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Degradation Pathways of Trichloroethylene and 1,1,1-Trichloroethane by *Mycobacterium* sp. TA27

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We analyzed the kinetics and metabolic pathways of trichloroethylene and 1,1,1-trichloroethane degradation by the ethane-utilizing *Mycobacterium* sp. TA27. The apparent V_{max} and K_m of trichloroethylene were 9.8 nmol min^{-1} mg of cells^{-1} and 61.9 μM, respectively. The apparent V_{max} and K_m of 1,1,1-trichloroethane were 0.11 nmol min^{-1} mg of cells^{-1} and 3.1 μM, respectively. 2,2,2-trichloroethanol, trichloroacetic acid, chloral, and dichloroacetic acid were detected as metabolites of trichloroethylene. 2,2,2-trichloroethanol, trichloroacetic acid, and dichloroacetic acid were also detected as metabolites of 1,1,1-trichloroethane. The amounts of 2,2,2-trichloroethanol, trichloroacetic acid, chloral, and dichloroacetic acid derived from the degradation of 3.60 μmol trichloroethylene were 0.16 μmol (4.4%), 0.11 μmol (3.1%), 0.02 μmol (0.6%), and 0.02 μmol (0.6%), respectively. The amounts of 2,2,2-trichloroethanol, trichloroacetic acid and dichloroacetic acid derived from the degradation of 1.73 μmol 1,1,1-trichloroethane were 1.48 μmol (85.5%), 0.22 μmol (12.7%), and 0.02 μmol (1.2%), respectively. More than 90% of theoretical total chloride was released in trichloroethylene degradation. Chloral and 2,2,2-trichloroethanol were transformed into each other, and were finally converted to trichloroacetic acid, and dichloroacetic acid. Trichloroacetic acid and dichloroacetic acid were not degraded by strain TA27.

Key words: biodegradation; *Mycobacterium*; kinetics; pathway

Volatile chlorinated compounds such as trichloroethylene (TCE) and 1,1,1-trichloroethane (TCA), which have been widely used as organic solvents and degreasers, occur widely in wastewater and groundwater. Bioremediation, which is the practice of clean-up of contaminated sites by using microbial degradation activities for the pollutants, is one of the most promising and cost-effective new technologies for cleaning up groundwater contamination. For effective bioaugmentation, which is the practice of clean-up of contaminated sites by amending cultured microorganisms which are able to degrade the pollutants, it is important to measure the kinetic parameters and degradation products of TCE and TCA by the microbial inocula. TCE degradation products can be oxidized by aerobic bacteria that are able to utilize methane,[1–6] toluene,[7,8] phenol,[9] propane,[10–12] propylene,[13] and ammonia.[14] There have been few reports on the metabolites of TCE and TCA. *Methylosinus trichosporium* OB3b is known to metabolize TCE through production of TCE epoxide and chloral by a soluble methane monooxygenase.[4] Chloral is oxidized to trichloroacetic acid (TCAA) or reduced to 2,2,2-trichloroethanol (TCAol), while TCE oxide is hydrolyzed to dichloroacetic acid (DCAA), glyoxylic acid, formic acid, and carbon monoxide. As the degradation products of TCE by toluene dioxygenase are formic acid and glyoxylic acid, the degradation pathways might be different from the monooxygenase pathway.[7]

In earlier studies we isolated the ethane-utilizing bacterium, *Mycobacterium* sp. TA27, which can co-metabolically degrade TCE and TCA.[15–17] Only a few bacteria can degrade both TCE and TCA.[5,18] In this study, we investigated the kinetic parameters and degradation pathways of TCE and TCA by strain TA27.

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Abbreviations: TCE, trichloroethylene; TCA, 1,1,1-trichloroethane; TCAA, trichloroacetic acid; TCAol, 2,2,2-trichloroethanol; DCAA, dichloroacetic acid; OD, optical density; GC-FID, gas chromatography with a flame ionization detector

Materials and Methods

Chemicals. TCE, TCA, TCAA, DCAA, chloral, and TCAol were purchased from Wako Pure Chemicals (Osaka, Japan). [1,2-^{14}C] TCE (45 mCi mmol^{-1}, DuPont, Bostone, MA) was dissolved in methanol to make a stock solution of 1.58 mg of [1,2-^{14}C] TCE ml^{-1}. ACSII (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was a scintillation fluid used to measure radioactivity.

Kinetic experiments on TCE and TCA oxidation. Mycobacterium sp. TA27 isolated from soil was used throughout this experiment. This strain was grown in a mineral-salt medium in an ethane-air (2:8, vol vol^{-1}) atmosphere at 30°C for 3 days, as described in a previous paper.[15] The cells were harvested at late-log phase and washed twice with 0.05 M phosphate buffer (pH 7.0). The kinetic parameters of TCE and TCA oxidation were measured with resting cells of strain TA27. Five milliliters of reaction mixture containing strain TA27 cells and TCE or TCA in 0.05 M phosphate buffer at pH 7.0 were added to each 27-ml bottle, which was crimped with a butyl rubber stopper and an aluminum cap. The final cell concentration was 0.64 mg ml^{-1} (OD_{660nm} = 1.0). The oxidation rate was measured at 5 concentrations between 0.5 and 50 mg l^{-1} of TCE and TCA. The water concentrations of the TCE and TCA were calculated by using Henry's constant.[19] Reactions were carried out with shaking at 120 rpm at 25°C. The amounts of TCE and TCA degraded were measured by the head-space gas method using gas chromatography with a flame ionization detector (GC-FID). Kinetic parameters were calculated by the graphical methods of a Lineweaver-Burk plot using the values of oxidation rate (V) and substrate concentration (S). All kinetic tests were measured in duplicate.

Measurements of degradation products of TCE and TCA. Experiments on TCE, TCA, and their degradation products (TCAol, chloral, TCAA, and DCAA) were conducted in serum bottles (155 ml), each containing 30 ml of 0.05 M phosphate buffer (pH 7.0) with strain TA27. The cell concentration was 0.64 mg ml^{-1} in the degradation experiments of TCE and its degradation products, and was 3.2 mg ml^{-1} in the case of TCA degradation experiments. The bottle was crimped with a butyl rubber stopper and an aluminum cap. After the addition of TCE (6.83 μmol), TCA (4.12 μmol), chloral (2.04 μmol), TCAol (2.02 μmol), DCAA (2.33 μmol), or TCAA (1.84 μmol), the reaction mixture was incubated at 30°C for 24 h. All of the data are means of triplicate samples.

Quantities of TCAA, DCAA, TCAol and chloral in the reaction mixture were extracted into ethyl acetate as derivatives of 2,4-difluoroaniline and measured by GC-MS.[20,21] The reaction mixture in the serum bottle was centrifuged for 10 min at 10,000 × *g* and acidified to pH 1.7. The supernatant was transferred into a 100-ml separating funnel. One gram of NaCl was added to the funnel, which was shaken to dissolve the NaCl. Then 0.4 ml of 1 M 2,4-difluoroaniline in ethyl acetate, 0.4 ml of 1 M dicyclohexylcarbodiimide in ethyl acetate, and 15 ml of ethyl acetate were added, and the funnel was shaken for 40 min on a reciprocal shaker. After 5 g of NaCl had been added and dissolved, the aqueous layer was separated and extracted again with 5 ml of ethyl acetate. The combined ethyl acetate extract was successively washed with 3 M HCl, saturated $NaHCO_3$, and saturated NaCl, then dehydrated by anhydrous Na_2SO_4. The solution was evaporated to dryness and dissolved in 2 ml of benzene twice. The solution was analyzed by GC-MS. Detection limits of TCAA, DCAA, TCAol, and chloral were 0.2, 0.6, 10, and 10 μg l^{-1}, respectively. The amount of chloride released by TCE degradation was measured by using a chloride electrode (MA235 pH, Mettler Toledo, Osaka, Japan).

Radiolabeled TCE biodegradation. For the radioisotope-labeled TCE degradation experiment, 5.6 μCi of [1,2-^{14}C] TCE and 9 mg l^{-1} of unlabeled TCE were added to a 155-ml serum bottle containing 30 ml of 0.05 M phosphate buffer (pH 7.0) with strain TA27 (OD_{660nm} = 1.0). Measurement of ^{14}C activity in various fractions from reaction mixtures after 24 h was based on the method of Little *et al.*[1] The fractionation of CO_2, residual TCE, water-soluble, and cell fractions was carried out as follows. After the reaction mixture was alkalified with 1 N NaOH, aeration was started and residual TCE was removed by the addition of 30 ml of *n*-hexane 3 times. Next, the water fraction was acidified with 6 N HCl and the CO_2 evolved was adsorbed into 5 ml of 1 N NaOH solution (CO_2 fraction). The reaction mixture was alkalified with 1 N NaOH, and 1 ml of head-space gas was transferred to a vial with 5 ml of scintillation solution (residual TCE gas fraction). The reaction mixture was acidified with 1 N HCl, and then centrifuged at 7,000 rpm for 10 min. The radioactivity of the supernatant was measured (water-soluble fraction). The cells were washed with phosphate buffer (pH 7.0) twice, and their radioactivity was measured (cell fraction).

Gas chromatograph and GC-MS conditions. A gas chromatograph (model GC-14B, Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector and an electron ionization detector was used for measurement of volatile chlorinated compounds. The glass column (0.3 × 200 cm) was packed with Silicone DC550 (GL Science Inc., Tokyo, Japan). The temperatures of the injection port, oven, and detec-

tor were 250°C, 120°C, and 250°C, respectively. Nitrogen gas was used as a carrier. A QP5050 GC-MS system (Shimadzu Co, Kyoto, Japan) with an OV-17 capillary column (0.32 mm × 30 m; GL Science, Tokyo, Japan) was used with the following program: initial temperature 40°C for 5 min; temperature gradient of 5°C min^{-1} up to 100°C; temperature gradient of 10°C min^{-1} up to 230°C, and final temperature 230°C for 10 min. The temperatures of injection and the detector were kept at 200°C and 260°C, respectively.

Results

Kinetic parameters of TCE and TCA oxidation

The resting cells of strain TA27 degraded 13% and 54% of 10 mg l^{-1} TCA and TCE in 4 h, respectively. The values of 1/V and 1/S were calculated and plotted on Fig. 1. The apparent V_{max} and K_m of TCE oxidation were 9.8 nmol min^{-1} mg $cells^{-1}$ and 61.9 μM, respectively, by the graphical methods of Lineweaver-Burk. The apparent V_{max} and K_m of TCA oxidation were 0.11 nmol min^{-1} mg $cells^{-1}$ and 3.09 μM, respectively. The V_{max}/K_m of 0.033 for TCA oxidation was lower than that of 0.158 for TCE oxidation. Both values are on the same order level.

Identification of TCE and TCA degradation products

Table 1 shows the degradation products from TCE, TCA, chloral, TCAol, DCAA, and TCAA. Of the 6.83 μmol of TCE added, 52.7% was degraded by strain TA27. Small amounts of TCAol, TCAA, DCAA and chloral were produced from TCE. Eighty-six% of degraded TCA was transformed into TCAol. Small amounts of TCAA and DCAA were produced from TCA. Recovery of the 3 degradation products of TCA was almost 100%. It has been reported that the degradation product of TCA by *Methylosinus trichosporium* OB3b, *Nitrosomonas europaea*, and *Pseudomonas putida* G786 is TCAol.[2,18,22] Ours is the first study to report that the TCA degradation products are not only TCAol, but also TCAA and DCAA. In addition, we examined the degradation of the 4 metabolites. Chloral and

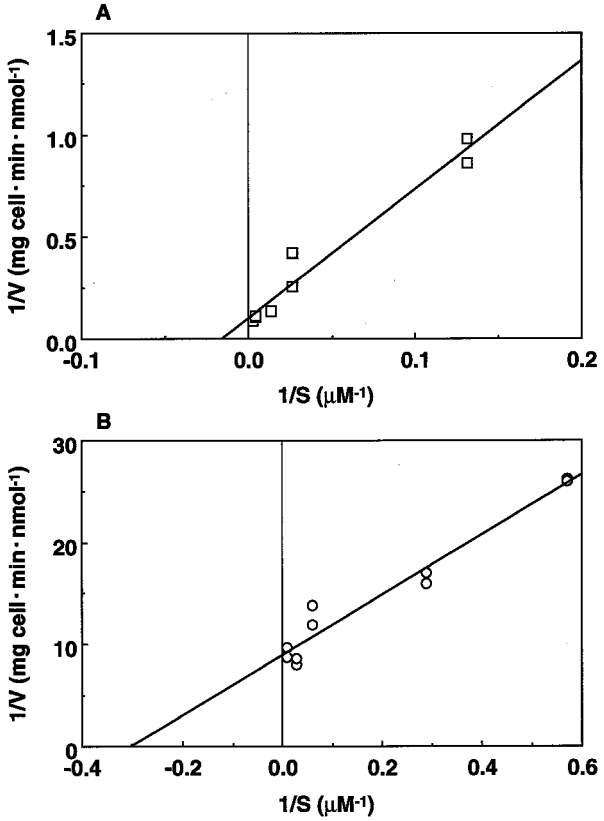

Fig. 1. Lineweaver-Burk Plot Used to Determine TCE (A) and TCA (B) Degradation Rates (V_{max}) and K_m.

Symbols: □, TCE; ○, TCA. Biodegradation was examined at 25°C and pH 7.0. Initial TCE concentrations were 1, 5, 10, 30, and 50 mg l^{-1}. Initial TCA concentrations were 0.5, 1, 5, 10, and 30 mg l^{-1}.

Table 2. Chloride Release from TCE and TCA Degradation by Strain TA27

Substrate	Amount degraded (μmol)	Theoretical amount of chloride released (μmol)	Free chloride released (μmol)	Chloride released (%)
TCE	2.38	7.14	6.59	92.1
TCA	1.73	5.19	0.02	0.3

The initial quantities of TCE and TCA were 6.83 μmol and 4.12 μmol, respectively.

Table 1. Degradation of TCE, TCA, and Their Products by Strain TA27

Substrate	Amount added (μmol)	Degradation (%)	Amount degraded (μmol)	Product amount (μmol)				
				Chloral	TCAol	DCAA	TCAA	total
TCE	6.83	52.7	3.60 ± 0.05	0.02 ± 0.01	0.16 ± 0.05	0.02 ± 0.00	0.11 ± 0.02	0.33 ± 0.07
TCA	4.12	42.2	1.73 ± 0.02	$<2.04\times10^{-3}$	1.48 ± 0.22	0.02 ± 0.00	0.22 ± 0.02	1.72 ± 0.23
Chloral	2.04	95.7	1.96 ± 0.02	—[a]	1.59 ± 0.20	0.06 ± 0.01	0.36 ± 0.02	2.02 ± 0.22
TCAol	2.02	12.5	0.25 ± 0.01	0.15 ± 0.05	—[a]	0.03 ± 0.00	0.09 ± 0.01	0.27 ± 0.07
DCAA	2.33	0.0	0.00 ± 0.00	$<2.04\times10^{-3}$	$<2.02\times10^{-3}$	—[a]	$<3.67\times10^{-5}$	—[b]
TCAA	1.84	0.0	0.00 ± 0.00	$<2.04\times10^{-3}$	$<2.02\times10^{-3}$	$<1.40\times10^{-4}$	—[a]	—[b]

Values are means (standard error). a: Degradation substrate, b: below detection limit. Abbreviations for compounds: TCAol, 2,2,2-trichloroethanol; DCAA, dichloroacetic acid; TCAA, trichloroacetic acid.

TCAol were transformed into each other and finally became TCAA and DCAA. The acidic compounds such as TCAA and DCAA were not degraded by strain TA27. The recovery percentages from chloral and TCAol degradation were almost 100%. Therefore, it was confirmed that the degradation of TCA did not result in mineralization.

Distribution of ^{14}C labeled TCE degradation products

[1,2-^{14}C] TCE was used to measure TCE mineralization. Thirty-five per cent of the total amount of TCE in the gas and liquid fractions remained undegraded. The distributions of radioactivity in the cell, CO_2, and water-soluble fractions represented 80.8%, 15.6%, and 3.6% of the degraded TCE (100%), respectively. Therefore, TCE was mineralized by the ethane-utilizing bacterium, strain TA27. Also, a compound corresponding to 0.14% of the formic acid in degraded TCE produced was detected by HPLC analysis to be a water-soluble product of TCE degradation (data not shown).

Chloride release by TCE degradation

Significantly high radioactivity of 80.8% for degraded TCE was observed in the cell fraction. We examined the amount of chloride released by TCE and TCA degradation (Table 2). If all of the chloride were to be released from the degraded TCE, the amount released would be 7.14 μmol. After 24 h of incubation, 6.59 μmol of chloride was measured. The recovery percentage was 92.1%. In TCA degradation by strain TA27, little chloride was released.

Discussion

Several reports have supplied kinetic data for aerobic TCE-degrading bacteria (Table 3). By varying the TCE concentration from 5 mM to 75 mM, the V_{max} and K_m of toluene- and phenol-oxidizing bacteria were calculated as 8 to 20 nmol min^{-1} mg protein^{-1} and 4 to 10 μM for *Pseudomonas cepacia* G4, *P. mendocina* KR1 and *P. putida* F1.[9] V_{max} and K_m of 509 ± 174 nmol min^{-1} mg protein^{-1} and 145 ± 61 μM were obtained for *Methylosinus trichosporium* OB3b grown in continuous culture under copper stress with 20 mM formate.[5,9] The V_{max} and K_m of strain TA27 for TCE degradation were 9.81 nmol min^{-1} mg cells^{-1} (28.3 nmol min^{-1} mg protein^{-1}) and 61.9 μM. These values were similar to those for other bacteria, apart from *Methylosinus trichosporium* OB3b. It seems that the high V_{max} obtained for OB3b was based on the addition of formate as an energy source.

We calculated the V_{max} and K_m of strain TA27 for TCA degradation (Table 3). The kinetic parameters of TCA degradation had previously been reported only for *Methylosinus trichosporium* OB3b by Oldenhuis.[5] The V_{max} for TCA degradation by strain TA27 was much lower than that by OB3b. However, strain TA27 has a strong ability to degrade TCA under growing conditions.[15] Therefore, it seems that degradation activity is increased by the presence of an energy source. Strain TA27 had a higher affinity for TCA than did OB3b.

Figure 2 shows a hypothetical pathway for TCE and TCA degradation by strain TA27. Some reports have described TCE degradation pathways in aerobic bacteria. TCE is initially converted to chloral and TCE oxide by methane-oxidizing bacteria.[4,21,24,25] Then, TCE oxide and its metabolites are converted to carbon dioxide, while chloral is oxidized to TCAA and DCAA, or reduced to TCAol. The quantities of metabolites and the pathways of TCE degradation by strain TA27 were similar to those of degradation by methane-oxidizing bacteria. TCAol and chloral were detected as TCE degradation products of a propane-oxidizing bacterium, *Mycobacterium vaccae* JOB-5, and these products accounted for 25% of the degraded TCE.[12] However, strain TA27, like the metanotrophs, produced less TCAol and chloral than *M. vaccae* JOB-5.

TCE mineralization by several bacteria has been

Table 3. Comparison of Michaelis-Menten Kinetic Parameters for TCE and TCA Degradation

Microorganisms	Substrate	Enzyme	Growth substrate	V_{max}	K_m (μM)	Reference
Mycobacterium sp. TA27	TCE	Ethane monooxygenase	ethane	28.3[a]	62	This study
Methylosinus trichosporium OB3b	TCE	Methane monooxygenase	methane	509[a]	145	5
				37.5[a,c]	75	9
Xanthobacter sp. strain Py2	TCE	Alkene monooxygenase	propene	16[a]	116	13
Rhodococcus corallinus B-276	TCE	Alkene monooxygenase	NBG medium	2.4[a]	187	23
Pseudomonas cepacia G4	TCE	Toluene 2-monooxygenase	phenol	9[a]	4	9
Pseudomonas mendocina KR1	TCE	Toluene 4-monooxygenase	phenol	20[a]	10	9
Pseudomonas putida F1	TCE	Toluene dioxygenase	toluene	8[a]	5	9
Nitrosomonas europaea	TCE	Ammonia monooxygenase	ammonia	—	30	14
Mycobacterium sp. TA27	TCA	Ethane monooxygenase	ethane	0.1[b]	3	This study
Methylosinus trichosporium OB3b	TCA	Methane monooxygenase	methane	214[b]	24	5

[a] (nmol min^{-1} mg protein^{-1}).
[b] (nmol min^{-1} mg cell^{-1}).
[c] Initial V (nmol min^{-1} mg protein^{-1}).

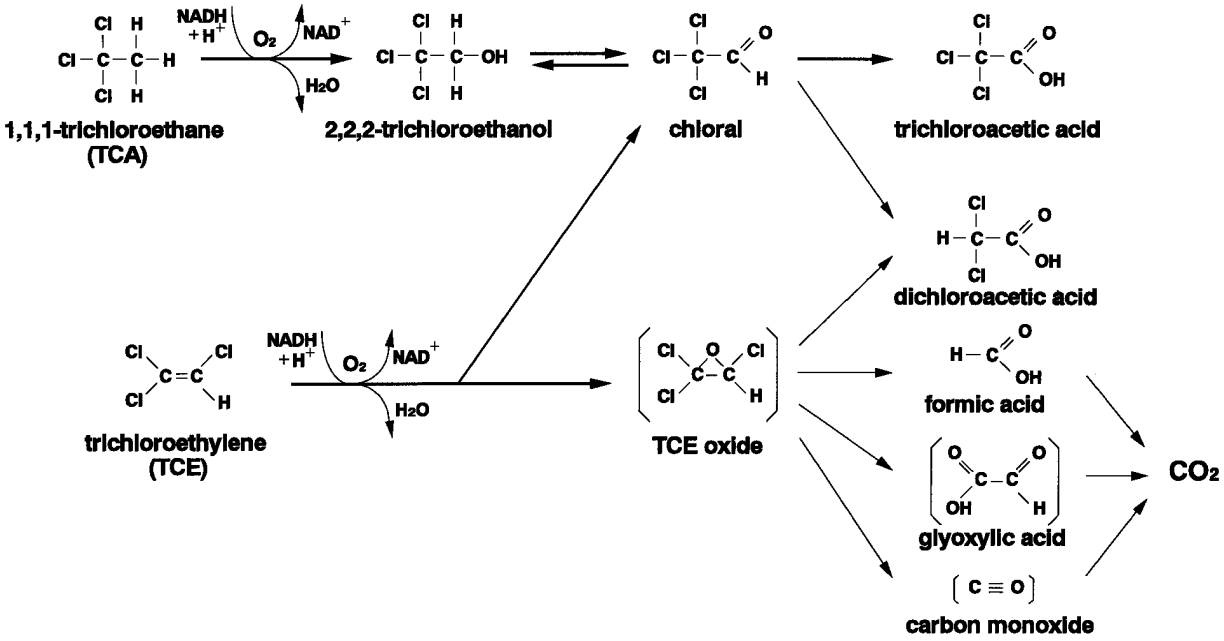

Fig. 2. Proposed Pathway of TCA and TCE Degradation by Strain TA27.

studied. Of the degradation products of *Rhodococcus rhodochrous* and *Rhodococcus* sp. strain Sm-1 with propane monooxygenase, CO_2 constituted 30%, the aqueous fraction 50%, and biomass 10%.[10] For *M. vaccae* JOB-5 degradation of TCE, the distribution of radioactivity was 12% in the CO_2 fraction, 86% in the water-soluble fraction, and 3% in the cell-associated fraction.[12] The distribution of radioactivities was 32% to 41% in the CO_2 and 3.7% to 36% in the cell-associated fractions for methane-oxidizing bacteria.[1,25] With strain TA27, more than 80% of the radioactivity was in the cell fraction, and this was very high compared with that of other bacteria. About 90% of the amount of chloride that would theoretically be released was released from TCE. From these results, it seemed that TCE was converted to less toxic compounds.

There have been a few reports on TCA degradation pathways. It was reported that TCAol was the only product of degradation by *Methylosinus trichosporium* OB3b, *Nitrosomonas europaea*, and *Pseudomonas putida* G786.[2,18,22] The production of TCAA from TCA by cytochrome P450 has been reported in humans.[26] In our experiment, strain TA27 produced TCAA and DCAA from TCA. However, strain TA27 was unable to release chloride through TCA degradation. *Xanthobacter autotrophicus* GJ10 and an aerobic TCAA-decomposing bacterium, TCA1, are able to degrade DCAA and TCAA, respectively.[27,28] Therefore, complete degradation of TCA by a mixed culture of strain TA27 and TCAA-degrading bacteria will be possible.

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