Biodegradation of triclosan by a wastewater microorganism

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ABSTRACT

Triclosan, a synthetic antimicrobial agent, has been considered as an emerging environmental contaminant. Here we reported a triclosan-degrading wastewater bacterial isolate, Sphingopyxis strain KCY1, capable of dechlorinating triclosan with a stoichiometric release of chloride. The stain can degrade diphenyl ether but not 2,4,4′-tribromodiphenyl ether and 2,2′,4,4′-tetrabromodiphenyl ether, despite all these three compounds are structurally similar to triclosan. While strain KCY1 was unable to grow on triclosan and catechol, it could grow with glucose, sodium succinate, sodium acetate, and phenol. When grown with complex nutrient medium containing a trace amount of triclosan (as low as 5 μg/L), the strain could retain its degradation ability toward triclosan. The maximum-specific triclosan degradation rate (qm) and the half-velocity constant (Km) are 0.13 mg-triclosan/mg-protein/day and 2.8 mg-triclosan/L, respectively. As triclosan degradation progressed, five metabolites were identified and these metabolites continue to transform into non-chlorinated end products, which was supported by a sharp drop in androgenic potential. The activity of catechol 2,3-dioxygenase in the cell extract was detected. No triclosan degradation was observed in the presence of 3-fluorocatechol, an inhibitor of meta-cleavage enzyme, suggesting that triclosan degradation proceed via meta-cleavage pathway. Based on all the observations, a degradation pathway for triclosan by strain KCY1 was proposed.

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1. Introduction

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol) is a common synthetic antimicrobial agent that has been incorporated into more than 700 different industrial and personal care products. These products, including deodorants, soaps, toothpastes, and various plastic products, contain 0.1–0.3% triclosan (Sabaliunas et al., 2003; Schweizer, 2001; Singer et al., 2002). Not surprisingly, triclosan, ranging from 0.14 to 2.3 μg/L, was detected in 58% of 139 U.S. streams (Kolpin et al., 2002). The widespread of triclosan in the environment has raised a great concern, because the trace level of triclosan might promote the development of antimicrobial-resistant microorganisms (Braudaki and Hilton, 2004), and cause adverse effects on the ecosystem (Tatarazako et al., 2004). When exposed to UV, triclosan can potentially be transformed into more toxic chemicals like chlorodioxins (Latch et al., 2003; Rule et al., 2005). Triclosan has shown weak androgenic activity in aquatic species (Foran et al., 2000) and both estrogenic and androgenic responses in human breast cancer cells (Gee et al., 2008), suggesting triclosan itself is an endocrine-disrupting compound.

Biodegradation of triclosan in the environment and wastewater has recently become an interesting research topic (Hay et al., 2001; Kim et al., 2011; Meade et al., 2001; Roh et al., 2009; Sabaliunas et al., 2003; Schweizer, 2001; Singer et al., 2002). A previous study reported that approximately 79% of triclosan was removed by biological wastewater treatment processes (Singer et al., 2002), suggesting that (i) biodegradation can be an important removal mechanism in wastewater...
and (ii) triclosan-degrading bacteria are present in the activated sludge. These two aspects were supported by two later studies. For example, biodegradation of triclosan was observed by two wastewater microorganisms, Sphingomonas sp. Rd1 (Hay et al., 2001) and Nitrosomonas europaea (Roh et al., 2009), and by nitrifying activated sludge (Roh et al., 2009). Still, our knowledge about wastewater microorganisms capable of degrading triclosan is limited. Recently, a known diphenyl ether degrader, Sphingomonas sp. PH-07, showed an ability to partially degrade triclosan and produce three metabolites (hydroxylated triclosan, 4-chlorophenol, and 2,4-dichlorophenol) (Kim et al., 2011). To date, no complete dechlorination of triclosan was observed and triclosan degradation kinetics and pathways still remain unclear.

In this study, we report isolation and characterization of a wastewater triclosan-degrading bacterium, Sphingopyxis strain KCY1. This isolate showed complete dechlorination of triclosan based on stoichiometric release of chloride. We also determined triclosan degradation kinetics, proposed possible degradation pathway for triclosan, and assessed the potential significance of this isolate to triclosan biodegradation in wastewater.

2. Material and methods

2.1. Chemicals

Triclosan (TCS) (97% pure) was purchased from Aldrich Chemical Inc. (Milwaukee, WI). Dimethylformamide (DMF) was purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ). N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and bicinechnic acid (BCA) protein assay kit were purchased from Pierce Biotechnology Inc. (Rockford, IL). Diphenyl ether (DE), 2,4,4’-Tribromodiphenyl ether (tri-BDE) and 2,2’,4,4’-tetrabromodiphenyl ether (tetr-BDE, 1 mg/L) were purchased from Accustandard (New Haven, CT). 3-Fluorocatechol was purchased from Alfar Aesar (Ward Hill, MA). Fast DNA kit was purchased from Q-Biogene (Carlsbad, CA). Stock solution of 1 g/L triclosan was prepared in acetone.

2.2. Isolation and identification of triclosan-degrading bacteria

A triclosan-degrading consortium, originally inoculated with activated sludge, was used as a source for the isolation of triclosan-degrading bacteria. Briefly, a loopful of the consortium was streaked onto nitrate mineral salts (NMS)-triclosan (5 mg/L) agar plates that were incubated at 30 °C (Chu and Alvarez-Cohen, 1996). Due to slow and poor growth of colonies on the NMS-triclosan agar plates, colonies with different morphology were picked and re-streaked onto 1/10-strength R2A-triclosan agar plates (containing 5 mg/L of triclosan, 10% of R2A broth, and 10.8 g/L BBL selected agar). Only one of three isolates, named strain KCY1, was later confirmed with an ability to degrade triclosan in liquid medium. The 16S rRNA gene of strain KCY1 was sequenced as previously described (Yu et al., 2007). The sequence was aligned with other known triclosan-degrading bacteria using Clustal X2. A phylogenetic tree was constructed using TreeView software. The GenBank accession number for 16S rRNA sequence of strain KCY1 was DQ983313.2. Detail descriptions of enrichment, isolation, and confirmation of triclosan degradation ability of this isolate are available in Supplementary information (SI). The cell morphology of the isolate was observed by transmission electron microscopy (TEM) (Fig. S1 in SI).

2.3. Determination of degradation ability toward triclosan and compounds structurally similar to triclosan

The isolate was initially grown in 20% R2A medium with 5 mg/L triclosan for three days. The cell suspension was harvested by centrifugation and the pellet was washed with 50 mM phosphate-buffered saline and then resuspended in fresh NMS medium for experimental use. The degradation tests were conducted in 250 mL flasks containing the resting cell suspension and 5 mg/L of triclosan. The flasks were incubated on a rotary shaker at 150 rpm and at 30 °C, and liquid samples were collected over time for triclosan measurements. A subset of collected liquid samples was used for BLYES and BLYAS assays. Liquid samples collected from degradation experiments were also used to measure concentrations of chloride.

A parallel set of experiments was conducted to determine whether the isolate could degrade compounds that are structurally similar to triclosan. Three compounds, diphenyl ether (DE, 1 g/L), 2,4,4’-tribromodiphenyl ether (tri-BDE, 1 mg/L), and 2,2’,4,4’-tetrabromodiphenyl ether (tetr-BDE, 1 mg/L) (Kim et al., 2007; Robrock et al., 2009), were selected (Fig. S2 in SI). Particularly, PDBEs, used as flame retardants, are toxic and known as endocrine disruptors (Meerts et al., 2000). The degradation experiments were conducted similarly as described above, except using resting cell suspension (OD600 = 0.6–0.8) and each of these three compounds.

2.4. Resistance to other antimicrobial agents

Experiments were performed to determine whether strain KCY1 could resist to three common antimicrobial agents: kanamycin, trimethoprim and ampicillin (See SI for experimental details).

2.5. Determination of Monod kinetic parameters for triclosan degradation

Monod degradation kinetic model \( \frac{q}{q_m} = \frac{S}{K_s} + S \), was used to describe triclosan degradation by strain KCY1. The kinetic experiments were conducted in a series of 40 mL EPA glass vials containing resting cells of strain KCY1 (OD600 = 0.4) and triclosan (ranging from 0.3 to 5 mg/L) in NMS medium. The vials were incubated on a rotary shaker at 150 rpm and at 30 °C for 3 h, and then used for triclosan and protein measurements. The incubation duration (3 h) was determined in the laboratory where initial degradation rates remained linear. Experimental data obtained from kinetic tests were plotted as specific triclosan degradation rates (\( q \), mass of substrate/mass of cell protein/time) against triclosan concentrations (S, mass/volume). The maximum specific triclosan degradation rate (\( q_m \), mass of triclosan/mass of cell protein/time) and the half-velocity constant (\( K_{v0} \), mass of triclosan/volume) were determined by curve fitting using Sigmplot 8.0 (SPSS Inc.) as
previously described (Roh and Chu, 2010). All kinetic experiments were conducted in duplicate.

2.6. Determination of degradation/utilization ability toward other organics

Experiments were performed to determine whether the isolate could grow on three common macro-organics in wastewater: glucose (300 mg/L), sodium acetate (175 mg/L), and sodium succinate (300 mg/L) (Roh and Chu, 2010). These compounds were selected for the experiments because glucose is a common carbohydrate, and sodium succinate and sodium acetate are components present inside the tricarboxylic acid cycle (TCA cycle). Cell growth expressed as optical density (OD600), protein contents, and volatile suspension solids, was monitored over time. Doubling times were determined from the exponential growth phase curves. Autoclave-killed cells were used as negative controls.

2.7. Effect of complex nutrients on triclosan degradation

Cells grown in a complex nutrient medium without prior exposure to triclosan were tested for their ability to retain its biodegradation of triclosan. Experiments were conducted as follows. Strain KCY1 was grown in 100% R2A (fully nutrient-rich) medium without or with (5 or 500 μg/L) triclosan and transferred to its respective growth medium every two days. After four consecutive transfers, the cells were harvested as described above for degradation tests. The degradation experiments were conducted in glass vials containing 5 mg/L triclosan and the resting cells in NMS medium.

2.8. Bioluminescent androgenic/estrogenic screening assays

To evaluate androgenic and estrogenic potential of triclosan degradation metabolites and end products, the bioluminescent androgenic and estrogenic screening (BLYES and BLYAS) assays were performed as described previously (Eldridge et al., 2007; Sanseverino et al., 2005).

2.9. Determination of enzymes responsible for triclosan degradation

The isolate was screened for the presence of catechol 2,3-dioxygenase and/or catechol 1,2-dioxygenase using a spectrophotometric method as described previously (Klecka and Gibson, 1981; Nakai et al., 1988). In addition, triclosan degradation via meta-cleavage pathway was tested by adding 3-fluorocatechol (50 mg/L) or in the absence of it. 3-Fluorocatechol is an inhibitor of catechol 2,3-dioxygenase that catalyzes meta-cleavage reactions (Bartels et al., 1984; Toyama et al., 2010). Lack of triclosan degradation in the presence of 3-fluorocatechol would, therefore, suggest that a meta-cleavage reaction is essential for triclosan degradation.

2.10. Chemical analysis

Chloride concentrations were measured using a DX-80 Ion Chromatography (IC) system (Dionex, Sunnyvale, CA) equipped with an IonPac AS14A-5 μm analytical column (3 x 150 mm). Triclosan, tri-BDE, tetra-BDE, and DE concentrations and degradation metabolites were determined using a GC (Agilent 6890/MS (Agilent 5973) equipped with DB-5 column. In addition, to detect possible degradation metabolites, LC/MS analysis was performed using a Surveyor HPLC system (ThermoFinnegan, San Jose, CA) interfaced with quadruple ion trap mass spectrometer (LCQ-DECA; ThermoFinnegan). Detailed description of chemical analysis is available in SI.

3. Results and discussion

3.1. Identification of a triclosan-degrading microorganism, strain KCY1

Among three presumptive triclosan-degrading colonies, one isolate (yellow-mucoid), designated strain KCY1, showed the ability to degrade triclosan in NMS medium. Strain KCY1 is a short, rod-shaped (0.5 μm x 1.7 μm) Gram-negative bacterium with a flagellum (Fig. S1 in SI). Based on its 16S rRNA gene sequence, strain KCY1 is a member of the genus Sphingopyxis. As shown in Fig. 1, strain KCY1 had 98% similarity to Sphingopyxis sp. M20, a known phenanthrene-degrading bacterium (Bodour et al., 2003). Strain KCY1 also has 93% similarity to two DE degraders (Sphingomonas sp. PH-07 and Sphingomonas sp. SS3) and one known triclosan degrader (Sphingomonas sp. Rd1) (Hay et al., 2001; Kim et al., 2007; Schmidt et al., 1992). In addition, strain KCY1 has 80% homology to two well studied biphenyl degraders, Burkholderia xenovorans LB400 and Rhodococcus sp. RHA1, indicating no substantial relationship between strain KCY1 and these PCB-degrading strains (see Fig. S5 in SI). It is not surprising to observe the high homology between strain KCY1 and related species (family: Sphingomonadaceae). The phylogenetic tree was constructed using the neighbor-joining method with bootstrapping and rooted by referring to Methanococcus thermolithotrophicus. Bootstrap support values from 1000 replicates are indicated at branch nodes. The scale bar corresponds to 10 substitutions per 100 nucleotide positions. 1Triclosan-degrading bacteria; 2Diphenyl-ether degrading bacteria; 3Phenanthrene-degrading bacteria.
of strain KCY1 to other known pollutant degraders, since many members in the genera Sphingopyxis, and the sphingomonads were known for their ability to degrade a wide range of xenobiotic-pollutants (Stolz, 2009; Tani et al., 2011).

3.2. Characteristics of strain KCY1

Since strain KCY1 can grow rapidly on R2A agar, it is expected that the strain can also grow on glucose, sodium succinate, and sodium acetate (Fig. 2). Higher growth yield was observed when sodium acetate was supplied as a carbon source. The observed average yields (Y) ranged from 0.10 to 0.22 mg-protein/mg-BOD₅. The doubling times ranging between 5 and 9 h were observed. The ability of strain KCY1 to grow in these organics suggests that the strain can grow in the presence of common organics in wastewater. Although strain KCY1 can degrade triclosan (see below), no biomass/protein increase was observed for more than 30 days when the strain was supplied with triclosan as a sole carbon source in NMS medium (data not shown). This result suggested that strain KCY1 was unable to grow with triclosan. Furthermore, strain KCY1 was found to be kanamycin and trimethoprim resistant, but ampicillin sensitive (data not shown).

3.3. Degradation of triclosan by strain KCY1

As shown in Fig. 3, strain KCY1 degraded approximately 90% of initial triclosan (5 mg/L) in 24 h and reached about 100% removal on day 2. The initial specific degradation rate for triclosan ranged from 0.099 to 0.103 mg-triclosan/mg-protein/day (Fig. 3). Strain KCY1 was capable of completely degrading triclosan at a faster degradation rate than those of known triclosan degraders (Hay et al., 2001; Kim et al., 2011; Meade et al., 2001; Roh et al., 2009). Because previous studies did not contain sufficient data for the calculation of specific degradation rates, only qualitative comparison can be made below. For example, a much longer time (4–9 days) was needed for two soil triclosan-degrading bacteria, Pseudomonas putida and Alcaligenes xylosoxidans subsp denitrificans to degrade 0.15–0.18 mg/L of triclosan (Meade et al., 2001). Incomplete triclosan degradation was reported by Sphingomonas sp. Rd1 and triclosan-degrading consortium (35% of triclosan (500 mg/L) in 14 days) (Hay et al., 2001), and by N. europaea (50% of triclosan (1 mg/L) in 1 day) (Roh et al., 2009). A recent study showed that Sphingomonas sp. PH-07 can degrade 25% of triclosan (10 mg/L) in 8 days (Kim et al., 2011).

Since triclosan is a chlorinated compound, the amount of chloride release from triclosan biodegradation could be used to assess the degree of dechlorination. Assuming that 5 mg/L (0.017 mmol/L) triclosan is completely dechlorinated, a theoretical chloride concentration of 1.84 mg/L (0.052 mmol/L) is expected (note: the calculation is based on 3 mol of chloride is theoretically released per 1 mol of triclosan degraded).

#### Fig. 3 – Biodegradation of triclosan by the resting cells of strain KCY1. Reduction of androgenic potential in samples was observed during triclosan degradation. The relative androgenic responses of the samples over time were determined by dividing the initial response measured at time zero. Triclosan remaining (open circles); relative androgenic responses (open diamonds); chloride release (open squares). '3 mol of chloride is theoretically released per 1 mol of triclosan degraded.'
sensitivities between the yeast cells and human breast cancer cells. As shown in Fig. 3, the androgenic potential of the liquid samples decreased at three different paces: a very rapid decrease in the first 6 h, followed by a much slower decrease rate between hour 6 and day 2, and then declined at a moderate rate between day 2 and day 3. This decline pattern did not correspond well to the trend of triclosan degradation – approximately 95% of triclosan was degraded on day 1 and triclosan concentration was below detection limit on day 2. Some unknown intermediates with higher or less androgenic potential might have been produced during triclosan transformation. Nevertheless, at the end of triclosan degradation, these known metabolites and unknown intermediates converted to end-products/metabolites with non- and/or less androgenic potential.

Since triclosan is a chlorinated organic compound, the decrease of androgenic potential over time might correlate to the extent of dechlorination during the triclosan degradation. The decline of androgenic potential could be explained by the decrease in initial triclosan concentration and the transformation into less-chlorinated metabolites that were detected in this study before day 1 (see identification of metabolites below). Between day 1 and day 2, the reduction rate of androgenic activity became slower than the triclosan degradation rate, suggesting that (i) other androgenic metabolites might be produced during this period or (ii) androgenic metabolites might be transformed at a slower rate than the triclosan degradation rate. Interestingly, the androgenic response (~40% of original response) was observed despite that triclosan was no longer detected after day 2. This indicated a slow transformation of triclosan metabolites with androgenicity, like 2,4-dichlorophenol.

Previous studies have reported that 2,4-dichlorophenol exhibits androgenic activity in zebrafish embryos (Sawle et al., 2010) and in human prostate cancer cells (Kim et al., 2005). The statement is supported by 84% of the stoichiometric chloride release on day 2. On day 3, no androgenic potential of the sample was detected and greater than 100% recovery of chlorides was obtained, suggesting dechlorination and breakdown of the chlorine-containing metabolites into none-chlorinated end products with no androgenic activity.

3.6. Degradation kinetic parameters for triclosan

The results of triclosan degradation tests were used to develop the relationship between specific triclosan degradation rate (q)

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**Table 1 – Effects of growth medium on specific triclosan degradation rates by strain KCY1.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 0–1</th>
<th>Day 1–2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 100% R2A</td>
<td>No degradation</td>
<td>No degradation</td>
</tr>
<tr>
<td>B. 100% R2A + 5 µg/L triclosan</td>
<td>0.009 ± 0.004</td>
<td>0.060 ± 0.015</td>
</tr>
<tr>
<td>C. 100% R2A + 500 µg/L triclosan</td>
<td>0.022 ± 0.023</td>
<td>0.057 ± 0.022</td>
</tr>
<tr>
<td>D. 100% R2A + 5 mg/L triclosan</td>
<td>0.059 ± 0.008</td>
<td></td>
</tr>
<tr>
<td>E. 20% R2A + 5 mg/L triclosan</td>
<td>0.101</td>
<td></td>
</tr>
</tbody>
</table>

a Cells (A, B, and C) were subcultured four times (every 2 days) into the respective growth medium with different concentrations of triclosan ranging from 0, 5, or 500 µg/L before used for triclosan (5 mg/L) degradation experiments in NMS medium.

b Cells (D and E) were not subcultured before used for triclosan (5 mg/L) degradation experiments in NMS medium.

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and triclosan concentrations (Fig. 4). The Monod kinetic parameters were calculated by fitting nonlinear regression to the experimental data (SigmaPlot, SPSS Inc, version 16.0). Initial degradation rates were calculated by dividing the amount of triclosan degraded within 3 h over the initial biomass. No significant change in biomass was observed at the end of the 3-h kinetic experiments. The maximum specific triclosan degradation rate ($q_m$) and the half-velocity constant ($K_m$, mass of substrate/volume) were 0.13 mg-triclosan/mg-protein/day (or 1.7 mg-BOD$_5$/mg-protein/day) and 2.8 mg-triclosan/L (or 3.7 mg-BOD$_5$/L), respectively. The $K_m$ value is much higher than ambient concentrations of triclosan in wastewater (ranging from 0.61 to 5.1 µg/L) (Thompson et al., 2005; Yu and Chu, 2009), suggesting that triclosan degradation by strain KCY1 would follow the first order degradation kinetics in wastewater.

3.7. Degradation ability toward compounds which are structurally similar to triclosan

Since two BDEs (tri- and tetra- BDEs) and DE are structurally similar to triclosan and strain KCY1 can degrade triclosan, we hypothesized that strain KCY1 could degrade these compounds as well. Strain KCY1 was able to degrade approximately 78% of DE (1 g/L) within 5 days, but unable to use DE as a sole carbon source (data not shown). Although earlier it was reported that C–Br and C–Cl bonds are at least equally viable for enzymatic reaction (Dos Santos et al., 1999), strain KCY1 was unable to degrade tri-BDE and tetra-BDE. The inability of strain KCY1 to degrade these two BDEs could be due to a combination of various factors, including the difference in electron-withdrawing effects that would result from the difference between the halogen species (Cl vs Br) and the absence of hydroxy group in both BDEs that could contribute to selectivity of the enzymes. The reason why strain KCY1 can degrade DE but not tri- and tetra- BDEs was unclear in this study.

3.8. Enzymes involved in triclosan biodegradation

Another set of experiments was conducted to examine whether 3-fluorocatechol, a meta-cleavage inhibitor, would affect triclosan biodegradation. As shown in Fig. 5, the degradation of triclosan ceased after the addition of 3-fluorocatechol at 23-hr, suggesting that strain KCY1 might use a meta-cleavage pathway to degrade triclosan. In addition, only the activity of catechol 2,3-dioxygenase, not catechol 1,2-dioxygenase, was detected in the cell extract of strain KCY1 grown on triclosan (specific enzyme activity = 337 nmol/min/mg-protein). The overall result strongly suggests that a meta-cleavage pathway was involved in triclosan degradation. More studies are needed to further elucidate oxygenase enzymes that are responsible for triclosan degradation.

3.9. Degradation metabolites and possible degradation pathway for triclosan

During the triclosan biodegradation, five metabolites were identified. These metabolites are monohydroxy-triclosan, 6-chloro-3-(2,4-dichlorophenoxyl)-4-hydroxy-cyclohexa-3,5-diene-1,2-dione, 3-chloro-4-(5,7-dichloro-3-oxo-2,3-dihydrobenzo[1,4]dioxin-2-yl)-2-oxobut-3-enal, 3,5-dichloro-6-dihydroxycyclohexa-3,5-diene-1,2-dione, and 2,4-dichlorophenol (Fig. S3 and S4 in SI). 2,4-Dichlorophenol was confirmed using the authentic standard. The authentic standards for the other four metabolites are not commercially available. These metabolites were tentatively identified based on mass spectra and the fragmentation patterns. The two identified metabolites (monohydroxy-triclosan and 2,4-dichlorophenol) were among the seven triclosan metabolites previously observed during triclosan degradation by a diphenyl ether degrader, Sphingomonas sp. PH-07 (Kim et al., 2011). The other three identified metabolites have not been reported before. All the five metabolites were observed in the first 8-hr degradation time. However, past this time (8 h), only 2,4-dichlorophenol was observed in 24-hr and 32-hr samples, indicating continuous degradation of these metabolites. At the end of triclosan degradation experiments, a stoichiometric release of chloride ions was observed, suggesting complete dechlorination of triclosan by strain KCY1.

Here we proposed a biodegradation pathway for triclosan by strain KCY1 (Fig. 6). Based on the results of inhibition tests and enzyme activity assays, triclosan degradation is likely to follow a meta-cleavage pathway. Given that strain KCY1 was
able to degrade DE and that DE has been known to be degraded via a meta-cleavage pathway (Kim et al., 2007; Pfeifer et al., 1989), we proposed that an initial attack of a regioselective dioxygenase at the 2,3-position of triclosan which has resulted in the formation of 6-chloro-3-(2,4-dichlorophenoxy)-4-hydroxycyclohexa-3,5-diene-1,2-dione, and monohydroxy-triclosan (detected) and dihydroxy-triclosan (not detected) (reactions a and a1 in Fig. 6). Pfeifer et al. (Pfeifer et al., 1989) had reported that a further dioxygenation with simultaneous ether-bond cleavage occurred during diphenyl ether degradation. Thus, it is possible that the monohydroxy- and dihydroxy-triclosan were further attached by 2,3-dioxygenase and then subjected to an ether cleavage to produce 2,4-dichlorophenol (shown as reaction a2 or a3 or a4 in Fig. 6). Two other ring-fission intermediates might be formed; however, none of them were detected in this study. The detection of 3,5-dichloro-4,6-dihydroxycyclohexa-3,5-diene-1,2-dione also suggested another degradation route involving the occurrence of an ether bond cleavage of 6-chloro-3-(2,4-dichlorophenoxy)-4-hydroxycyclohexa-3,5-diene-1,2-dione (shown as reaction a5 in Fig. 6). Interestingly, strain KCY1 can grow on phenol, but not on catechol, suggesting that the utilization of phenol by strain KCY1 may not follow a catechol pathway, but a hydroquinone pathway (Bae et al., 1996). The complete recovery of chloride ions suggests that 2,4-dichlorophenol and 3,5-dichloro-4,6-dihydroxycyclohexa-3,5-diene-1,2-dione were completely dechlorinated (the reaction a6 in Fig. 6). The chlorinated metabolites might explain the delayed decrease of androgenic activity even after triclosan concentration decreased to zero on day 2 (see Fig. 3).

4. Conclusions

In this study, a wastewater isolate, Sphingopyxis strain KCY1, showed an ability to completely degrade triclosan with a stoichiometric release of chloride. This strain can grow with glucose, sodium succinate, and sodium acetate but not triclosan (data not shown) or catechol. Also, its triclosan degradation ability can be retained when the growth medium contained trace amount of triclosan, as low as 5 mg/L. Given that ambient triclosan concentrations in wastewater typically range from 0.61 to 5.1 μg/L and wastewater is full of readily available organics, strain KCY1 may play an important role in triclosan biodegradation in wastewater. Five different transformation metabolites were detected during triclosan biodegradation.
Our results also suggested that 2,3 dioxygenases and meta-cleavage are important for triclosan degradation. Future studies are needed to investigate the abundance of strain KCY1 in different built and natural environments, the significance of its role during triclosan degradation in wastewater, and the possibility of bioaugmentation with this strain for enhanced triclosan biodegradation in engineered systems.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2012.05.025.

References


Sanseverino, J., Gupta, R.K., Layton, A.C., Patterson, S.S., Ripp, S.A., Saidak, L., Simpson, M.L., Schultz, T.W., Sayler, G.S.,
2005. Use of Saccharomyces cerevisiae BLYES expressing bacterial bioluminescence for rapid, sensitive detection of estrogenic compounds. Applied and Environmental Microbiology 71 (8), 4455–4460.


Supplementary Information
for
Biodegradation of Triclosan by a Wastewater Microorganism

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Supporting Information: 12 pages, 5 figures
Enrichment and Isolation of Strain KCY1

Five mL of the activated sludge was added into a 250 mL flask containing 95 mL of nitrate mineral salt (NMS) medium (Chu and Alvarez-Cohen, 1996) and 5 mg/L of triclosan. The flask was incubated on a rotary shaker (150 rpm) at 30 °C. After 20 days of initial incubation, 10 mL of the cell suspension was transferred to a new flask containing the same growth medium. Every 10 days, the enrichment culture was transferred to a new flask and this procedure was repeated four times. The specific degradation rate of triclosan of the enrichment culture increased from 0.03 to 0.17 nmol/min/mg of protein. One loopful of the consortium was streaked onto noble agar plates containing NMS medium and 5 mg/L of triclosan. The plates were incubated at 30 °C. Since the colonies grew slowly on the NMS-triclosan noble agar plates, these colonies were picked and re-streaked onto 1/10-strength R2A agar plates containing triclosan (5 mg/L triclosan, 10% of R2A broth, and 10.8 g/L BBL selected agar). After numerous streaking, three colonies with different morphology were selected to examine their ability to degrade triclosan in NMS medium containing 5 mg/L of triclosan (triclosan was used as a sole carbon source). The concentrations of biomass were determined as optical density at absorbance 600 nm using UV-visible spectrophotometer (HP G1130A) and as protein content using a BCA protein assay kit, respectively.

Transmission Electron Microscopy (TEM)

Cell morphology was examined by transmission electron microscopy (TEM) performed with JEOL 1200EX equipped with the SIA-15C camera (JEOL, Tokyo Japan), using the exponentially growing cells. Strain KCY1 was prepared with the gram-stain, and then observed by TEM.
Resistance to Different Antimicrobial Agents

Strain KCY1 was examined for the resistance of three antimicrobial agents, kanamycin, trimethoprim and ampicillin. Strain KCY1 was streaked onto three 20% R2A agar plates containing 50 mg/L of kanamycin, 10 mg/L of trimethoprim, and 10 mg/L of ampicillin, respectively (Boehme et al., 2004). Also strain KCY1 was streaked onto 10% R2A agar plates containing 5 mg/L of triclosan as a positive control of the cell growth. The plates were then incubated at 30°C.

Chemical Analysis

Liquid samples were collected and acidified to reach pH 2-3 by adding concentrated sulfuric acid. The acidified liquid samples were extracted with ethyl acetate (1:1 v/v) at 350 rpm for 60 min, before adding anhydrous sodium sulfate. Then ethyl acetate layer (i.e. the upper-layer) was transferred to a new glass vial, purged with nitrogen gas to dryness, and then reconstituted in 450 µL of acetone. For GC/MS analysis, the reconstituted samples were derivatized with BSTFA (50 µL) in acetone. Triclosan concentrations were determined using a GC/MS in selective ion monitoring (SIM) mode as described by Roh et al. (Roh et al., 2009). In addition, different GC oven temperatures (Masai et al., 1997) in full scan mode was used to detect triclosan biodegradation metabolites.

The reconstituted samples were used directly for LC/MS analysis. The LC analysis was performed on a Surveyor HPLC system (ThermoFinnigan, San Jose, CA) interfaced with quadruple ion trap mass spectrometer (LCQ-DECA; ThermoFinnigan) APCI-MS. An Aquasil C18 column (2.1 × 150 mm, 3 µm; Thermo Hypersil-Keystone, Bellafonte, PA) was used for separation with water (A) and methanol (B) both containing 0.1% formic acid as mobile phases.
The elution gradient was as follows: 5% B for 2 minutes increased to 100% B in 25 minutes and held for 5 minutes. An APCI probe in negative ion mode was used for ionization. The MS operating conditions were optimized as follows: sheath gas and auxiliary gas flow rate, 50 and 10 arbitrary units, respectively; APCI vaporizer temperature, 450°C; corona current, 5 μA; and transfer capillary temperature, 150°C.

Liquid samples of tri- and tetra-BDE, and DE were prepared as described previously. (Kim et al., 2007; Robrock et al., 2009). The GC oven temperature program for BDEs concentrations was the same as previously described (Li et al., 2009). Standard concentration curves for BDEs ranging from 0.1 to 3 mg/L were used.

The chloride concentrations were measured using a DX-80 ion chromatography (IC) (Dionex, Sunnyvale, CA) equipped with an IonPac AS14A-5μm analytical column (3 × 150 mm). An eluent solution containing 0.16 M Na₂CO₃ and 0.02 M NaHCO₃ was used. The detection limit for chloride ion was 0.05 mg/L.

**Enzyme Activity Assays**

Cells of strain KCY1 grown on triclosan were harvested by centrifugation, washed twice with 50 mM phosphate buffer, and resuspended in the same buffer (Min et al., 2009). The cells were lyzed by ultrasonication (Min et al., 2009; Toyama et al., 2010) and the cell lysates were centrifuged at 15,000 × g at 4 °C for 30 min. The supernatant was used for enzyme assay and protein concentration measurement.

Activity assays for catechol 2,3-dioxygenase and catechol 1,2-dioxygenase were conducted at 25 °C as describe previously (Klecka and Gibson, 1981; Nakai et al., 1988). The reaction mixture contained 1.9 mL of 20 mg/L of catechol and 0.1 mL of cell extract in a final
volume of 2 mL. Formation of 2-hydroxymuconic semialdehyde was determined at 375 nm and

cis-cis-muconic acid was determined at 260 nm with a spectrophotometer (HP G1130A, USA).

Protein content was measured using a BCA protein assay kit. Enzyme activity was expressed as
a micromole of substrate per minute per mg of protein (Toyama et al., 2010).

**Bioluminescent Androgenic/Estrogenic Screening Assays**

The bioluminescent androgenic and estrogenic screening (BLYES and BLYAS) assays were performed as described previously (Eldridge et al., 2007; Sanseverino et al., 2005). The *S.
cerevisiae* BLYES and BLYAS were obtained from the University of Tennessee, Knoxville, Tennessee. After 6 hours of incubation, the bioluminescence was measured using a BioTek Synergy 4 Microplate Reader (BioTek, Winooski, VA). Testosterone (from 0.7 µM to 0.3 nM) and 17β-estradiol (E2) (from 0.1 µM to 5.4 nM) were used as reference compounds for androgenic and estrogenic responses, respectively. The 50% effective concentration (EC₅₀) of the reference compounds and samples were determined as described by Sanseverino et al. (Sanseverino et al., 2009).

**Ability of Strain KCY1 to Grow on Catechol**

The ability of strain KCY1 to grow on catechol was determined using agar plates containing 1.5% noble agar, catechol, and NMS medium. The plates were prepared as follows. After agar was solidified, the strain was streaked on the plates and crystal of catechol was placed on one side of the plate so that a concentration gradient of catechol was developed. The plates were incubated at 30 °C. No growth was observed on the plates, suggesting that the strain was unable to grow on catechol.
Fig. S1. Transmission electron micrograph of *Sphingopyxis* strain KCY1. The strain is rod-shaped (with a diameter of 1.5 µm and a length of 2.0 µm) and has a flagellum. Bar: 10 µm

Fig. S2. Chemical structures of 2,4,4’-tribromodiphenyl ether (tri-BDE) and 2,2’,4,4’-tetrabromodiphenyl ether (tetra-BDE)
Fig. S3. (a) Gas chromatogram of triclosan degradation metabolites as the TMS derivatives; GC mass spectrum of TMS-derivatized (b) 2,4-dichlorophenol (peak 1), (c) triclosan (peak 2), (d) 6-chloro-3-(2,4-dichlorophenoxy)-4-hydroxycyclohexa-3,5-diene-1,2-dione (peak 3) and (e) monohydroxy-triclosan (peak 4)
m/z = 390

m/z = 448
Fig. S4. (a) APCI-LC/MS of triclosan degradation metabolites; APCI mass spectrum at retention time (b) 25.3 min (peak 1), 3,5-dichloro-4,6-dihydroxycyclohexa-3,5-diene-1,2-dione; (c) 26.5 min (peak 2), monohydroxy-triclosan; (d) 27.0 min (peak 3), 3-chloro-4-(5,7-dichloro-3-oxo-2,3-dihydrobenzo[1,4]dioxin-2-yl)-2-oxobut-3-enal; (e) 27.9 (peak 4), triclosan.
Fig. S5. A phylogenetic tree showing relative relationship between the triclosan-degrading isolate, strain KCY1, and known triclosan degraders. The phylogenetic tree was constructed using the neighbor-joining method with bootstrapping and rooted by referring to Methanococcus thermolithotrophicus. Bootstrap support values from 1000 replicates are indicated at branch nodes. The scale bar corresponds to 10 substitutions per 100 nucleotide positions.

*Methanococcus thermolithotrophicus* [M59128]

\[ \textit{Rhodococcus jostii} \text{ RHA1}^{1,3} \] \textbf{Actinobacteria}

\[ \textit{Nitrosomonas europaea} \text{ [AB070983]}^{2,3} \]

\[ \textit{Alcaligenes} \text{ sp. Swo} \text{ [AF292240]}^3 \]

\[ \textit{Burkholderia xenovorans} \text{ LB400}^{1,3} \]

\[ \textit{Rhodanobacter} \text{ sp. Sy} \text{ [AF292242]}^3 \]

\[ \textit{Pseudomonas} \text{ sp. Ly} \text{ [AF292237]}^3 \]

\[ \textit{Pseudomonas} \text{ sp. Spd} \text{ [AF292239]}^3 \]

\[ \textit{Agrobacterium} \text{ sp. Sws} \text{ [AF292241]}^3 \]

\[ \textit{Sphingopyxis} \text{ Strain KCY1} \text{ [DQ983313.2]}^{3,4} \]

\[ \textit{Sphingomonas} \text{ sp. PH-07} \text{ [DQ185574]}^{3,4} \]

\[ \textit{Sphingomonas} \text{ sp. SS3} \text{ [HQ260903]}^4 \]

\[ \textit{Sphingomonas} \text{ sp. Rd1} \text{ [AF292238]}^3 \]

1 PCBs-degrading bacteria; 2 Ammonia oxidizing bacteria; 3 Triclosan-degrading bacteria; 4 Diphenyl-ether degrading bacteria; 5 PBDEs-degrading bacteria.
REFERENCES


fuliginis strains from Phragmites australis rhizosphere sediment. Applied and Environmental Microbiology 76(20), 6733-6740.