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Effects of growth substrate on triclosan biodegradation potential of oxygenase-expressing bacteria

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HIGHLIGHTS

- *Mycobacterium vaccae* JOB5 degraded triclosan when expressing propane monooxygenase.
- *Rhodococcus jostii* RHA1 degraded triclosan when expressing propane or alkane monooxygenase, or biphenyl dioxygenase.
- Biphenyl-grown *R. jostii* RHA1 showed the highest triclosan transformation capacity.
- *R. jostii* RHA1 has a finite transformation capacity for triclosan due to product toxicity.
- 2-Chlorohydroquinone was first detected in triclosan degradation by biphenyl-grown RHA1.

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ABSTRACT

Triclosan is an antimicrobial agent, an endocrine disrupting compound, and an emerging contaminant in the environment. This is the first study investigating triclosan biodegradation potential of four oxygenase-expressing bacteria: *Rhodococcus jostii* RHA1, *Mycobacterium vaccae* JOB5, *Rhodococcus ruber* ENV425, and *Burkholderia xenovorans* LB400. *B. xenovorans* LB400 and *R. ruber* ENV425 were unable to degrade triclosan. Propane-grown *M. vaccae* JOB5 can completely degrade triclosan (5 mg L^{-1}). *R. jostii* RHA1 grown on biphenyl, propane, and LB medium with dicyclopropylketone (DCPK), an alkane monooxygenase inducer, was able to degrade the added triclosan (5 mg L^{-1}) to different extents. Incomplete degradation of triclosan by RHA1 is probably due to triclosan product toxicity. The highest triclosan transformation capacity (T_c, defined as the amount of triclosan degraded/the number of cells inactivated; 5.63×10^{-3} ng triclosan/16S rRNA gene copies) was observed for biphenyl-grown RHA1 and the lowest T_c (0.20×10^{-3} ng-triclosan/16S rRNA gene copies) was observed for propane-grown RHA1. No triclosan degradation metabolites were detected during triclosan degradation by propane- and LB + DCPK-grown RHA1. When using biphenyl-grown RHA1 for degradation, four chlorinated metabolites (2,4-dichlorophenol, monohydroxy-triclosan, dihydroxy-triclosan, and 2-chlorohydroquinone (a new triclosan metabolite)) were detected. Based on the detected metabolites, a *meta*-cleavage pathway was proposed for triclosan degradation.

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1. Introduction

Triclosan is a broad-spectrum antimicrobial agent that has been added in soaps, deodorants, toothpastes, and various plastic products in the past decades (Latch et al., 2003; Dann and Hontela, 2011). Due to the long-term and widespread usage of these triclosan-containing products, triclosan has been detected in surface water, wastewater, and soil, as well as in human breast milk, blood, and urine samples (Adolfsson-Erici et al., 2002; Kolpin et al., 2002; McAvoy et al., 2002; Singer et al., 2002; Bester, 2003, 2005; Allmyr et al., 2006; Kinney et al., 2008; Queckenberg et al., 2010; Dann and Hontela, 2011). The occurrence of triclosan in the environment and humans has raised a great concern, because triclosan is a weak endocrine disrupting compound (Foran et al., 2000) and can be potentially transformed into more toxic chlorinated compounds in the environment (Latch et al., 2003). Furthermore, trace levels of triclosan have been suggested to promote the development of cross-resistance to antibiotics among bacteria (Schweizer, 2001). While triclosan is not regulated in the United States, the Canada and Japan restrict the use of triclosan in cosmetics and Germany banned the use of triclosan in food contact plastics (Japan Ministry of Health, 2006; Health Canada, 2007; European Commission, 2010). Several European countries, including Denmark, Sweden, Norway and Finland, have issued national consumer advisories for the use of triclosan (Winter, 1994; Allmyr et al., 2008; European Commission, 2010; Dann and Hontela, 2011; Latosińska et al., 2012).

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In addition to the growth-linked degradation of triclosan (Meade et al., 2001), triclosan can be cometabolically degraded by several oxygenase-expressing aerobic bacteria (Roh et al., 2009; Kim et al., 2011; Lee et al., 2012). Roh et al. (2009) reported that ammonia-oxidizing bacteria can degrade triclosan and suggested that ammonia monooxygenase (AMO) is responsible for the degradation. Kim et al. (2011) also suggested that diphenyl ether dioxygenase is involved in triclosan degradation based on the detection of hydroxylated triclosan. Recently, Lee et al. (2012) reported cometabolic biodegradation of triclosan and diphenyl ether by catechol 2,3-dioxygenase in *Sphingopyxis* strain KCY1 when the strain was pregrown with 20% R2A medium containing triclosan.

Oxygenases play an important role in the degradation/detoxification of pollutants in the environment. Mono- or di-oxygenases with a broad substrate specificity, including AMO (Hyman et al., 1988; Arciero et al., 1989; Rasche et al., 1991; Keener and Arp, 1994; Chang et al., 2002; Shi et al., 2004; Sayavedra-Soto et al., 2010), propane monooxygenase (PMO) (Wackett et al., 1989; Steffan et al., 1997; Sharp et al., 2005; Vainberg et al., 2006) and biphenyl dioxygenases (Furukawa et al., 2004; Fritsche and Hofrichter, 2005; Haritash and Kaushik, 2009; Robrock et al., 2011), have shown the ability to cometabolize a wide range of aliphatic and/or aromatic compounds. Accordingly, it is possible that other oxygenase-expressing bacteria can degrade triclosan via cometabolic reactions.

In this study, as triclosan is a polychlorinated hydroxydiphenyl ether, four oxygenase-expressing bacteria capable of degrading ethers and/or biphenyl were selected to examine their biodegradation potential of triclosan. The selected bacteria are *Mycobacterium vaccae* JOB5 (Perry, 1968), *Rhodococcus ruber* ENV425 (Steffan et al., 1997), *Rhodococcus jostii* RHA1(Seto et al., 1995a), and *Burkholderia xenovorans* LB400 (Seeger et al., 1997) (hereafter referred as JOB5, ENV425, RHA1, and LB400, respectively). These strains can grow on a wide range of carbon sources and express different oxygenases in responding to their growth substrates. When grown on propane, JOB5 and ENV425 can express propane monooxygenases (hereafter referred as PMO) to degrade different ethers, including methyl *tert*-butyl ether (MTBE) (Wackett et al., 1989; Steffan et al., 1997; Smith et al., 2003; Vainberg et al., 2006). Biphenyl-grown RHA1 and LB400 are known for their ability to express biphenyl dioxygenases (hereafter referred as BPDO) to degrade polychlorinated biphenyls (PCBs) (Masai et al., 1997; Arnett et al., 2000) and polybrominated diphenyl ethers (PBDEs) (Robrock et al., 2009). Interestingly, RHA1 can also grow on propane to express PMO (Sharp et al., 2007) and contains alkane monooxygenase (AlkMO) gene clusters (McLeod et al., 2006). JOB5 can express butane monooxygenase when grown on butane (Hamamura et al., 1999). Lopes Ferreira et al. (2007) demonstrated that AlkMO was induced in JOB5 when grown on alkanes. Thus, we investigated the effects of different growth substrates (propane, biphenyl, and complex medium like Luria–Bertani (LB) medium) on triclosan degradation potential of these four model strains.

In addition, product toxicity is one of unfavorable phenomena commonly observed during cometabolic degradation. The transformation metabolites of the cometabolic substrate can inactivate degradative microorganisms and/or enzymes to result in a finite transformation capacity for the cometabolic substrate, T_c (defined as the mass of cometabolic substrate degraded over the mass of biomass inactivated) (Chu and Alvarez-Cohen, 1998). Accordingly, when triclosan is a cometabolic substrate, the degradative microorganims and/or enzymes may be inactivated by the triclosan transformation products, leading to a complete cease of triclosan degradation. Viability of cells, before and after exposure to triclosan, was determined by counting the colonies on plates or by quantifying the 16S rRNA gene copies of viable cells, using real-time PCR with propidium monoazide (PMA). PMA, a DNA intercalating dye, can readily penetrate into membrane-compromised cells (presumably dead) to form covalent bonds with the DNA of the dead cells. The modified DNA of the dead cells cannot be PCR amplified (Nocker et al., 2007; Kobayashi et al., 2010); thus, samples pretreated with PMA will allow for discriminating viable from dead cells.

This is the first study to evaluate the triclosan biodegradation potential of oxygenase-expressing bacteria under different growth conditions. The degradation potential was evaulated in terms of its initial degradation rate, degree of triclosan dechlorination, and transformation capacity (T_c) if product toxicity occurs. Further, triclosan degradation metabolites and possible pathways were determined.

2. Materials and methods

2.1. Chemicals

Triclosan (97%) and biphenyl (99%) were purchased from Aldrich Chemical Inc. (Milwaukee, WI). Propane gas (>99.9%) and sodium formate were purchased from MP Biomedicals Inc. (Solon, Ohio). Tetrazotized *o*-dianisidine was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). Dicyclopropylketone (DCPK) (95%), and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO). 3-Fluorocatechol and naphthalene (99.6%) was purchased from Alfar Aesar (Ward Hill, MA). Propidium monoazide (PMA) was purchased from Biotium Inc. (Hayward, CA). Bicinchoninic acid (BCA) protein assay reagent kit and *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) were purchased from Pierce Biotechnology Inc. (Rockford, IL). Stock solution of triclosan ($1\ g\ L^{-1}$) was prepared in acetone.

2.2. Strains and culture conditions

Strains JOB5 and ENV425 were kindly provided by Dr. Robert Steffan, CB&I (Lawrenceville, NJ). LB400 was kindly provided by Dr. Rebecca Parales, University of California, Davis, CA. RHA1 was kindly provided by Dr. Bill Mohn, University of British Columbia, Canada.

Different growth substrates (propane, biphenyl, and LB medium) were used to induce the expression of oxygenases in the four selected strains (Table 1). Detailed description of the cultivation procedure is available in Supplementary information (SI). The activity of non-specific monooxygenase enzyme in the pregrown cells was confirmed using colorimetric naphthalene oxidation assay as applied previously (Chu and Alvarez-Cohen, 1998). Detailed description of the assay procedure is available in SI.

2.3. Triclosan degradation tests with resting bacterial strains

Triclosan degradation ability of four model strains was examined. The resting cells of theses strains were prepared and the degradation tests were conducted similarly as described by Lee et al. (2012) (See SI for the detail). Briefly, model strains were pregrown on different growth substrates and harvested in the late exponential growth phase by centrifugation at 10000 g for 5 min. The harvested cells were resuspended in fresh NMS medium to reach $OD_{600} \sim 1.0$ for degradation tests. Parallel sets of experiments with acetylene (an inhibitor of non-specific monooxygenases) were used to assess whether non-specific monooxygenases are responsible for triclosan degradation (Chu and Alvarez-Cohen, 1998) (See SI for the detail).

2.4. Chemical analysis

As triclosan is a polychlorinated compound, the amount of chloride released from triclosan biodegradation can be used to assess

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