Isolation of Dichloromethane-Degrading Bacteria from Drainage Water

Hiroshige Kawata,[a] Chizuko Nakayama,[a] Miwa Sakamoto,[a] Hisayoshi Ikatsu,[b] Shin-ichi Miyoshi,[a] Ken-ichi Tomochika[a] and Sumio Shinoda*,[a]

[a]*Laboratory of Environmental Hygienics, Faculty of Pharmaceutical Sciences and* [b]*Center for Environmental Science and Technology, Okayama University, Tsushima, Okayama 700-8530, Japan*

(Receive October 14, 1999; Accepted December 28, 1999)

The biodegrading ability of drainage water from research laboratories to dichloromethane (DCM) and chloroform (CF) was surveyed. When DCM was used as a sole carbon source in a synthetic mineral salt medium, some water samples showed ability to degrade DCM, and DCM-degrading bacteria were isolated from them, whereas no samples showed CF degradation activity. Two isolates, strain P3310, a *Flavimonas* sp., and strain G31, a *Chryseobacterium* sp., were used for further investigations. Both strains were able to use DCM as a carbon source for growth and also grow in complex media containing other carbon sources, suggesting they were facultative methylotroph. Both strains needed 6 days at 30℃ to completely degrade 200 mg/l of DCM with the first isolated cells, but this was shortened to 2 days with the first subculture, suggesting they were acclimatized. Although the DCM-degrading activity of strain G31 was inhibited by addition of other carbon sources such as peptone or glucose, that of strain P3310 was not affected. Thus, strain P3310 may be a useful candidate for bioremediation to eliminate DCM from drainage.

Key word —— dichloromethane, biodegradation, methylotroph

INTRODUCTION

Environmental pollution by chlorinated organic compounds is a serious problem at present because of their residual tendency in the environment, due to stability against biodegradation and long-lasting toxicity. For example, aromatic chlorinated organic compounds, including polychlorinated dibenzo-*p*-dioxins (PCDD, so-called dioxins), polychlorinated biphenyls (PCB), and dichloro-diphenyl trichloroethane (DDT) are easily accumulated in organisms by bioconcentration because they can not easily be metabolized or excreted. Many chlorinated aliphatic compounds are also resistant to biodegradation and have toxicity, although accumulation tendency is lower than with aromatic analogs. Several one- and two-carbon chlorinated-compounds, such as chloroform (CF), dichloromethane (DCM), trichloroethane and tetrachloroethylene, are restricted by laws including "Water Supply Law" and "Water Pollution Control Law" in Japan. These compounds are used not only for industrial purposes including washing of machine parts and dry cleaning, but also as organic solvents in chemical research. CF and DCM are especially useful solvents in organic synthesis or analytical chemistry. Continuous usage of these compounds in research laboratories is anticipated because of their usefulness. The total volume used needs to be reduced because of restriction by the laws. Under this situation, a study of biodegradation of these compounds by environmental microorganisms is important, especially for the control of waste water from research institutions.

There have been some reports on biodegradation of CF and DCM. Two bacterial groups able to degrade DCM have been reported. One is a methylotroph which is able to use C_1 compounds such as methanol or methylamine (but not methane) as a carbon source for growth.[1-3] Bacteria in this group metabolize DCM to formaldehyde, formic acid, and then carbon dioxide

*To whom correspondence should be addressed: Laboratory of Environmental Hygienics, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700-8530, Japan. Tel.: +81-86-251-7966; Fax: +81-86-251-7926; E-mail: shinoda@pheasant.pharm.okayama-u.ac.jp

under aerobic conditions. Formaldehyde formed in this process may be assimilated. Degradation of DCM to formaldehyde is catalyzed by gluthathion-*S*-transferase which is induced by addition of DCM as the sole carbon source. The other is a group of acetic acid bacteria which produce formic acid or acetic acid from DCM under anaerobic conditions.[4)]

A few reports on biodegradation of CF have also been published but no reports on utilization of CF as a carbon source for growth. Under aerobic conditions, monooxygenases, such as methane monooxygenase or toluene monooxygenase, produced by methane- or toluene-utilizing bacteria are able to degrade CF coupled under coexistence of methane or toluene.[5)] Under anaerobic conditions, a group of methanogenic bacteria generate methane from CF through DCM by reductive dechlorination, although the degradation rate is very low.[6–8)]

Though DCM- or CF- degrading bacteria are already known, information is still insufficient and activity is not enough to eliminate these compounds from the environment. This paper presents results on the ability of microorganisms in drainage water from chemical laboratories to degrade CF or DCM and on the isolation of the DCM-degrading bacteria.

MATERIALS AND METHODS

Collection of Water Samples——Water samples were collected from July to December, 1997 at drainage ditches on the campus of Okayama University where there are many chemical and biochemical laboratories.

Substrates and Media——DCM, CF, methylethylketone and *o*-xylene were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and peptone was purchased from Difco Laboratories, Detroit, U.S.A.

Peptone medium (0.1% peptone, pH 7.0) and mineral salt (MS) medium were used for cultivation of environmental microorganisms. MS medium contained the following constituents (gram per liter): $(NH_4)_2SO_4$, 0.5; K_2HPO_4, 0.5; $Na_2HPO_4 \cdot 12H_2O$, 0.5; $NaH_2PO_4 \cdot 2H_2O$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.001; $CaCl_2$, 0.001; pH 7.0).

Detection of DCM and CF——Concentrations of DCM and CF were determined by a Shimadzu GC-6AM gas chromatograph equipped with a flame ionization detector (GC-FID). A glass column (2.0 m by 3.0 mm) packed with Silicone DC 550/chromosorb WAW DMCS (60—80 mesh) was used. N_2 gas was used as the carrier gas at a flow rate of 30 ml/min. Temperatures of the column and injector were 100 and 110℃, respectively. Pressures of H_2 and air were 0.45 and 1.25 kg/cm^2, respectively.

Degradation Tests——Degradation test in peptone medium was carried out using a modification of the method of Kondo *et al.*[9)] A 0.5 ml water sample as a source of environmental microorganisms, or a 0.5 ml of suspension of isolated organisms and 1.0 ml of test substrate solution were added to 5 ml of 2X peptone medium in a 50 ml serum vial bottle. The volume of the mixture was adjusted to 10 ml by adding sterilized water. The bottle was sealed by a rubber stopper and aluminum cap, and incubated at 30℃ in the dark by shaking at 140 rpm. Residual amount of test substrate was measured by GC-FID after incubation. A control mixture without microorganisms was also incubated under the same conditions to monitor the change of the test substrates.

To test the ability of microorganisms to utilize the test compounds as carbon sources for growth in synthetic inorganic medium, peptone medium was replaced by MS medium in the above assay system.

Bacteriological Methods——Colony-forming units (CFU) were determined by inoculating 0.1 ml of sample in peptone agar medium containing 0.1% peptone and 1.5% agar (pH7.0), incubating at 30℃ overnight and counting the colonies formed. Identification of the isolated bacteria was performed with a BBL Crystal™ Identification System (Becton Dickinson Co. Ltd., U.S.A.).

RESULTS AND DISCUSSION

Biodegradation of DCM and CF by Aquatic Organisms

Among many methods to evaluate biodegrading activity of aquatic microorganisms, the method reported by Kondo *et al.*[9)] was first applied to test the ability of microorganisms in drainage water from chemical or biochemical laboratories to biodegrade DCM and CF. In this method, a small amount of nutrient, 0.1% peptone, is added to the medium to support growth of various heterotrophic organisms. Therefore, the method is useful to evaluate the ability of microorganisms to degrade general organic substrates since enough of the bacterial cells can

grow. However, no degradation of DCM (200 mg/l) or CF (10 mg/l) was observed during cultivation at 30℃ for 3 days (Table 1). When this method was adopted to reference compounds,[10,11] methylethylketone (easy/moderate degradability), benzene (moderate degradability) and *o*-xylene (moderate/hard degradability), water samples showed activity to degrade these compounds to some extent (Table 1), suggesting that the water samples have enough activity to biodegrade general organic compounds, whereas DCM and CF may be extremely hard to degrade.

Table 1. Biodegradation of Organic Compounds in a Peptone Medium by Drainage Water from Research Laboratories

Organic compound	Degradation rate (%)		
	0—20	21—80	81—100
Dichloromethane	26	0	0
Chloroform	26	0	0
Methylethylketone	8	11	7
Benzene	8	8	10
o-Xylene	22	4	0

Drainage water samples were incubated with each compound in 0.1% peptone medium as described in the text. After 3 days incubation at 30℃, residual amount of the compound was measured and degradation rate was calculated. The total number of water samples was 26. According to degradation rate, the water samples were divided into 3 groups, and the number of the samples in each group is indicated in the table. Original concentration of the compounds (mg per liter) : dichloromethane, 200 ; chloroform, 10 ; methylethylketone, 10 ; benzene, 20 ; *o*-xylene, 20.

On the other hand, some samples showed degrading activity toward DCM when DCM was used as the sole carbon source in MS medium (Fig. 1). Two samples, Nos. 3 and 14, showed especially high degradability, with 85 and 99% degradation of 200 mg/l DCM, respectively. However, CF was not degraded although the test was carried out at lower concentration (10 mg/l) than DCM because of the low solubility and high toxicity of CF to bacteria. No reports were found on utilization of CF as a carbon source for microbial growth. We have tried to isolate CF-assimilating microorganisms from various soil and water samples but have not yet succeeded. DCM and CF are both useful organic solvents in

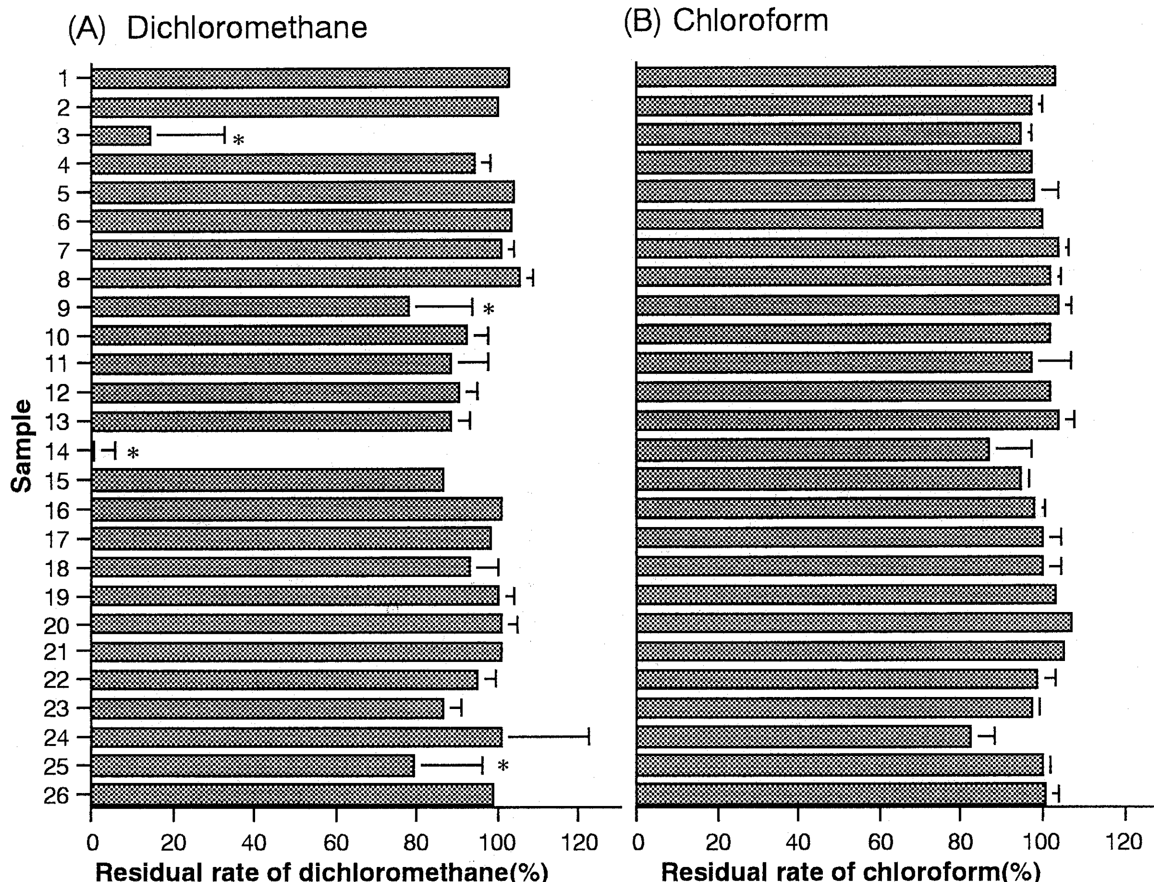

Fig. 1. Biodegradation of DCM and CF by Discharged Water from Research Laboratories

0.5 ml of water sample was added to MS medium (final volume of 10 ml) containing 200 mg/l DCM or 10 mg/l CF, incubated at 30℃ for 7 days, and the residual amounts of DCM and CF measured.

* : Residual rate less than 80 %.

chemical and biochemical research, however the law on the standard of effluent DCM has taken effect, whilst that for CF has not yet.

Isolation of Microorganisms and Their Ability to Degrade DCM

Aliquots of the cultures which showed degrading activity to DCM were inoculated in 0.1% peptone agar medium or MS agar medium containing DCM (200 mg/l), and the plates were incubated at 30℃ for 2—7 days. Colonies formed were collected and tested for ability to degrade DCM. Among the 78 isolates (58 from peptone agar and 20 from MS agar), 13 isolates showed activity to reduce concentration of DCM (200 mg/l) by more than 20% after 7 days incubation. Isolates from peptone agar were generally more active than those from MS agar. Among these isolates, P3310 and G31, which degraded 100 and 98% of DCM under the above conditions, respectively, and showed different colony morphology, were used in further experiments. Strain P3310 was a Gram-negative rod and was suspected to be *Flavimonas* species by preliminary identification, whereas G31 was a Gram-negative short rod and suspected to be *Chryseobacterium* species. *Flavimonas* is a genus separated from *Pseudomonas*,[11] whereas *Chryseobacterium* is from *Flavobacterium*,[12] and both are often isolated from clinical specimens. No reports have been found on DCM biodegradation by bacteria with these genera and their methylotrophic property is unclear. Therefore investigation of the DCM-biodegrading property of these isolates is of interest.

The two strains were inoculated into MS medium (10^3—10^4 CFU/ml) containing various concentrations of DCM and their degrading ability was determined. As shown in Table 2, both strains showed sufficient activity at 500 mg/l DCM but not at 1000 mg/l. Figure 2 shows the time course of the decrease of DCM and the growth of bacteria in MS medium containing 200 mg/l DCM. An increase in CFU was accompanied by a decrease in DCM with both strains. Six days were required for complete disappearance of DCM in both strains. When aliquots of these cultures were inoculated to a fresh medium, 2 days was enough for complete degradation in both strains (data not shown),

Table 2. Biodegradation of Dichloromethane by Strains P3310 and G31

Dichloromethane mg/l	Degrader P3310 %	Degrader G31 %
100	87	100
200	98	97
500	82	87
1000	0	0

Bacteria were inoculated into MS medium with various concentrations of dichloromethane, incubated at 30℃ for 7 days, and the residual amount of dichloromethane was measured.

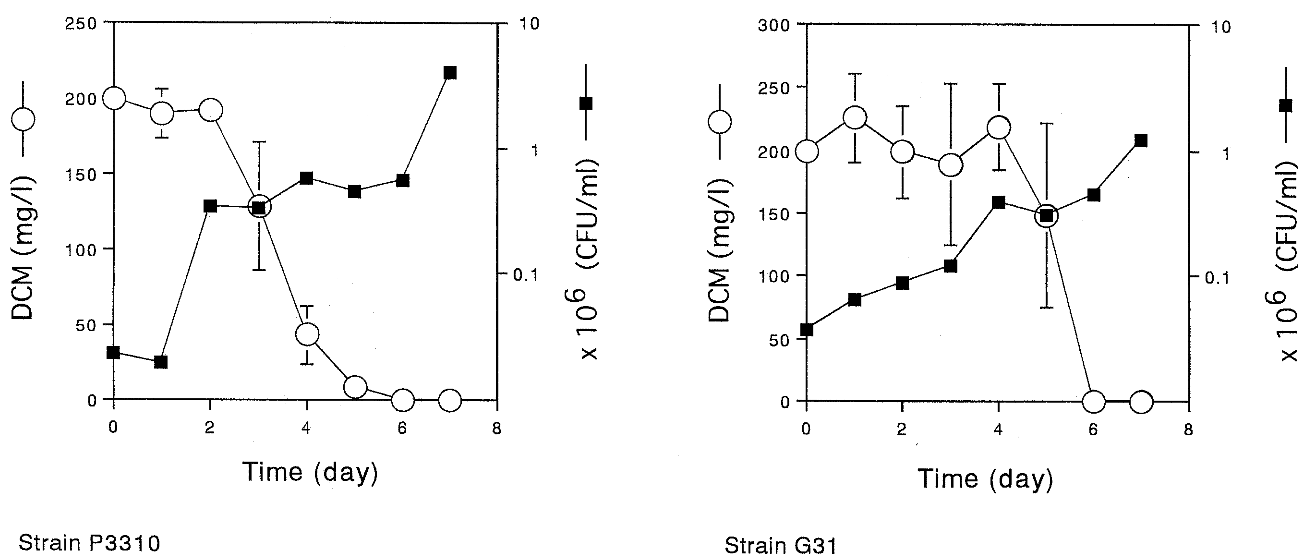

Fig. 2. Time Course of Bacterial Growth and Decrease of DCM Concentration by Incubation with Isolated Bacteria in MS Medium

Bacteria were inoculated into MS medium containing 200 mg/l DCM, and incubated at 30℃ for 7 days. Aliquots of the culture were withdrawn at regular intervals and DCM concentration and the number of bacteria (CFU) were determined.

suggesting acclimatization of the organisms.

Both strains were isolated from peptone agar plates as shown above. They could also utilize DCM for their growth, suggesting that they were facultative methylotrophs.

Although both strains showed similar degrading ability to DCM, the inhibitory effect of co-existing organic compounds was different between the strains. Addition of 10 and 30 mg/l of peptone showed 18 and 100% inhibition of degradation of 200 mg/l DCM by strain G31 after 7 days incubation in MS medium, whereas the same concentrations of peptone did not affect the activity of strain P3310 and sufficient degrading activity was observed, even with 1000 mg/l peptone (data not shown). Similar results were obtained by addition of glucose, suggesting an inhibitory effect of nutrient on degrading activity of strain G31 but not on P3310. The water sample from which P3310 was isolated did not show DCM degrading activity in peptone medium as shown in Table 1, although it showed enough activity in MS medium in which no carbon source other than DCM was added. It is considered that P3310 did not show activity in the peptone medium because of interference by other organisms, since P3310 may be a minor species in the water sample. In any case, P3310 is an useful candidate for elimination of DCM from drainage water, if enough cells are used. An increase in DCM-degrading activity by acclimatization was observed. Furthermore, DCM-degrading enzyme was detected in the culture supernatant (unpublished observations). Application of these bacterial cells or enzyme protein from strain P3310 to eliminate DCM may be possible.

Further characterization of the isolates and the enzyme are in progress in our laboratory.

REFERENCES

1) Braus-Stromeyer S.A., Hermann R., Cook A.M., Leisinger T., *Appl. Environ. Microbiol.*, **59**, 3790—3797 (1993).
2) Leisinger T., *Environ. Health Perspect*, **103** (Suppl 5), 33—35 (1995).
3) Brunner W., Staub D., Leisinger T., *Appl. Environ. Microbiol.*, **40**, 950—958 (1980).
4) Leisinger T., Bader R., Hermann R., Schmid-Appert M., *Appl. Environ. Microbiol.*, **55**, 2819—2826 (1989).
5) Oldenhius R., Oedzes J.Y., van der Waarde J.J., Uilleumier S., *Biodegradation*, **5**, 237—248 (1994).
6) McClay K., Fox B.G., Steffan R.J., *Appl. Environ. Microbiol.*, **62**, 2716—2722 (1996).
7) Oldenhius R., Vink R.L.J.M., Janssen D.B., Witholt B., *Appl. Environ. Microbiol.*, **57**, 7—14 (1991).
8) Patel R.N., *Appl. Environ. Microbiol.*, **44**, 1130—1137 (1982).
9) Kondo M., Nishihara T., Shimamoto T., Watabe K., Fujii M., *Eisei Kagaku*, **34**, 115—122 (1988).
10) Kondo M., Nishihara T., Shimamoto T., Koshikawa T., Iio T., Sawamura R., Tanaka K., *Eisei Kagaku*, **34**, 188—195 (1988).
11) Holmes B., Steigewalt A.G., Weaver R.E., Brenner D.J., *Int. J. Syst. Bacteriol.*, **37**, 245—250 (1987).
12) Vandamme P., Bernard J.-F., Segers P., Kersters K., Holmes B., *Int. J. Syst. Bacteriol.*, **44**, 827—831 (1994).