
<tr><th colspan="2">Major metabolites in 6:2 FTOH degradation</th></tr>
<tr><td colspan="2"> 
</td></tr>
<tr><th colspan="2">Kinetic models</th></tr>
<tr><td colspan="2">$$\frac{d[A]}{dt} = -k1 \times [A]$$
$$\frac{d[B]}{dt} = k1[A] - k2[B]$$
$$\frac{d[C]}{dt} = k2[B] - k3[C] - k4[C]$$
$$\frac{d[D]}{dt} = k3[C]$$
$$\frac{d[E]}{dt} = k4[C]$$
Where, A = 6:2 FTOH, B = 6:2 FTCA, C = 6:2 FTUCA, D = 5:2 ketone, E = 5:3 acid</td></tr>
<tr><th colspan="2">Calculation of k values and concentrations</th></tr>
<tr><td><u>Step 1</u>

k1 and [A]</td><td>(1) Integrate dA/dt
$$k1 = \frac{\ln[A]0 - \ln[A]}{t - t0}$$
(2) Find k1 by using the concentrations for A from the real data
(3) $[A] = [A]_0 * \exp(-k1*t)$
Using k1 and $[A]_0$, calculate the concentration of [A] at t</td></tr>
</table>

<u>Step 2</u> k2 and [B]	(1) Integrate dB/dt $$[\mathrm{B}] = \left([\mathrm{B}]0 - \frac{\mathrm{k1} \times [\mathrm{A}]0}{\mathrm{k2} - \mathrm{k1}}\right) \times \exp(-k2 \times t)$$ $$+ \frac{\mathrm{k1} \times [\mathrm{A}]0}{\mathrm{k2} - \mathrm{k1}} \times \exp(-\mathrm{k1} \times \mathrm{t})$$ (2) Assume $[\mathrm{B}]_0 = 0$; Use k1 and $[\mathrm{A}]_0$ from the above (3) On an Excel sheet, find k2 which produces the best fitting concentration of [B] at each time point, comparing to [B] from the real data
<u>Step 3</u> k3, k4 and [C]	(1) Integrate dC/dt $$[\mathrm{C}] = ([\mathrm{C}]0 - \mathrm{P} - \mathrm{Q}) \times \exp(-(\mathrm{k3} + \mathrm{k4}) \times \mathrm{t})$$ $$+ \mathrm{P} \times \exp(-\mathrm{k2} \times \mathrm{t}) + \mathrm{Q} \times \exp(-\mathrm{k1} \times \mathrm{t})$$ Where, $$\mathrm{P} = \frac{\mathrm{k2}\ ([\mathrm{B}]0 - \frac{\mathrm{k1} \times [\mathrm{A}]0}{\mathrm{k2} - \mathrm{k1}})}{(\mathrm{k3} + \mathrm{k4}) - \mathrm{k2}}$$ $$\mathrm{Q} = \frac{\mathrm{k2} \times \frac{\mathrm{k1} \times [\mathrm{A}]0}{\mathrm{k2} - \mathrm{k1}}}{(\mathrm{k3} + \mathrm{k4}) - \mathrm{k1}}$$ (2) Assume $[\mathrm{C}]_0 = 0$; Use k1, k2, $[\mathrm{A}]_0$ and $[\mathrm{B}]_0$ from step 2 (3) On an Excel sheet, find k3 and k4 which provide the best fitting concentration of [C], comparing to [C] from the real data
<u>Step 4</u> [D] and [E]	(1) Integrate dD/dt and dE/dt $D = D_0 + k3 * [C] * t$ $E = E_0 + k4 * [C] * t$ (2) Calculate the concentration of [C] and [D]

(a)

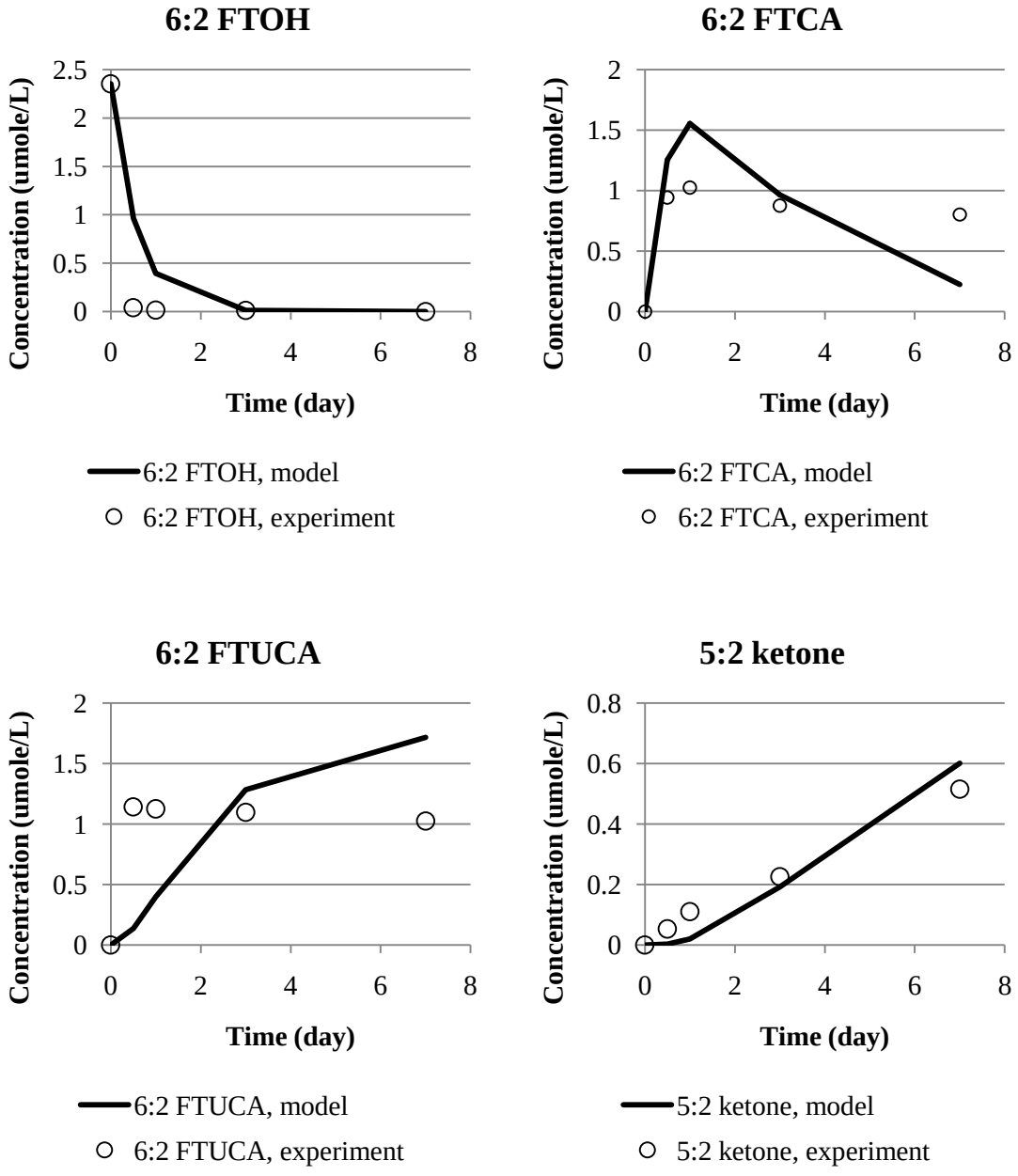
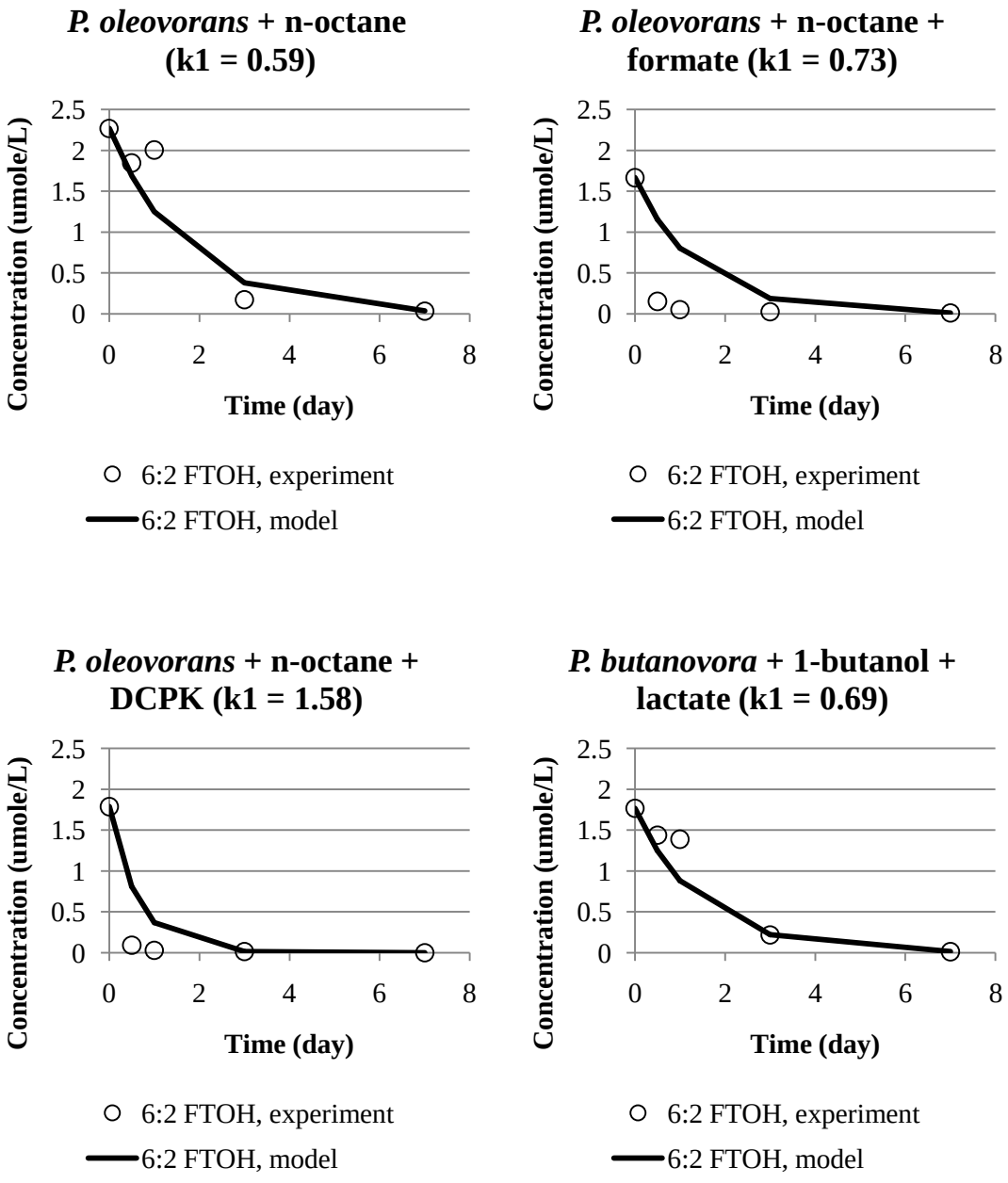


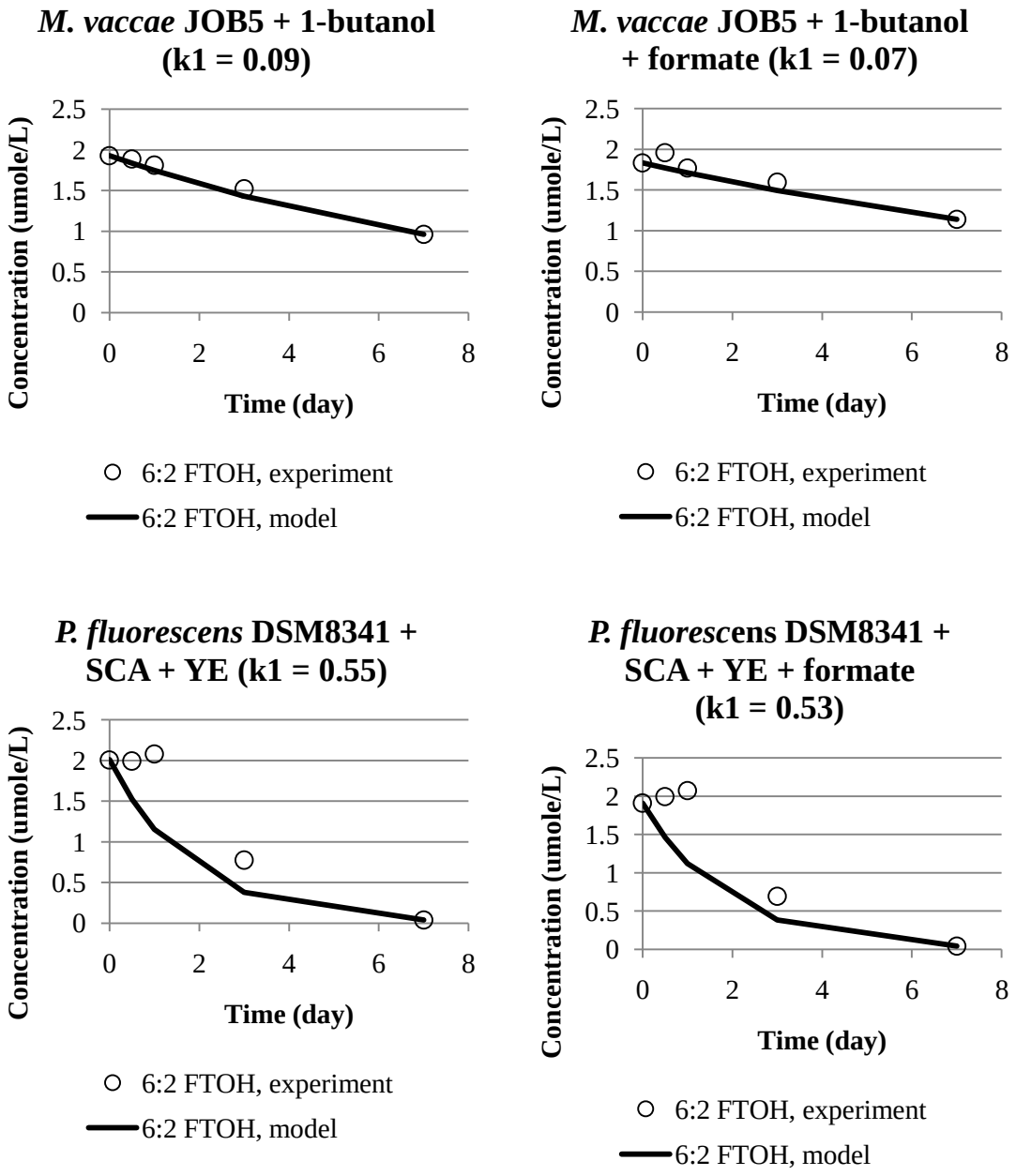
Fig. A4.1 Validation of the kinetic models. (a) 6:2 FTOH and the major metabolites by the models and experiment for *P. butanovora* in the presence of 1-butanol, (b) 6:2 FTOH by the models and experiment for different strains in various conditions

(b)



.Fig. A4.1 Continued

(b)



.Fig. A4.1 Continued