Original Article

Isolation and Characterization of PCNB Degrading Bacterium, *Pseudomonas aeruginosa* Strain I-41

Katsunori TAMURA,* Yuki HASEGAWA,* Toshiaki KUDO
and Isamu YAMAGUCHI

Microbial Toxicology Laboratory, The Institute of Physical and Chemical Research (RIKEN),
and *Molecular Ecology Evolution Group, Research Development Corporation of
Japan (JRDC), Hirosawa, Wako 351-01, Japan

(Received September 2, 1994; Accepted February 20, 1995)

Pentachloronitrobenzene (PCNB) degrading microorganisms were screened from soil samples. Each soil sample was cultured at 28°C for 7 days with or without shaking in a mineral salt medium containing 0.1% of yeast extract and 50 µg/ml PCNB. In 92 of 114 samples, 20 to 80% of PCNB degradation was observed in still cultivation, suggesting that PCNB degrading microorganisms exist in most soil environments irrespective of previous application of PCNB to the field. From the soil samples indicating a high degradation rate, an isolate I-41 was selected to be the strongest degrader of PCNB and it was identified as *Pseudomonas aeruginosa*. The bacterium degraded PCNB almost completely at the concentration of 10 µg/ml under still conditions within a week, while it degraded only 18% of PCNB under shaking conditions, suggesting that I-41 can metabolize PCNB more efficiently under anaerobic conditions. The principal metabolic product of PCNB by I-41 was proved to be pentachloroaniline (PCA) by GC/MS. The results suggest that the main degradation pathway of PCNB in soil environment would be reduction of PCNB to PCA. The bacterial growth reached the stationary phase within 2 days, and the reduction of PCNB began incidentally to the bacterial growth. Both rates of the bacterial growth and PCNB metabolism were highest at the yeast extract concentration of 0.5–1.0%. When more than 200 µg/ml of PCNB was added to the culture medium, both bacterial growth and PCNB metabolism were suppressed possibly due to high concentration of PCNB and highly accumulated PCA in the culture medium as well. I-41 metabolized not only PCNB but also its related compounds such as nitrochlorobenzenes. By a test with cell free extract of I-41, this reductive degradation was confirmed to have been processed by an enzymatic reaction.

INTRODUCTION

The pentachloronitrobenzene (PCNB) has been widely used as a fungicide, especially for the control of soil-borne diseases caused by *Plasmodiophora brassicae* and *Rhizoctonia solani*. This fungicide is physicochemically stable and generally resistant to microbial degradation, and reportedly remains in soil for many years after application,^{1,2)} though repetitive use of PCNB in some cabbage or chinese cabbage field appeared to bring about reduction of PCNB efficacy on clubroot disease due to problem soil.³⁾ Since large quantities of PCNB have been continually

applied to the cultivated fields, residual PCNB or its metabolites may affect on non-target soil microorganisms leading to their ecological hazards.

So far several reports on degradation of PCNB in soil have suggested that soil microorganisms were associated with it.^{1,2,4-6)} However, there are few reports on PCNB degrading microorganisms with their degradation characteristics. In order to understand the microbial degradation of PCNB in soil environment, it is essential to isolate PCNB degrading microorganisms, to study degradation mechanisms, and then to investigate the ecology of those microorganisms. These un-

derstandings might also lead us to clarification of a part of microbial evolution in the presence of artificially synthesized chemicals such as chlorinated aryl compounds.

The present study is dealing with screening of soil microorganisms capable of degrading PCNB and its metabolic characterization.

MATERIALS AND METHODS

1. Chemicals

PCNB, 2,3,5,6-tetrachloronitrobenzene (2,3,5,6-TeCNB), 2,4,5-trichloronitrobenzene (2,4,5-TCNB), 2,3,5,6-tetrachloroaniline (2,3,5,6-TeCA), 2,4,5-trichloroaniline (2,4,5-TCA) were purchased from Kanto Chemical Co., Inc., Tokyo. Pentachlorophenol (PCP) and hexachlorobenzene (HCB) were purchased from Wako Chemical Co., Tokyo. Pentachloroaniline (PCA) and pentachloroaniline (PCTA) were kindly supplied by Mitsui Toatsu Chemicals Inc., Tokyo. 2,4,5,6-Tetrachloroisophthalonitrile (TPN) was obtained from Kumiai Chemical Industry Co., Ltd., Tokyo.

2. Media and Culture Conditions

The liquid medium used contained 0.5 g K_2HPO_4 , 0.5 g $NaNO_3$, 12.5 mg $MgSO_4 \cdot 7H_2O$, 5 mg $CaCl_2 \cdot 2H_2O$ in 1 l of distilled water. For solid medium, agar was added to the above medium at the concentration of 1.5%. Having been autoclaved, filter-sterilized yeast extract solution in distilled water (20 mg/ml) was added into the above liquid or agar medium at the final concentration of 0.1% unless otherwise noted. PCNB and other chemicals dissolved in dimethylsulfoxide (DMSO) at the concentration of 20 mg/ml were added to the liquid or solid medium to give 50 μ g/ml or appropriate concentrations. Each 0.1 g of soil samples was put into 5 ml of liquid medium in a test tube (2.5 $\times$ 16 cm), and incubated for 7 days at 28°C with or without shaking. Then each 0.1 ml of culture broth was inoculated into a new liquid medium, and further incubation was performed for 7 days at 28°C. All incubations were carried out in the dark.

3. Isolation of PCNB Degrading Microorganisms

Soil samples were collected not only from

the cultivated fields where PCNB was applied but also from several places including mountain districts, volcanic zones, beach areas and an industrial activated sludge. After analyses of PCNB degradation (decrease of amount), broths which showed more than 20% of PCNB degradation were used for isolation of degrading microorganisms. Each culture broth was serially 10-fold diluted with a phosphate buffered saline, and 100 μ l of each dilute was spread on the solid medium described above. After incubation at 28°C for 2 to 3 days, each single colony was picked up and reincubated on the same medium. Then the isolates examined for the ability of PCNB degradation were stored, if necessary, in 10% skim milk medium at -20°C.

4. Analytical Methods

After extraction of residual PCNB and metabolites in culture broth with equal volume of *n*-hexane for three times, the extracts were combined and concentrated. Then they were analyzed by gas chromatography (GC), and the metabolites were identified by GC/Mass spectrometry. A Hitachi Model GC14B instrument equipped with a flame ionization detector and an open tube column 'G-205' (10 m $\times$ 1.2 mm i.d., 0.5 μ m of film thickness, Chemicals Inspection and Testing Institute, Japan) was used for GC analyses. GC conditions were as follows; carrier gas was He at 130 ml/min flow rate, and temperatures of column, injector, and detector were 170, 250, and 300°C, respectively. A Hewlett Packard Model HP5890 instrument equipped with mass selective detector HP5971 and a capillary column 'Ultra2' (50 m $\times$ 0.32 mm i.d., 0.17 μ m of film thickness, Hewlett Packard Co.) was used for GC/MS analyses. GC/MS conditions were; carrier gas He at 40 ml/min flow rate, and temperatures of column, injector and detector 170, 250, and 280°C, respectively. The detection limits of PCNB with GC or GC/MS were 0.01 and 0.001 μ g/ml, respectively. Chemicals listed in MATERIALS AND METHODS 1 were used as possible substrates and metabolites, and they were analyzed under same conditions as PCNB with those unique retention times and selected ion masses. Recovery of all the chemicals

tested in the technique was more than 90%.

5. Identification of Bacteria

The bacterial strains were identified by a method shown in "Bergey's Manual of Determinative Bacteriology," 8th ed.⁷⁾ Most tests for identification were performed by using a kit of Biotest No. 2 'Eiken' (Eiken Chemical Co., Tokyo).

6. Preparation of Washed Cell Suspension

PCNB degrading bacterium was incubated in 200 ml of aforementioned mineral salts plus yeast extract medium (MSY) containing 1 µg/ml PCNB. After incubation for 24 hr, cells were collected by centrifugation, washed twice with 50 ml of sterilized water, and suspended in MSY medium at the concentration of OD₆₆₀ = 1.0. This washed cell suspension was used for examination of metabolic specificity.

7. Preparation of Cell Free Extract and Its Reductase Activity for PCNB

Bacterial cells of I-41 incubated in 100 ml of Luria-Bertani (LB) broth⁸⁾ for 24 hr were collected by centrifugation at 8000 × *g* for 10 min, washed with 50 ml of 50 mM Tris-HCl (pH 7.0), and then suspended in 20 ml of the same buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Bacterial cells were disrupted by ultra-sonication for 10 min at 30 sec intervals per 1 min on ice bath. The suspension was then centrifuged at 18,000 × *g* for 20 min, and the supernatant was used as a cell free extract. One milliliter of cell free extract was added to 2 ml of reaction mixture which contained 50 mM Tris-HCl (pH 7.0) and 10 µg/ml of PCNB. Then the reaction mixture was incubated at 28°C for 1 hr, and PCNB and PCA were extracted and analyzed as described above.

RESULTS

1. Isolation of PCNB Degrading Microorganisms

Soil samples were collected from diverse field soils of various places. Under shaking conditions, no samples showed significant decrease of PCNB. Under still conditions, however, 92 samples among total 114 samples showed more than 20% of PCNB degradation, when com-

Table 1 PCNB degrading activity of soil samples.

Place	No. of samples ^{a)}	No. of samples with PCNB degradability ^{b)}
Koumi town ^{c)}	22	22
Minakami town	4	3
Wako city	14	12
Shinrin-Koen area	10	7
Kamogawa city	6	3
Nasu-Kogen area	12	7
Furano town	12	11
Yakushima area	10	5
Tsumagoi village ^{c)}	24	22
Total	114	92

^{a)} Samples were collected from diverse positions of each place.

^{b)} PCNB degradability means that more than 20% of PCNB was degraded.

^{c)} Cultivated fields where PCNB has been continually applied.

All cultures were incubated under still conditions.

pared with control which contained no soil samples (Table 1). Though the exact amount of volatilized PCNB during the incubation was not determined, these soil samples which caused less than 80% of residual PCNB were regarded as positive candidates for isolation of capable microbes. Bacteria were mainly isolated from these samples and they were subjected to further examination for the ability of PCNB degradation. A bacterium I-41 isolated from the soil sample of mountain district in Minakami town was capable of degrading PCNB almost completely when 10 µg/ml of the compound was applied to the medium.

2. Identification of PCNB Degrading Microorganism

The isolate I-41 was Gram-negative, non-sporeforming aerobic rods, and motile by a single polar flagellum. The colonies formed on nutrient agar plates were white, convex, semitransparent and circular with a smooth surface and entire margin. The present bacterium showed positive reactions in the following tests: reduction of nitrate to nitrite;

growth at 41°C; utilization of citrate; metabolism of acetoamide; gelatin liquefaction; activities of oxidase, catalase and arginine dihydrolase; production of acid from glucose, xylose and mannose. The following tests were negative: utilization of malonic acid; production of indole and hydrogen sulfate; metabolism of esculin and urea; activity of β -galactosidase; production of acid from arabinose, fructose, maltose, rhamnose, mannitol and sucrose. From these results, I-41 was identified as *Pseudomonas aeruginosa*.

3. Time-course of PCNB Degradation

The principal degradation product of PCNB by I-41 was identified as PCA by GC/MS. I-41 degraded PCNB almost completely when

Fig. 1 Time course of bacterial population (A) and metabolism of PCNB to PCA by *Pseudomonas aeruginosa* I-41 (B) under different culture conditions.

(A) The optical density (660 nm) of culture broth under shaking conditions (■) or still conditions (●). (B) ■: residual amount of PCNB under shaking conditions, ●: PCNB under still conditions, □: PCA produced under shaking conditions, ○: PCA under still condition. Initial concentration of PCNB was 10 $\mu\text{g/ml}$, and residual PCNB and produced PCA were extracted with *n*-hexane, and analyzed by GC.

Fig. 2 Effect of yeast concentration on metabolism of PCNB by *Pseudomonas aeruginosa* I-41.

Concentration of yeast extract added to the culture medium, ●: 0.05%, ○: 0.1%, ■: 0.5%, □: 1.0%. Initial concentration of PCNB was 10 $\mu\text{g/ml}$.

it was incubated with 10 $\mu\text{g/ml}$ PCNB under still conditions for 7 days, while it degraded less than 20% of PCNB under shaking condition (Fig. 1). In time-course study of PCNB degradation by this bacterium, the bacterial growth reached a stationary phase within 2 days, and the reduction of PCNB was correlated to the increase in the bacterial population in still conditions. During 7 days incubation, the amount of produced PCA was about 70% of the amount of applied PCNB.

4. Effects of Yeast Extract Concentration on Growth Rate and Metabolism of PCNB

Effects of the concentration of yeast extract added to the culture medium were examined on bacterial growth of I-41 and metabolism of PCNB. When yeast extract was added at the concentration of 0.5 and 1.0% of the medium, the optical density at 660 nm was over 1.0 and the complete degradation of PCNB was observed within 7 days incubation, while at less than 0.1%, lower growth and far less PCNB metabolism were observed (Fig. 2). Therefore, all cultures thereafter were performed in the presence of 0.5% yeast extract.

Fig. 3 Effects of PCNB concentration on growth rate (A) and PCNB metabolism (B) by *Pseudomonas aeruginosa* I-41.

Initial concentration of PCNB, ○: 10 µg/ml, ●: 50 µg/ml, □: 100 µg/ml, ■: 200 µg/ml.

Fig. 4 Effect of PCA concentration on growth rate of *Pseudomonas aeruginosa* I-41.

Initial concentration of PCA, ○: 10 µg/ml, ●: 50 µg/ml, □: 100 µg/ml, ■: 200 µg/ml.

5. Effects of Concentration of PCNB and PCA on Growth Rate and Metabolism of PCNB

To determine whether the concentration of PCNB added to the culture medium affects the growth of I-41 or PCNB metabolism, time-course of bacterial growth and PCNB degradation were studied at various concentrations of PCNB. While the bacterial growth was not affected significantly at the concentrations less than 100 µg/ml of PCNB, it was markedly suppressed at 200 µg/ml of PCNB (Fig. 3). To determine whether this suppression is due to a high concentration of PCNB or to a highly accumulated PCA in culture medium, an effect of PCA on growth of I-41 was also examined. In contrast to the good bacterial growth at less than 50 µg/ml of PCA, it was significantly suppressed at 200 µg/ml (Fig. 4). The degradation rates of PCNB were also suppressed by 200 µg/ml PCNB or PCA, although actual amount of PCNB degradation per cell appeared larger at the high concentration of PCNB or PCA.

6. Metabolic Specificity

Because the metabolism of PCNB was suggested to be incident to the growth of I-41

Table 2 Metabolism of PCNB and its related compounds by washed cells^{a)} of *Pseudomonas aeruginosa* I-41.

Compounds	Rate of metabolism (%)
Pentachloronitrobenzene	100
2,3,5,6-Tetrachloronitrobenzene	34
2,4,5-Trichloronitrobenzene	51
Pentachloroaniline	46
Pentachlorothioanisole	0
Pentachlorophenol	0
Hexachlorobenzene	0
Tetrachloro-isophthalonitrile	0

^{a)} Bacterial cells of isolate I-41 were grown for 24 hr in MSY medium containing PCNB (1 µg/ml), collected by centrifugation, washed twice with sterilized water. They were resuspended in MSY medium ($OD_{660} \approx 1.0$) and incubated with PCNB or its related compounds (10 µg/ml) for 2 days, and residual amount of each compound was analyzed.

Table 3 Metabolism of PCNB by cell free extract of *Pseudomonas aeruginosa* I-41.

Reaction mixture	Residual amount of PCNB ($\mu\text{g/ml}$)	Amount of produced PCA ($\mu\text{g/ml}$)
Cell free extract ^{a)} + PCNB	4.8	3.6
Cell free extract (heated ^{b)}) + PCNB	8.5	ND ^{c)}
Buffer + PCNB	9.6	ND

^{a)} Cell free extracts were prepared as described in the text. PCNB dissolved in DMSO was added to the reaction mixtures at the concentration of 10 $\mu\text{g/ml}$ and incubations were performed at 28°C for 1 hr.

^{b)} Cell free extract was kept at 95°C for 10 min.

^{c)} Not detected.

from the above results, the washed cells with adjusted OD₆₆₀ were used for comparative metabolism tests of PCNB or its related compounds. I-41 degraded not only PCNB completely, but also it metabolized 2,3,5,6-TeCNB and 2,4,5-TCNB at the ratios of 34 and 51%, respectively (Table 2). The major metabolites of these compounds were confirmed to be PCA, 2,3,5,6-TeCA and 2,4,5-TCA, respectively (data not shown). I-41 did not metabolize PCTA, PCP, HCB and TPN.

7. Metabolism of PCNB by Cell Free Extract

Cell free extract was prepared from I-41 to confirm whether the metabolism of PCNB by I-41 is enzymatic reaction. While cell free extract reduced PCNB to PCA, a heat treated extract did not reduce it (Table 3).

DISCUSSION

Several lines of studies on PCNB degradation in soil suggested that the soil microorganisms were associated with PCNB degradation.^{1,2,4,6)} To our knowledge, however, this is the first report on the PCNB degrading soil microorganism isolated and characterized. In this work, PCNB degradation was observed in most soil samples collected from various area, and PCNB degrading microorganisms were ubiquitously isolated from these samples. It could be argued that these microbes might have come from PCNB powder as it was not

sterilized in our experiments, but such possibility appeared to be ruled out because more than 90% of PCNB was always recovered from the incubated mixtures which contained no soil samples or isolated microorganisms. Since the samples included not only PCNB applied soils but also soils from uncultivated fields such as mountain district, beach area and marsh, it could be concluded that PCNB degrading microorganisms exist in most soil environments irrelevant to the pre-treatment of PCNB. This was supported by the fact that the present isolate I-41, the strongest degrader of PCNB among the microorganisms obtained, was isolated from mountain district and was identified as *P. aeruginosa*, which commonly exists in soil and water environments.⁹⁾ In addition, PCA was detected in most samples which showed PCNB degradation and the principal metabolite of PCNB by I-41 was confirmed to be PCA. Furthermore, several reports also indicated that PCA was the major metabolite in continually PCNB-applied soils.^{10,11)} Our preliminary analytical experiment on PCNB metabolites in field soils also confirmed such results. Therefore, the main degradation pathway of PCNB in soil environments is considered to be the conversion of PCNB to PCA.

I-41 reduced not only PCNB to PCA but also TeCNB to TeCA and TCNB to TCA, respectively. The enzyme system which catalyzes the reduction of PCNB would have a comparatively low substrate specificity among nitroaryl compounds. In fact, nitroaryl reductase system had reportedly low specificity to the substrates.¹²⁾ Thus the reduction of PCNB by I-41 would be incidental rather than specific for PCNB. Moreover, the degradation of PCNB to PCA has been observed in some fungi,^{4,10)} plants^{11,13-15)} and animals,^{16,17)} suggesting that the conversion of PCNB to PCA is a non-specific biological ability.

PCNB is a potent fungicide especially active against *P. brassicae* and *R. solani*.¹⁸⁾ However, we found that more than 200 $\mu\text{g/ml}$ of PCNB also suppressed the growth of bacterium I-41 and its metabolism of PCNB. In addition, we observed that 200 $\mu\text{g/ml}$ PCA also significantly suppressed the growth of I-41. Although it is not known how PCA suppresses the growth of

I-41, Ko & Farley reported that PCA has a growth inhibitory activity against several isolated microorganisms,¹⁾ and Murthy & Kaufman¹⁰⁾ showed that PCA caused inhibition of soil respiration under anaerobic conditions. These facts indicate that a high concentration of PCNB and a highly accumulated PCA would somehow affect the populations of soil microorganisms in addition to the target plant pathogens in the fields where PCNB was continually applied at a large amount.

ACKNOWLEDGMENTS

We are grateful to Messrs. K. Takeda and J. Yumoto, Nagano Prefectural Experimental Station for their kind help to collect samples. We also thank Drs. M. Maeda and S.-Y. Chung, Research Development Corporation of Japan (JRDC), and Mr. M. Kimura, The Institute of Physical and Chemical Research (RIKEN) for their valuable discussions. The authors are indebted to Mitsui Toatsu Chemicals Inc. and Kumiai Chemical Co., for their kind supply of the test samples.

REFERENCES

- 1) W. H. Ko & J. D. Farley: *Phytopathology* **59**, 64 (1969)
- 2) T. Nakanishi: *Ann. Phytopathol. Soc. Jpn.* **38**, 249 (1972)
- 3) K. Suyama & H. Okazaki: Abstr.; 19th Annu. Meet. Pestic. Sci. Soc. Jpn., D111, 1994 (in Japanese)
- 4) C. I. Chacko, J. L. Lockwood & M. Zabik: *Science* **154**, 893 (1966)
- 5) T. Nakanishi & H. Oku: *Ann. Phytopathol. Soc. Jpn.* **35**, 339 (1969)
- 6) T. Nakanishi & H. Oku: *Phytopathology* **59**, 1761 (1969)
- 7) R. E. Buchanan & M. G. Gibbons: "Bergey's Manual of Determinative Bacteriology," 8th ed., The Williams & Wilkins Company, London, p. 217, 1975
- 8) T. Maniatis, E. F. Fritsch & J. Sambrook: "Molecular Cloning, A Laboratory Manual," Cold Spring Harbor Laboratory, New York, 1982
- 9) N. R. Krieg & J. G. Holt: "Bergey's Manual of Systematic Bacteriology," Vol. 1, 8th ed., The Williams & Wilkins Company, London, p. 164, 1984

- 10) N. B. Murthy & D. D. Kaufman: *J. Agric. Food Chem.* **26**, 1151 (1978)
- 11) M. Ogiso & H. Tanabe: *J. Pesticide Sci.* **7**, 391 (1982)
- 12) I. Yamashita: *Biochem. J.* **27**, 85 (1954)
- 13) S. Gorbach & U. Wagner: *J. Agric. Food Chem.* **15**, 654 (1967)
- 14) G. L. Lamoureux, J.-M. Gouot, D. G. Davis & D. G. Rusness: *J. Agric. Food Chem.* **29**, 996 (1981)
- 15) D. G. Rusness & G. L. Lamoureux: *J. Agric. Food Chem.* **28**, 1070 (1980)
- 16) P. W. Aschbacher & V. J. Feil: *J. Agric. Food Chem.* **31**, 1150 (1983)
- 17) E. J. Kuchar, F. O. Geenty, W. P. Griffith & R. J. Thomas: *J. Agric. Food Chem.* **17**, 1237 (1969)
- 18) T. Nakanishi & H. Oku: *Ann. Phytopathol. Soc. Jpn.* **36**, 67 (1970)

要 約

PCNB 分解菌 *Pseudomonas aeruginosa* I-41 株の分離および特性

田村勝徳, 長谷川 志, 工藤俊章, 山口 勇
殺菌剤 PCNB を分解する微生物を各種土壌試料から選抜した。PCNB を 50 µg/ml 添加した 0.1% 酵母エキス含有無機塩培地中で各試料を 28°C で 7 日間静置培養すると, 114 試料中 92 試料において 20~80% の PCNB の分解が認められた。高分解率を示した試料から *Pseudomonas aeruginosa* に同定される強分解菌 I-41 株を選抜した。本菌は静置培養条件下では一週間以内に 10 µg/ml の PCNB を完全に分解したが, 振とう条件下では 18% の分解に留まったことから, 嫌気的条件下でより効率的に分解が進むことが示唆された。GC/MS 分析により I-41 株の PCNB 分解産物はベンタクロロアニリン (PCA) と同定された。以上より, PCNB 分解菌は PCNB の存在の有無にかかわらずほとんどの土壌環境に生息し, おもな代謝経路は PCA への還元であることが判明した。I-41 株の PCNB 代謝における酵母エキス添加濃度は 0.5~1.0% が最適であり, 200 µg/ml の高濃度 PCNB 存在下では菌の生育および代謝が抑制された。さらに本菌は PCNB 以外にもニトロクロロベンゼン類を還元すること, および PCNB 代謝が酵素反応によることを認めた。