Biodegradation of Dodecyltrimethylammonium Bromide by *Pseudomonas fluorescens* F7 and F2 Isolated from Activated Sludge

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Two dodecyltrimethylammonium bromide (DTAB)—degrading bacteria were isolated from activated sludge of a wastewater treatment plant (WWTP). The bacteria identified as *Pseudomonas fluorescens* was called F2 and F7. The BOD biodegradability of DTAB by these bacteria were relatively high and similar to that of the activated sludge. F7 grew in a basal medium containing DTAB as the sole carbon source, producing trimethylamine (TMA) from DTAB by a N-dealkylation process. Then, the intermediate TMA was degraded to dimethylamine (DMA) by N-dimethylation as a second step. The pathway of the degradation of DTAB by this strain was the same as that of the activated sludge. On the other hand, *Ps. fluorescens* F2 degraded DTAB to TMA, but TMA was not further degraded. As for *Ps. fluorescens* F7 degrading activity, anionic compounds, such as fatty acids and sodium dodecyl sulfates (SDS), enhanced the degradation and dodecanoate became the carbon and energy source for this bacteria strain. These results demonstrate that monoalkyl quaternary ammonium compounds are ultimately degraded through N-dealkylation and mineralization, by a bacterium isolated from activated sludge.

Key words: quaternary ammonium compounds, surfactant, biodegradation

Quaternary ammonium compounds (QACs), major surfactants, have been widely used as disinfectants, dispersion agents and other products. QACs have one or two hydrophobic alkyl chains at a quaternary nitrogen atom. When QACs are discharged into the aquatic environment, attention should be paid to their effects, because such chemicals have an antimicrobial action at relatively low concentrations (Kakiuchi, 1993, Stephans *et al.*, 1994, Kikuchi *et al.*, 1996). A study on the biodegradation of QACs showed they were ultimately biodegradable when present at low concentrations in river surface water and/or sediments (Larson and Vashon, 1983). However, their biodegradation mechanism is little known, although there are some reports on monoalkyl or dialkyl-QACs degrading bacteria.

Dean-Raymond and Alexander (1977) reported that a mixture of *Pseudomonas* and *Xanthomonas* isolated from soil and sewage grew in a medium consisting of decyltrimethylammonium salt as a sole carbon source, and that *Xanthomonas* alone in the presence of yeast extract and casamino acids oxidized the terminal carbon of the long alkyl chain of the salt. van Ginkel (1992) demonstrated the cleavage of the N-C bond of hexadecylammonium salt by *Pseudomonas* sp. Nishiyama *et al.* (1995) showed the biodegradation pathway of monoalkyl-QACs by activated sludge obtained from a WWTP (domestic sewage mainly). Recently, Nishihara *et al.* (2000) found the biodegradation pathway of didodecyltrimethylammonium salt and broad assimilation activity for QACs using a degrading bacteria isolated from activated sludge of a WWTP. Thus, it is worthwhile studying the microbial contribution to the degradation of monoalkyl-QACs. In this paper, we report the biodegrada-

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tion pathway of DTAB—degrading bacteria isolated from activated sludge from a WWTP.

Materials and Methods

Isolation of bacteria and degradation assay

DTAB—degrading bacteria were isolated from the activated sludge of a municipal wastewater treatment plant by using enrichment and agar plate cultures. The sludge was collected from a WWTP in Utsunomiya, Tochigi, Japan in March 1993. The basal medium used for enrichment culture consists of 15 mg of DTAB, 30 mg of activated sludge, 65.3 mg of K_2HPO_4 , 26.0 mg of KH_2PO_4 , 134 mg of $Na_2PO_4/12H_2O$, 5.1 mg of NH_4Cl , 67.5 mg of $MgSO_4/7H_2O$, 82.5 mg of $CaCl_2$ and 0.75 mg of $FeCl_3/6H_2O$ per liter. The pH of the medium was adjusted to 7.2. Incubation was carried out at 25°C under shaking. When growth was visible, the enrichment culture was streaked on agar plate containing the basal medium.

Chemicals

Dodecyltrimethylammonium bromide was purchased from Tokyo Kasei Kogyo Co., Ltd., Japan. Other reagents were of the highest purity available.

Analytical methods

1H nuclear magnetic resonance spectrometry (1H -NMR), Ion chromatography and dissolved organic carbon analysis were used for the quantification and identification of DTAB and its degradation products.

1H -NMR analysis: The cultured medium in each flask was filtrated through a GF/C filter and evaporated to dryness. The residue was dissolved in d_4 -methanol, passed through a 0.45 μm filter, and again evaporated to dryness. This was followed by the addition of D_2O containing disodium fumarate as an internal standard. The conditions for this analysis were as follows: frequency, 270 MHz; temperature, 40°C; frequency range, 6000 Hz; scan, 128 times; apparatus, JMN-EX270 (JEOL, Japan).

Ion chromatography analysis: The cultured medium in each flask was analyzed by ion chromatography after filtration. The conditions for ion chromatography were as follows: separation column, TSKgel IC-Cation (4.6 mm i.d. $\times$ 50 mm, TOSOH, Japan); mobile phase, 2 mM HNO_3 , 1.2 mL/min; temperature, 40°C; apparatus, IC 7000 ion chromatographic analyzer (Yokogawa analytical systems, Japan).

DOC analysis: The concentration of dissolved organic carbon (DOC) in the filtrated culture medium was deter-

mined with a TOC-5000 total organic carbon analyzer (Shimadzu Corp., Japan).

Biological oxygen demand (BOD) was determined using an automatic oxygen consumption measuring apparatus (Courometer, Okura Electric Co., Ltd., Japan) according to OECD modified MITI test (I) (1981). The volume of basal culture volume was 300 mL and the concentration of DTAB was 30 mg/L.

Results

Isolation and identification of DTAB—degrading bacteria

Two strains of bacteria were isolated from the enrichment culture of the activated sludge. They were identified as *Pseudomonas fluorescens* according to Bergey's manual of Determinative Bacteriology (1974), based on morphological and physiological characteristics as well as electronmicroscopic findings (Table 1, respectively). These strains were called *Ps. fluorescens* F2 and *Ps. fluorescens* F7.

Degradation activities of *Ps. fluorescens* F7 and F2

The DTAB degradation activity of the isolates was represented as a ratio of the biochemical oxygen demand (BOD) to the theoretical oxygen demand (ThOD). Figure 1 shows the BOD degradability curve of F7, F2 and the activated sludge obtained from a WWTP. DTAB degradation by *Ps. fluorescens* F7 and F2 started within 48 hours, and the BOD degradability was 60% within a week. The two strains as well as the activated sludge had high-degrading activity.

Degradation products of DTAB treated with *Ps. fluorescens* F7 and F2

DTAB and its degradation products in the cultured medium were identified and determined by 1H -NMR and ion chromatography. In the case of F7, trimethylamine (TMA) and dimethylamine (DMA) were detected. Figure 2 shows the time course changes in the concentration of DTAB and its intermediates during treatment with *Ps. fluorescens* F7. After 7 days of culture, TMA was initially detected and after 8 days dimethylammonium (DMA) was detected. After 9 days, the sum of molar concentrations of DTAB and its intermediates accounted for 32% of the initial concentration of DTAB.

From these results, we propose that the pathway of biodegradation of DTAB by *Ps. fluorescens* F7 is as follows: DTAB is initially degraded to TMA through an N-C bond cleavage (N-dealkylation); then, TMA is degraded to DMA by another N-C bond cleavage (N-demethylation) (Fig. 3).

Table 1. Morphological and physiological characteristics of *Pseudomonas fluorescens* F2 and F7.

	F2	F7
Shape	rod	rod
Gram reaction	—	—
Spore	—	—
Motility	+	+
Flagella	Polar (multitrichous)	Polar (multitrichous)
Oxidase	+	+
Catalase	+	+
O-F test	O	O
Color of colony	NP	NP
Fluorescent pigments	+	+
Water soluble pigments	+	+
Accumulation of PHB	—	—
Growth at 41°C	—	—
Levan formation from sucrose	—	+
Arginine dihydrolase	+	+
Denitrification	+	+
Nitrate reduction	+	+
Gelatin liquefaction	+	—
Starch hydrolysis	—	—
Lecithinase	+	—
Lipase (Tween 80 hydrolysis)	+	—
Quinones	Q-9	Q-9
DNA base ratio G+C, moles%	63	62
Carbon sources for growth:		
Glucose	+	+
Trehalose	+	+
2-Ketogluconate	+	+
Inositol	—	+
Geraniol	+	—
L-Valine	+	+
β-Alanine	+	+
DL-Arginine	+	+
L-Tartaric acid	—	—
Ethanol	+	+
D-Alanine	+	+
Sucrose	—	+
Mannitol	—	+
Sorbitol	—	+
Rhamnose	—	—

Although we did not detect any other product by the chemical analyses performed, it is probable that fatty acids, aliphatic aldehyde and/or alcohol were also produced but were immediately assimilated by this bacterium.

Figure 4 shows the time course changes in the concentration of DTAB and its intermediate by another DTAB de-

Fig. 1. BOD degradability curves of DTAB by isolated bacteria and activated sludge. Symbols are as follows: ▲, *Pseudomonas fluorescens* F2; ■, *Pseudomonas fluorescens* F7; and ○, activated sludge.

Fig. 2. Time course of DTAB degradation by *Pseudomonas fluorescens* F7. ■, DTAB; □, TMA; △, DMA; and ○, OD₆₆₀.

grading bacteria *Ps. fluorescens* F2. This strain stoichiometrically produced TMA without further N-demethylation. DTAB was degraded by this bacterium to TMA as the terminal product, but in the environment TMA might be subjected to N-demethylation by other bacteria, for instance the *Ps. fluorescens* F7 strain described above (Fig. 3).

Effects of co-existing chemicals on DTAB degradation by *Ps. fluorescens* F7

The effects of co-existing chemicals on DTAB degradation caused by *Ps. fluorescens* F7 were evaluated. The co-existing chemicals used were dodecanal and dodecanoate as predicted intermediates of DTAB, yeast extracts and glucose which are generally used in the culture of bacteria, sodium dodecyl sulfates (SDS) and linear alkylbenzene sulfonates (LAS) of anionic surfactant.

Dodecanoate, SDS and LAS remarkably enhanced the degradation of DTAB by *Ps. fluorescens* F7 (Fig. 5). In the

Anionic compounds may accelerate the degradation of DTAB, because these chemicals form an ionic pair complex with DTAB, of cationic compound, reducing thereby its antimicrobial action. It is interesting to note that dodecanoate, one of the predicted intermediates, accelerates the degradation and becomes the sole source of carbon and energy for

In contrast, DTAB degradation was not affected by the presence of dodecanal or yeast extracts, and glucose delayed the degradation of DTAB.

Ps. fluorescens F7 pre-incubated in nutrient broth or basal medium was inoculated in basal medium. We assumed that if DTAB degradation by *Ps. fluorescens* F7 depended on an inducible enzyme, it affected the rate of DTAB. As shown in Figure 6, no difference in the rate of DTAB degradation between nutrient broth or basal medium for pre-incubation was observed, suggesting no involvement of inducible enzyme.

Ps. fluorescens F7 and F2 isolated from activated sludge of a WWTP degraded DTAB to TMA by N-dealkylation, as the first step of the degradation process. This process was similar to that for alkyl (C10-C18) in activated sludge of a WWTP, as reported by Nishiyama *et al.* (1995). The BOD degradability of DTAB was also relatively high and similar to that of the activated sludge. These results suggested that

Fig. 5. Effects of dodecanoate (A), SDS (B) and LAS (C) on the degradation of DTAB. ■, DTAB; □, TMA; △, DMA; and ○, OD₆₆₀.

Ps. fluorescens F7 and F2 played an important role in DTAB degradation by activated sludge of a WWTP.

As illustrated in Figure 3, there was a difference in the degradation of DTAB between *Ps. fluorescens* F7 and F2. The TMA intermediate was degraded by *Ps. fluorescens* F7, but not by *Ps. fluorescens* F2. van Ginkel *et al.* (1992) have reported that *Pseudomonas* sp. isolated from activated sludge produced the final degradation product TMA from

Fig. 6. Effect of DTAB degradation by pre-incubation, in the basal medium (A), nutrient broth (B). ■, DTAB; □, TMA; △, DMA; and ○, OD₆₆₀.

hexadecyltrimethylammonium chloride. In our study, *Ps. fluorescens* F2 also produced TMA from DTAB. Although these microorganisms may play a role in the aquatic environment, mineralization of DTAB was not completely achieved. Consequently, for complete mineralization these microorganisms need the help of other microorganisms, which can degrade TMA.

On the other hand, *Ps. fluorescens* F7 produced TMA from DTAB through a N-dealkylation process, and then TMA was transformed to DMA. It appeared that the microorganisms, which ultimately degraded QACs having long alkyl chains alone as *Ps. fluorescens* F7 has the potential for, complete the mineralization without any help for other microorganisms. A kind of microorganism able to completely degrade QACs by itself, might be useful for the biological treatment of wastewater containing QACs.

The acceleration of DTAB degradation in the presence of dodecanoate, LAS or SDS, implies a relationship between DTAB and anionic compounds in the medium. Nishihara *et al.* (1998) proposed that sodium laurate depresses the biocidal activity of didecyldimethylammonium chloride

(DDAC) against *Staphylococcus aureus*, because sodium laurate can form a less active complex with DDAC by both ionic and hydrophobic interactions. We thought that *Ps. fluorescens* F7 reduced DTAB degradation in medium containing 500 mg/L of DTAB without co-existing anionic chemicals, because the minimum inhibition concentration of DTAB was 125 mg/L for this microorganism. Masuda *et al.* (1976) showed that the biodegradability of QACs was stimulated by the addition of SDS to the medium. These findings suggested the DTAB degradation by *Ps. fluorescens* F7 might be accelerated by the formation of an DTAB/anionic co-existing chemical complex. Moreover, dodecanoate, one of the anionic co-existing chemicals tested, accelerates DTAB degradation and become the carbon source and energy source in the growth of *Ps. fluorescens* F7.

If the N-dealkylation of DTAB by *Ps. fluorescens* F7 depended on an inducible enzyme, DTAB N-dealkylation should be affected by the presence of DTAB in the pre-incubation medium. To determine the effect of the pre-incubation medium, the incubation was done in medium containing DTAB or nutrient broth. As shown in Figure 6, no effect was observed on the growth of *Ps. fluorescens* F7 pre-incubated in basal medium or nutrient broth. Although we cannot decide without investigating the enzyme level, this result suggested that N-dealkylation of DTAB did not depend on an inducible enzyme.

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