土壌中におけるクロロタロニル分解細菌の菌数変化と特性

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Original Article

Population Change and Characteristics of Chlorothalonil-Degrading Bacteria in Soil*

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Bacteria that degraded chlorothalonil (TPN, 2,4,5,6-tetrachloro-1,3-isophthalonitrile) were present in soils with no history of chlorothalonil application at population levels of 6×10^6 to 12×10^6 CFU/g-soil and were enriched by chlorothalonil application to higher population levels of 2×10^7 to 8×10^7 CFU/g-soil. The chlorothalonil-degrading bacteria were tolerant to high concentrations of chlorothalonil. Eleven strains of chlorothalonil-degrading bacteria were isolated and tentatively identified as *Azomonas*, *Flavobacterium*, *Moraxella* and *Pseudomonas* spp. The isolates required other carbon sources for degradation, except for those two from *Flavobacterium* strains that degraded chlorothalonil as a carbon and energy source. Substrate specificity was high in the two strains from *Azomonas* and *Flavobacterium*.

INTRODUCTION

In order to enhance the microbial degradation of organic pollutants for remediation of contaminated soils, it is essential to understand the enhancing mechanism, especially the relationship between the degradation of chemicals in soil and the behavior of degrading microorganisms.

A number of studies have shown that there are two types of microbial degradation of pesticides in soil. In the first type of degradation, repeated applications of a pesticide to soil enhance the degradation by enriching pesticide-degrading microorganisms. The enriched microorganisms often metabolize the pesticide as a carbon and energy source, which is designated as catabolism by Matsumura.[1] The degradation of 2,4-D,[2] pentachlorophenol,[3,4] dalapon,[5] propanil[6] and thiobencarb[7] belongs to this category.

In the second type of degradation, a population of degrading microorganisms in soil does not change even when a pesticide is repeatedly applied and no enhancement of degradation occurs. The microorganisms require other carbon sources to degrade the pesticide, which is called incidental metabolism by Matsumura[1] or cometabolism by Alexander.[8] The degradation of molinate[9] is classified into this category.

In the previous studies the degradation of the fungicide chlorothalonil (TPN, Daconil®, 2,4,5,6-tetrachloro-1,3-isophthalonitrile) in soil was suppressed by repeated applications,[10,11] but the degradation pattern does not belong to either of the above-mentioned two types. In order to understand this type of degradation, it is essential to study chlorothalonil-degrading microorganisms. Chlorothalonil-degrading bacteria were isolated by Sato & Tanaka,[12] and fungi by Oku *et al.*,[13] but no study has been done on the population and the characteristics of chlorothalonil-degrading microorganisms. In this study, we investigated population change in chlorothalonil-degrading bacteria in soils while repeatedly applying the fungicide and

* Microbial Degradation of the Fungicide Chlorothalonil (Part 2). For Part 1, see Ref. 10).
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examined microbiological characteristics of the bacteria.

MATERIALS AND METHODS

1. *Chemicals*

Chlorothalonil (99.9% purity) was obtained from SDS Biotech K.K. Isophthalonitrile (IPN, 98.0%) and terephthalonitrile (TePN, 95.0%) were purchased from Wako Pure Chemical, Osaka. 2,6-Dichlorobenzonitrile (2,6-DBN, 99.0%) and 2,3,5,6-tetrachloroterephthalonitrile (TTPN, 98.0%) were purchased from Tokyo Kasei, Tokyo. Pentachloronitrobenzene (PCNB, 99.9%) and pentachlorophenol (PCP, 99.0%) were purchased from Aldrich, WI, U.S.A. The chemical structures of these compounds are shown in Fig. 1. The compounds were used to examine the substrate specificity of chlorothalonil-degrading bacteria, while an Daconil® wettable powder containing chlorothalonil as active ingredient (a.i.) by 75% was used for agar plates to isolate degrading bacteria.

2. *Culture Media*

In order to count and isolate bacteria that degraded chlorothalonil through incidental metabolism and catabolism, the following two media were used. One was NB agar prepared for incidental metabolism-type bacteria. It was composed of 1/10 diluted nutrient broth (Eiken Chemical, Tokyo), 15 g/*l* of agar (Nakarai Tesque, Kyoto) and Daconil® wettable powder which had been suspended at 40 mg-a.i./*l* of concentration. pH was adjusted to 7.0.

The other was MS agar for bacteria that degraded through catabolism. It contained

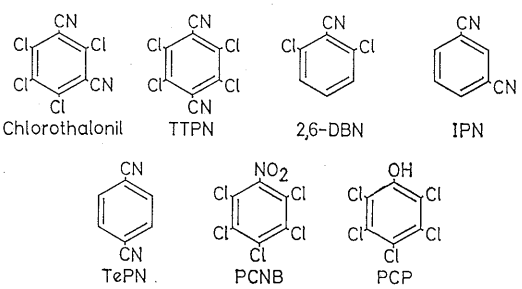

Fig. 1 Chemical structures of chlorothalonil and its related compounds.

the following mineral salts in 1 *l*: K_2HPO_4, 0.5 g; $NaNO_3$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.0125 g; $CaCl_2 \cdot 2H_2O$, 0.005 g; solution of trace elements, 10 ml; agar, 15 g and Daconil® wettable powder, 40 mg-a.i./*l*. One litre of trace element solution contained 10 mg of $ZnSO_4 \cdot 7H_2O$, 3 mg of $MnCl_2 \cdot 4H_2O$, 30 mg of H_3BO_3, 20 mg of $CoCl_2 \cdot 6H_2O$, 1 mg of $CuCl_2 \cdot 2H_2O$, 2 mg of $NiCl_2 \cdot 6H_2O$, and 3 mg of $NaMoO_4 \cdot 2H_2O$. pH was adjusted to 7.0 with 1 N-NaOH or 1 N-HCl.

In order to examine bacteria for chlorothalonil degrading ability, two kinds of broth were used: NB broth and MS broth. They had the same composition as the NB agar and the MS agar, respectively, except that chlorothalonil (99.9%) was dissolved at 0.3 mg/*l* instead of Daconil® wettable powder formulation. The NB broth was used for isolates from the NB agar and the MS broth for those from the MS agar.

3. *Soils*

Anjo, Ibaraki, Nagakute control-plot and Nagakute applied-plot soils were used. The first three had no history of chlorothalonil application, and the Nagakute applied-plot soil had been treated with chlorothalonil for 6 years. The soil properties were described in the previous paper.[10]

4. *Enumeration of Chlorothalonil-Degrading Bacteria in Soil*

Soils were incubated according to the method reported previously.[10] Soils with chlorothalonil (treated) or without chlorothalonil (control) were all incubated simultaneously. Soil samples were taken for bacterial enumeration after preincubation (before chlorothalonil application) and after incubation (after chlorothalonil application). The incubation periods were 50 days for Anjo soil (two times of chlorothalonil applications), 30 days for Ibaraki soil (three times) and 42 days for Nagakute control-plot soil (one time), respectively.

Samples were serially diluted to 10^{-4} to 10^{-6}. A dilution (0.1 ml) was spread on Petri dishes containing the NB agar or the MS agar and incubated at 30°C for 1 week. The number of formed colonies was counted (de-

signated as chlorothalonil-tolerant bacteria) and 20 or 50 colonies were selected at random and transferred to the same medium. The selected colonies were examined for chlorothalonil-degrading ability. Total aerobic bacteria were enumerated in the NB agar without Daconil® wettable powder.

5. Test of Colonies for Chlorothalonil Degradation Ability

One loopful of bacteria from a colony on the NB agar was inoculated into the NB broth, and those from the MS agar into the MS broth. After incubation at 30°C for 7 days on a reciprocal shaker, the cultures were examined for the remaining amount of chlorothalonil by the same method as previously reported.[10] The colonies that had degraded more than 80% of the initial amount of chlorothalonil were counted as positive.

6. Identification of Chlorothalonil-Degrading Bacteria

Colonies of bacteria that had degraded chlorothalonil was further transferred repeatedly on the medium to confirm the purity of the cultures. The isolated strains were examined for taxonomy.[14-16]

7. Degradation of Chlorothalonil-Related Compounds

Using the NB broth, we examined some isolates for an ability to degrade chlorothalonil-related compounds including IPN (0.5 mg/*l*), TePN (0.5 mg/*l*), 2,6-DBN (1.0 mg/*l*), TTPN (1.0 mg/*l*), PCNB (1.5 mg/*l*) and PCP (1.0 mg/*l*). After 7 days of incubation at 30°C, the remaining amount of a compound in the culture was determined. Chlorothalonil, TTPN, TePN, 2,6-DBN and IPN were analyzed with a Hewlett Packard model 5710 gas chromatograph equipped with N-P FID. The column (1.5 m×3 mm i.d.) was 1.5% silicon OV-17 on chromosorb W (AW-DMCS) 80/100 mesh. The column was set at 190°C, the detector at 300°C and the injector at 250°C. The carrier was 30 ml/min of nitrogen gas. The flow rates of air and hydrogen were 100 ml/min and 3 ml/min, respectively. The retention times of chlorothalonil, TTPN, 2,6-DBN, TePN and IPN were 6.1, 5.4, 1.2, 3.2, 1.5 min, respectively. PCP was measured by a modified method of Kuwatsuka & Igarashi[17] (retention time: 4.5 min) and PCNB by the method described in the previous paper[10] (retention time: 2.2 min).

RESULTS

Chlorothalonil-degrading bacteria in the Anjo soil was enumerated in the NB medium and the MS medium, as shown in Table 1. Although the number of colonies of chlorothalonil-tolerant bacteria was not significantly different between the two media, degrading bacteria were only countable in the NB medium. This suggested that the majority in the Anjo soil were bacteria that could degrade chlorothalonil through incidental metabolism. Results were the same in the Ibaraki soil. The NB medium was the one suitable for enumeration and isolation of chlorothalonil-degrading bacteria.

Using the NB medium, we counted degrading bacteria in soils before and after chlorothalonil application. Table 2 shows the population of degrading bacteria in soils before chlorothalonil application. In the Anjo, Ibaraki and Nagakute control-plot soils, into which chlorothalonil had never been applied, chlorothalonil-degrading bacteria were present at high population levels ranging from 6×10^6 to 12×10^6 CFU/g-soil, corresponding to 6 to 17% of the total bacteria counted. In the

Table 1 The number of total aerobic, chlorothalonil-tolerant and -degrading bacteria in Anjo soil counted on agar plates, before chlorothalonil application.

Media	Microbes	Population (10^7 CFU[a])	Percentage (%)
NB (without Daconil®)	Total	10.1	100
NB	Tolerant	0.80	7.9
	Degrading	0.72	7.1
MS	Tolerant	0.70	6.9
	Degrading	0[b]	0

[a] CFU represents a colony forming unit per one gram soil on a dry weight basis.

[b] Population 0 means less than 1×10^5 CFU/g-soil.

Nagakute applied-plot soil, the population of degrading bacteria was 11 times higher than in the Nagakute control-plot soil. This indicated that degrading bacteria in the soil had been enriched by long-term applications of chlorothalonil.

Table 2 The population of total aerobic bacteria (T) and chlorothalonil-degrading bacteria (D) in soils before chlorothalonil application.

Soil	T (10^7 CFU[a)])	D (10^7 CFU)	D/T (%)
Ibaraki	7.2	1.2	17
Anjo	10.1	0.72	7.1
Nagakute			
Control-plot	9.7	0.64	6.6
Applied-plot	11	7.9	72

a) Same in Table 1.

Table 3 The population of total aerobic bacteria (T) and chlorothalonil-degrading bacteria (D) in soils after incubation with (treated) or without (control) chlorothalonil.

Soil Incubation	T (10^7 CFU[a)])	D (10^7 CFU)	D/T (%)
Ibaraki			
Control	11	3.1	27
Treated	13	3.5	28
Anjo			
Control	8.5	0.6	7.1
Treated	13	4.2	33
Nagakute control-plot			
Control	5.3	0.4	7.5
Treated	5.0	2.2	44

a) Same in Table 1.

The enrichment of degrading bacteria was also confirmed in the laboratory incubation experiment as shown in Table 3. In the Anjo and Nagakute control-plot soils, degrading bacteria drastically increased in both population and percentage to the total bacteria when chlorothalonil was added, compared with the control. Although population was not significantly different between the control and treated Ibaraki soils after incubation with chlorothalonil, the population was higher in incubated soils than in soil before incubation (Table 2).

From chlorothalonil-degrading isolates, we selected 11 strains which had formed morphologically different colonies and examined some taxonomic characteristics of the strains. All the strains were Gram-negative and rod-shaped, as shown in Table 4. They were tentatively determined as *Azomonas* for A40-2, I40-1 and N40-1, *Flavobacterium* for A0-6 and NL0-1, *Moraxella* for A0-1, A0-5 and A40-3, and *Pseudomonas* for A0-2, A0-3, A40-1. Three isolates, A40-2, I40-1 and N40-1, grew in Winogradsky's nitrogen-free medium.

Degradation of chlorothalonil by the isolates was examined again in both NB and MS broth containing chlorothalonil (Table 5). Two strains of *Flavobacterium* sp. degraded chlorothalonil in the MS broth, indicating the strains were able to utilize chlorothalonil as a sole source of carbon and energy, which is catabolism-type degradation.[1)] Besides, *Flavobacterium* NL0-1 degraded chlorothalonil even in the nitrate-free MS broth, and this meant that the strain also utilized chlorothalonil as a sole source of nitrogen. Morphologically iden-

Table 4 Taxonomic characteristics of chlorothalonil-degrading isolates.

	A0-1	A0-2	A0-3	A0-5	A0-6	A40-1	A40-2	A40-3	I40-1	N40-1	NL0-1
Gram reaction	—	—	—	—	—	—	—	—	—	—	—
Morphology	SR	R	R	SR	R	R	R	R	R	R	R
Flagellation	non	polar	polar	non	non	polar	polar	non	polar	polar	non
Catalase	+w	+w	—	+	+	+	+	+	—	+	+
Oxidase	+w	—	+w	+	+	+w	+w	+w	+w	+	+
TSI	Alk/—	Alk/—	Alk/—	Alk/—	—/—	—/—	Alk/—	—/—	Alk/—	Alk/—	Alk/—
OF-test	—	O	O	—	—	—	O	—	O	O	—
NO_3^- reduction	+	—	+	+	—	—	—	+w	+	+	—

SR, short rod; R, rod; +w, weakly positive; Alk, alkali; O, oxidative.

Table 5 Chlorothalonil degradation by degrading isolates in 1/10 diluted nutrient broth (NB) and in a mineral salt medium (MS) after 48 hours of incubation.

Isolates		Degradation rate (%)	
		NB	MS
Azomonas	A40-2	89	0
	I40-1	93	0
	N40-1	80	0
Flavobacterium	A0-6	100[a)]	80
	NL0-1	100	100
Moraxella	A0-1	100	0
	A0-5	100	0
	A40-3	99	0
Pseudomonas	A0-2	87	0
	A0-3	100	0
	A40-1	24	0

a) One hundred percent of degradation rate represents that the remaining amount of chlorothalonil decreased to less than 0.3% of the initial amount.

Table 6 Degradation of chlorothalonil-related compounds by some of the degrading isolates after 7 days of incubation.

	Degradation rate (%)	
	Flavobacterium NL0-1	*Azomonas* A40-2
Chlorothalonil	100[a)]	100
TTPN	100	62
2,6-DBN	0	0
IPN	0	90
TePN	0	85
PCNB	0	0
PCP	0	0

a) One hundred percent of degradation rate represents that the remaining amount of chlorothalonil decreased to less than 0.3% and that of TTPN to less than 1.3% of the initial amount.

tical colonies to those of *Flavobacterium* spp. accounted for 0 to 36% of the colonies of chlorothalonil-degrading bacteria. All isolates, except the above-mentioned two strains in *Flavobacterium* spp., required other carbon sources for chlorothalonil degradation, which is incidental metabolism-type degradation.[1)]

Using two isolates, we examined the substrate specificity of chlorothalonil-degrading bacteria (Table 6). *Flavobacterium* NL0-1 degraded only TTPN in addition to chlorothalonil, while *Azomonas* A40-2 degraded IPN, TePN and TTPN. The substrate specificity of both isolates was relatively high, although the degree of specificity was different.

DISCUSSION

Chlorothalonil-degrading bacteria in the Anjo soil before chlorothalonil application were counted in both NB and MS media. The NB medium was prepared for counting bacteria that degraded chlorothalonil through incidental metabolism, while the MS medium was for bacteria that had a catabolism-type degradation ability.[18,19)] Chlorothalonil-degrading bacteria could be counted only in the NB medium and this result suggested that the majority of the bacteria had degraded chlorothalonil through incidental metabolism. However, two isolates of *Flavobacterium* spp. from the NB medium degraded chlorothalonil through catabolism, and this meant that the chlorothalonil-degrading bacteria isolated using the NB medium included not only incidental metabolism-type but also catabolism-type, although the majority were incidental metabolism-type.

The population of chlorothalonil-degrading bacteria was larger than 5×10^6 CFU/g-soil even in soils with no history of chlorothalonil application. The population levels were higher than several percentages of the total aerobic bacteria. This explained the previous results that chlorothalonil was degraded in soils without any lag period after application.[10)]

Application of chlorothalonil increased the population of degrading bacteria in soils. However, in the previous studies[10,11)] degradation of chlorothalonil in soils was suppressed by repeated applications. These findings indicated that the applications had made the soil conditions unsuitable for degrading bacteria. It has been reported that the amendments of soils with organic materials kept or enhanced the ability of the soils to degrade chlorothalonil.[10,11,20)] Therefore, incidental metabolism-type bacteria, which occupied the majority of the degrading bacteria enumerated, may play an important role for degradation in soils.

Degrading bacteria were isolated from chlorothalonil-tolerant colonies grown on the NB agar containing 40 mg/*l* of chlorothalonil as the suspension of Daconil® wettable powder, and the population was larger than 10^6 CFU/g-soil. Therefore, in the cases of the soils we used, it is unlikely that chlorothalonil-degrading bacteria were sensitive to chlorothalonil dissolved in soil solution at higher than 0.1 mg/*l*, and that the activity of the degrading bacteria was suppressed, as proposed by Takagi & Wada.[11]

The abundance of degrading bacteria in all soil samples suggested that the degradation ability of bacteria was due to enzymes common in many bacteria. All isolates in this study were Gram-negative and rod-shaped. Sato & Tanaka,[12] however, isolated non-spore forming Gram-positive rod-shaped bacteria as degraders. Degrading enzymes may exist in many species of bacteria. Chlorothalonil-degrading bacteria were not able to degrade chlorothalonil-related compounds such as 2,6-DBN, PCP and PCNB. The abundance was not therefore due to the adaptation or the induction of the bacterial enzymes by pesticides of related chemical structure. Besides, the degrading enzymes were rather specific, although the degree of specificity was different among the isolates. Thus, it seems that the chlorothalonil-degrading ability of bacteria in soils comes from specific enzymes present in many bacterial species and that it cannot be induced by related compounds.

We have elucidated the presence of chlorothalonil-degrading bacteria in soils at high population levels, the population increase by chlorothalonil application and some characteristics of the degrading enzymes. How chlorothalonil degradation in soils is suppressed by repeated applications in spite of a population increase in degrading bacteria, however, still remains to be solved.

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要　　約

土壌中におけるクロロタロニル分解細菌の菌数変化と特性*

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クロロタロニル（TPN，2, 4, 5, 6-tetrachloro-1, 3-isophthalonitrile）を分解する細菌はクロロタロニル施用歴のない土壌でも 6×10^6 から 12×10^6 CFU/g-soil 程度存在しており，クロロタロニル施用によって更に集積され 2×10^7 から 8×10^7 CFU/g-soil の菌数まで増加した．分解菌は高濃度のクロロタロニルに対して耐性であった．11株の分解菌が単離され *Azomonas*, *Flavobacterium*, *Moraxella*, *Pseudomonas* と仮に同定された．*Flavobacterium* の2株がクロロタロニルを唯一の炭素源および窒素源として分解した以外は，分解のために他の炭素源を必要とした．*Azomonas* および *Flavobacterium* に属する分解菌2株により，基質特異性が高いことが示された．

* 殺菌剤クロロタロニルの微生物分解（第2報）