Title: Assimilative and Co-metabolic Degradation of Chloral Hydrate by Bacteria and their Bioremediation Potential

Running title: CH degradation by a bacterial culture

Huhe[1], Nobuhiko Nomura[1], Toshiaki Nakajima[1], and Hiroo Uchiyama[1]*

[1] Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

*Phone and fax: 81-29-853-6626 E-mail: uchiyama.hiroo.fw@u.tsukuba.ac.jp

Abstract

Although the bacterial degradation of chloral hydrate (CH) has been recognized for several decades, its degradation pathway by assimilation has not been demonstrated. In this paper, we report the isolation of the LF54 bacterial strain, which utilizes CH as its sole carbon and energy source. LF54 converted CH into trichloroethanol (TCAol), which was dehalogenated to dichloroethanol (DCAol), and CO_2 was detected as the end product. Another strain that we isolated, RS20, co-metabolized CH into TCAol. Our 16S rRNA gene sequencing and taxonomic analyses revealed that the LF54 and RS20 strains belong to the *Pseudomonas* and *Arthrobacter* genera, respectively. When the two strains were inoculated into soil microcosms, both degraded 0.3 mM CH to undetectable levels (<0.01 mM) within 4 days. These results suggest that LF54 and RS20 could be used in the bioremediation of CH-contaminated environments.

20 **Key words:** Chloral hydrate; Biodegradation; Soil microcosm; Assimilative; Co-
21 metabolic

22 **Introduction**

23 Chloral hydrate (CH) is synthesized by the chlorination of ethanol and is usually used
24 as an intermediate in the production of insecticides and herbicides such as methoxychlor,
25 naled, trichlorfon, dichlorvos, and trichloroacetic acid (TCA) (1). Therefore,
26 environments near factories that produce CH and the abovementioned pesticides may be
27 contaminated. Xu *et al.* (2) reported that, in China, soil contamination by CH resulted
28 from the use of contaminated river water for field irrigation. In addition, CH is formed
29 when drinking water is disinfected by chlorine (3, 4) and is the third most prevalent
30 disinfection by-product in drinking water after trihalomethanes and haloacetic acids (3).
31 In the United States, median CH concentrations in finished water have been reported to
32 range from 1.0×10^{-5} to 1.5×10^{-5} mM, whereas the maximum concentrations in each
33 report ranged from 1.3×10^{-4} to 2.8×10^{-4} mM (5, 6). Surveys conducted in 1995 and
34 1997 showed that the CH levels in Canadian drinking water supplies ranged from
35 7.3×10^{-6} to 2.3×10^{-5} mM in winter and from 2.2×10^{-5} to 5.1×10^{-5} mM in the summer,
36 with a maximum level of 1.4×10^{-4} mM observed in winter (7, 8).

37 The high water solubility and low volatility of CH preclude significant exposure by
38 inhalation from the water solution (9). However, according to the World Health
39 Organization (WHO) guidelines, CH should be limited in drinking water because of its

40 adverse effect on health; the current maximum contaminant level is set at 6.1×10^{-5} mM
41 (10). Additionally, CH has been reported as a potent genotoxic and carcinogenic
42 compound (4). Therefore, efforts should be made to minimize further CH release into
43 the environment and to clean contaminated soil and water.

44 Concerning the bacterial degradation of CH, Nakajima *et al*. (11) and Oldenhuis *et al*.
45 (12) reported that the methanotrophic-degrading bacteria *Methylocystis* sp. M and
46 *Methylosinus trichosporium* OB3b could convert trichloroethylene into CH, which was
47 further degraded to trichloroethanol (TCAol) and TCA. This degradation was carried
48 out by a co-metabolism process. However, CH degradation by an assimilation pathway
49 has not been reported to date. In this paper, we report the isolation of a bacterial strain,
50 LF54, which can dechlorinate CH as its sole carbon and energy source under defined
51 growth conditions. Furthermore, we describe CH degradation by another co-metabolic
52 non-methanotrophic bacterial strain, RS20. Moreover, we surveyed the feasibility of
53 using the LF54 and RS20 strains in CH-contaminated soil using soil microcosm
54 experiments.

55 **Materials and methods**

56 **Mineral salts**

57 The cultivation media used in our experiments contained mineral salts (MS) in the
58 following quantities: 2.7 g KH_2PO_4, 2.7 g Na_2HPO_4, 1.0 g NH_4NO_3, 0.11 g
59 $MgSO_4\cdot7H_2O$, 0.6 g $Ca(NO_3)_2\cdot4H_2O$, 0.2 g $ZnSO_4\cdot7H_2O$, 0.1 g $FeSO_4\cdot7H_2O$, 0.03 g

60 $MnSO_4 \cdot 5H_2O$, 7.8 mg $CuSO_4 \cdot 5H_2O$, 4.5 mg $NiSO_4 \cdot 6H_2O$, 4.96 mg $Co(NO_3)_2 \cdot 6H_2O$,

61 0.23 mg H_3BO_3, 0.18 mg Na_2SeO_4, 0.32 mg $Na_2WO_4 \cdot 2H_2O$, and 0.25 mg

62 $Na_2MoO_4 \cdot 2H_2O$ (all in 1 l of deionized water). The pH was adjusted to 7.0 with 1 M

63 NaOH. Those chemicals were obtained from Wako Pure Chemicals Ltd., (Osaka,

64 Japan).

65 **Isolation of CH-degrading bacteria**

66 CH-degrading bacteria were isolated by the enrichment culture method using CH

67 from three samples collected at different sites, namely river sand, paddy field soil, and

68 lotus field soil in Tsukuba, a region in Japan that has no CH pollution history. The

69 enrichment culture was carried in two different media: MS medium supplemented with

70 0.3 mM CH as the sole carbon and energy source, and MS medium supplemented with

71 0.08 g l^{-1} Luria-Bertani (LB) broth (Nacalai Tesque, Inc., Kyoto, Japan) and 0.3 mM

72 CH. The 100-ml enrichment cultures were incubated at 30°C in 200-ml Erlenmeyer

73 flasks closed with silicone stoppers and shaken at 150 rpm. Their CH concentrations

74 were regularly monitored by gas chromatography (GC) (GC-2010, Shimadzu, Kyoto,

75 Japan) as described below. When CH was degraded to levels below the detection limit

76 (<0.01 mM) in the culture supernatant, 1% transfers to fresh enrichment medium were

77 performed, totaling five serial transfers. Finally, these cultures were diluted 10-fold and

78 spread on solidified MS medium containing 0.3 mM CH or on MS medium including

79 0.08 g l^{-1} LB agar and 0.3 mM CH to obtain pure isolates. Then, taxonomic

80 classification targeted to the single colonies was carried out by PCR-restriction

81 fragment length polymorphism (PCR-RFLP) analysis.

82 **CH degradation by isolated strains**

83 The CH degradation experiments with growing cells were performed as described

84 below. The LF54 strain (approximately 2×10^6 cells ml^{-1}) was suspended in a 100-ml

85 Erlenmeyer flask containing 25 ml of MS medium and 0.3 mM of CH in the liquid

86 phase using a syringe. The CH dechlorination activity was monitored in comparison to

87 respective controls without cells, and the cultures were incubated at 30°C and shaken at

88 150 rpm. Samples were taken at regular intervals to monitor the CH concentration,

89 degradation product, amount of chloride, and total protein concentrations.

90 To investigate the upper limit concentration of CH degraded, an initial inoculum of 2

91 $\times 10^6$ cells ml^{-1} of the LF54 or RS20 (MS medium including 0.08 g l^{-1} LB) strain was

92 cultured at different CH concentrations (0.3, 0.6, 3, or 6 mM) as described above. The

93 CH concentration was measured by GC.

94 To study the kinetics of strains LF54 and RS20, the following first order reaction

95 kinetic equation was used:

96 $$-\ln(C_t/C_0) = k_1 \chi_t t$$

97 where C_t and C_0 are the CH concentration (mM) at time t (day) and time t = 0,

98 respectively; k_1 (l/mg·day) is the pseudo-first-order specific rate reaction constant, and

99 χ_t is the biomass at time t (day) (13). We converted the protein measurements to
100 biomass by assuming that the cells are 55% protein by mass (dry weight) as in
101 *Escherichia coli* according to Neidhardt et al. (22).

102 **Classification of CH-degrading bacteria by PCR-RFLP**

103 The analysis of polymorphisms associated with the 16S rRNA gene fragments was
104 performed as described previously (14). Briefly, the 16S rRNA gene fragments were
105 amplified by PCR in a thermocycler (BIO-RAD Laboratories Inc., Hercules, California,
106 USA) at 95°C for 30 s, 55°C for 1 min, and 72°C for 2 min for a total of 35 cycles. The
107 primers used for PCR amplification of the 16S rRNA gene were 27f (5'-
108 AGAGTTTGATCCTGGCTCAG -3') and 1494r (5'-
109 TGACTGACTGAGGYTACCTTGTTAC -3'). The *Ex Taq* polymerase (TAKARA Bio.
110 Inc., Shiga, Japan) was used in this study. The amplified fragments were digested with
111 the *Hae*III, *Rsa*I, and *Hpa*II restriction enzymes (Toyobo, Co., Osaka, Japan) at 37°C
112 for 2 h; next, the digestion products were separated on 2% agarose (Nacalai Tesque, Inc.,
113 Kyoto, Japan) gels (100 V, 30 min) and visualized with ethidium bromide staining. A
114 100-bp DNA ladder (Bioneer, Daejon, Korea) was included as a size marker.

115 **Identification of the LF54 and RS20 strains**

116 Phylogenetic analysis was carried out with genomic DNA from the LF54 and RS20
117 strains; the DNA was extracted using the method described by Mizuguchi *et al*. (15).
118 The primers used for PCR amplification of the 16S rRNA gene were 27f and 1494r, as

119 described above. The purified PCR product was used directly in 16S rRNA gene

120 sequencing reactions with the primers 27f, 357f (5'- CCTACGGGAGGCAGCAG -3'),

121 518r (5'- GTATTACCGCGGCTGCTGG -3'), 907r (5'-

122 CCGTCAATTCCTTTGAGTTT -3'), 1387r (5'- GGGCGGWGTGTACAAGGC -3'),

123 and 1494r with a BigDye™ Terminator cycle sequence kit (Applied Biosystems,

124 Perkin-Elmer, Foster City, CA, USA). The complete sequence was assembled and

125 edited with DNASIS® Pro (Hitachi Software Engineering Co., Tokyo, Japan). The

126 assembled sequence was used to query the GenBank database using the BLAST

127 algorithm to identify the sequences most closely related to those of the isolated strains.

128 A phylogenic tree was generated using the NJPlot function in ClustalX (16).

129 **Measurement of CO_2**

130 We carried out a resting cell experiment to reliably measure the end product (CO_2).

131 The LF54 or RS20 strains were grown to mid-logarithmic phase in LB medium, washed

132 twice with chloride-free MS medium, and resuspended in a 69-ml serum bottle

133 containing 15 ml of MS medium and 9×10^8 cells ml^{-1}. The bottle was sealed with butyl

134 stoppers, which were crimped with aluminum caps, and the gas in the serum bottle was

135 replaced with pure air (<0.1 vol. ppm of CO, <0.1 vol. ppm of CO_2 and <0.1 vol. ppm

136 of total hydrocarbon) (Grade 1) (Saisan Co., Saitama, Japan) by a needle. After the

137 addition of 1 mM CH in the liquid phase using a syringe, the cultures were incubated at

138 30°C and shaken at 150 rpm. In the CO_2 analysis, the air in the serum bottle was

139 replaced with pure air (Grade 1) (Saisan Co., Saitama, Japan); the culture medium was
140 acidified and replaced with pure air, and the CO_2 was trapped with NaOH (17). The
141 amount of CO_2 from the culture was deducted from the amount of CO_2 from the control
142 without bacteria. CO_2 was quantified by a Shimadzu total organic carbon analyzer
143 (TOC-V_{CSH}). Three serum bottles were consumed each time.

144 **Soil microcosm experiment**

145 Trial polluted soil microcosm experiments were performed to measure the ability of
146 the LF54 and RS20 isolates to degrade CH in environmental samples. The experiments
147 were carried out as follows: 10 g of sandy soil (7.3 g of total organic carbon kg^{-1} and 0.9
148 g of total nitrogen kg^{-1}) which has no CH pollution history and 1 ml of distilled water
149 containing the bacteria (1×10^6 cells g^{-1} of dry soil) were added to 69-ml serum bottles,
150 which were sealed with butyl stoppers crimped with aluminum caps; then, CH was
151 added to a final concentration of 0.3 mmol kg^{-1} of soil. The negative control was
152 uninoculated. The cultures were incubated at 20°C in the dark without shaking.

153 **Analytical methods**

154 The disappearance of CH and degradation products TCAol and DCAol was
155 monitored by capillary GC as follows. Samples (1 ml) were extracted with 0.1 ml of
156 *t*-butyl methyl ether (Wako, Osaka, Japan) containing 0.3 mM 1,2,3-trichloropropane
157 (Wako, Osaka, Japan) as an internal standard. The extracts were analyzed by loading
158 1.0 µl samples into a 30 m × 0.25 mm DB-1 column (J & W Scientific Co., Florida,

159 USA) using nitrogen as the carrier gas. The column was installed in a model 2010 gas
160 chromatograph (Shimadzu) equipped with a flame ionization detector. The oven
161 temperature was programmed as follows: 7 min (isothermal) at 35°C, followed by a
162 temperature increase at a rate of 20°C min^{-1} to 250°C. The injector and detector
163 temperatures were 270°C. Chloride release was measured colorimetrically according to
164 the method of Bergmann and Sanik. The chloride was mixed with a reagent consisting
165 of mercury (II) thiocyanate and iron (III) nitrate, the red color appeared which was
166 measured at 463 nm (18).
167 Cell densities were established by measuring the total cellular protein content. The
168 culture was mixed well by vortexing; then, 1 ml of culture samples were harvested by
169 centrifugation, washed, resuspended in 500 μl of 20 mM Tris-HCl buffer (pH 7.5), and
170 sonicated with an ultrasonic processor (model UP50H; Stahnsdorf, Germany). The total
171 protein content was measured in 10 μl sub-samples by the Bradford method (19) using
172 serum albumin as the standard. All assays were performed in triplicate.
173 The morphology of the strain was examined through microscope (model BHA,
174 Olympus Optical Co., Tokyo, Japan).
175 **Accession numbers**
176 The 16S rRNA gene sequences of strains LF54 and RS20 were deposited in the
177 DDBJ, EMBL, and GenBank nucleotide sequence databases under accession numbers
178 AB525406 and AB525407, respectively. Samples of the strains LF54 and RS20 were

179 provided to the Japan Collection of Microorganisms (JCM) of RIKEN BioResource

180 Center (RBC) under the culture collection accession numbers JCM 17188 and JCM

181 17187, respectively.

182 **Results**

183 **Characterization of isolates**

184 Soil-free cultures of microbes showing CH-degrading activities were obtained by the

185 aerobic enrichment of soil samples that were repeatedly transferred to fresh medium.

186 Serial 1:10 dilutions to extinction finally yielded pure cultures; from those, different

187 colony morphologies were observed. Of these pure cultures, 53 colonies were purified

188 from MS agar medium containing 0.3 mM CH, and 87 colonies were purified from

189 supplemented MS agar medium including 0.08 g l^{-1} LB containing 0.3 mM CH. These

190 purified colonies were tested for their abilities to use CH as their sole carbon and energy

191 sources and for co-metabolism. Next, the similarity of the strains was compared using

192 the PCR-RFLP method as described in the Materials and Methods section. From the

193 DNA-banding profiles, the colonies were classified into six groups; a representative

194 strain was chosen from each group based on the best degradation activity in each group,

195 and these representative strains were used for further studies. These strains were

196 designated RS13, RS15, and RS20 from river sand; PF34 from paddy field soil; and

197 LF51 and LF54 from lotus field soil.

198 **Degradation of CH**

199 There are two types of metabolism in pollutant-degrading bacteria: one is
200 assimilation, and the other is co-metabolism. Among the strains described above, two
201 strains (LF54 and PF34) were grown in liquid culture containing 0.3 mM CH as the sole
202 carbon source. As strain LF54 grew, the increase in total cellular protein was coupled
203 with a decrease in CH and the release of chloride (Fig. 1A, B). Therefore, strain LF54
204 was proposed to utilize CH as its sole carbon and energy source. Although CH
205 degradation by strain PF34 was similar to the strain LF54, but the rate of degradation
206 was comparatively slow.

207 However, in strains RS13, RS15, RS20, and LF51, neither a decrease in CH nor
208 growth was observed when CH was used as the sole carbon source in MS medium (data
209 not shown). In contrast, when MS medium was supplemented with 0.08 g l^{-1} of LB,
210 these strains showed growth (total cellular protein), and CH was reduced to TCAol;
211 however, the release of chloride was not observed (Fig. 1C, D).

212 Strain LF54 was capable of degrading up to 3 mM CH without an appreciable lag
213 period, whereas concentrations of 6 mM CH were not degraded (Fig. 2A). Similarly,
214 strain RS20 was unable to degrade 6 mM CH, but at least 50% of the CH was degraded
215 within 10 days in cultures initially containing 3 mM CH (Fig. 2B). There was no
216 significant CH disappearance from the headspace in the abiotic controls.

217 The pseudo-first-order specific rate reaction constant of the representative six strains
218 were LF54, k_1 = 0.75; PF34, k_1 = 0.49; RS20, k_1 = 0.45; RS13, k_1 = 0.18; RS15, k_1 =

219 0.19 and LF51, k_1 = 0.28. Therefore in the following study, the LF54 and RS20 strains

220 displaying the fastest rates of CH removal were used as the representative CH

221 assimilative and co-metabolic strains, respectively.

222 **Identification of the CH-degrading bacteria, LF54 and RS20**

223 Strain LF54 was identified as a gram-negative, rod-shaped, motile bacterium, and its

224 cells were able to form flocs when grown in liquid medium supplemented with CH.

225 This ability may be due to the production of an extracellular matrix, making accurate

226 determinations of the cell numbers difficult by the optical density method, particularly

227 at low cell densities. Strain RS20 was identified to be a gram-positive obligate aerobe

228 that formed rods during exponential growth and cocci in the stationary phase. However,

229 strain RS20 did not belong to *Rhodococcus* based on 16S rRNA gene sequencing and

230 phylogenetic tree analysis (Fig. 3).

231 Analyses of 16S rRNA gene sequencing and taxonomy revealed that the LF54 and

232 RS20 strains belong to the *Pseudomonas* and *Arthrobacter* genera, respectively. The

233 closest relatives to the LF54 strain are *Pseudomonas monteilii* and *Pseudomonas fulva*

234 (99% sequence identity to each). Both have been placed in the *P. putida* group (20), and

235 this was confirmed in this experiment (Fig. 3). Therefore, LF54 was indicated to be a *P.*

236 *putida* strain. Strain RS20 is closely related to *Arthrobacter* sp. BS11 (Fig. 3); although

237 it had been separated from the dechlorinating bacteria *Desulfitobacterium*,

238 *Dehalobacter*, *Dehalococcoides*, *Clostridium*, *Methylosinus*, and *Methylocystis* in the

239 phylogenetic tree, the similarity was high compared to *Pseudomonas putida* F1, which
240 was reported to perform dehalogenation (21). Based on these results, we identified and
241 named strain LF54 *Pseudomonas* sp. LF54, whereas strain RS20 was identified and
242 named *Arthrobacter* sp. RS20.

243 **Time course of CH degradation products by LF54 and RS20**

244 From the resting cell assay, CO_2 was detected as the major end product (Fig. 4A). CH
245 (1 mM) was degraded to levels below the detection limit (<0.01 mM) in 40 h, and CO_2,
246 TCAol, and DCAol accounted for 62%, 29% and 9%, respectively, of the initial carbon
247 added. After approximately 10 more hours, almost all of carbon was transformed to CO_2
248 (Fig. 4A). However, in strain RS20, 75% of the initial carbon added was transformed to
249 TCAol in 40 h, and almost all of the CH was transformed to TCAol in 50 h; however,
250 CO_2 and DCAol were not observed (Fig. 4B).

251 **Bioaugmentation potential in the soil microcosm**

252 To assess the potential of the LF54 and RS20 strains for CH biodegradation in soil,
253 the microcosms were inoculated with 1×10^7 cells g^{-1} of soil with CH. Both LF54 and
254 RS20 strains degraded an initial concentration of 0.3 mmol of CH kg^{-1} of soil to
255 undetectable levels (<0.01 mM) in 5 days (Fig. 5A, B). TCAol and DCAol were
256 detected during the degradation described above, and a very small amount of TCA was
257 also detected. In the control, CH decreased by 15% in 5 days, and very small amounts
258 of TCA and TCAol were detected (Fig. 5C).

259 In all of the degradation experiments described above, the pseudo-first-order specific
260 rate constants (k_1) (l/mg･day) of the LF54 and RS20 strains were calculated as follows:
261 in liquid culture (obtained from Fig. 1A and C), $k_1 = 0.75$ and $k_1 = 0.45$, respectively,
262 and in soil microcosms (obtained from Fig. 5A and B), $k_1 = 0.55$ and $k_1 = 0.5$,
263 respectively.

264 **Discussion**

265 The ability of CH to support the growth of microorganisms has been questioned, and
266 it has been suggested that CH degradation is the result of the co-metabolism of
267 methanotrophic bacteria rather than direct growth-related mechanisms (11, 12).
268 However, the results of the present study clearly show the existence of bacteria that can
269 utilize CH as the sole carbon source without any requirement for co-substrates or
270 growth factors.

271 From the protein analysis in Fig. 1B, the biomass yield of strain LF54 grown in the
272 presence of CH was estimated. Approximately 3.9 mg l^{-1} of protein was additionally
273 produced per 0.3 mM (49.5 mg l^{-1}) of CH consumed in 10 days, this corresponds to a
274 yield of 0.12 mg of cells per mg of CH ($Y_{x/s}$) (1.4 g of biomass per mol of available
275 electrons). The specific growth rate (μ_{max}) was determined to be 0.005 h^{-1}. Furthermore,
276 the growth yield calculated based on the available electrons (2.2 g of biomass per mol of
277 available electrons) is a typical value for aerobic growth on different substrates (23).
278 These values were less than those obtained from growth on chlorinated hydrocarbons

279 and methyl *tert*-butyl ether reported by Yu *et al.* (23) and Hanson *et al.* (24). The low
280 growth rate and low yield found in this study could be explained by the possible
281 inhibitory feature of CH on bacterial growth, resulting in low-efficiency energy
282 production (25). In addition, CH may act as an uncoupler of ATP synthesis, or
283 metabolic intermediates may be toxic in the LF54 strain, as reported by Salanitro *et al.*
284 (26).

285 Regarding the intermediates of CH degradation by LF54, a small amount of
286 monochloroethanol (MCAol) formation was expected, but it was not detected under
287 either growing or resting cell conditions (Fig. 1, 4). Presumably, MCAol might be
288 rapidly degraded before reaching measurable levels.

289 Based on the results described above, we postulated the degradation pathway of CH
290 by strain LF54 as depicted in Fig. 6. We speculate that CH is first transformed into
291 TCAol by dehydrogenase; then, TCAol, DCAol and MCAol release one chlorine at
292 each step and are transformed into CO_2 by dehalogenase. This speculation is tentative,
293 and further study on enzymes involved in this pathway will be required to support it.

294 Although TCA was not detected in liquid culture with a single strain (Figs. 1 and 4),
295 a low concentration of TCA was detected in the soil microcosm (Fig. 5A, B and C).
296 TCAol was most likely oxidized and transformed to TCA by indigenous
297 microorganisms in the soil. Although TCA becomes a secondary contaminant, it may be
298 possible to degrade the TCA produced by co-culturing with TCA-degrading bacteria

299 such as *Xanthobacter autotrophicus*, which was reported to be a haloacetate-degrading

300 bacterium (27). Even in the control of the microcosm, TCAol and TCA were detected

301 (Fig. 5C), suggesting that other soil microorganisms that could metabolize CH may

302 exist.

303 Strains LF54 and RS20 were expected to use in CH contaminated water and soil

304 environment; we, therefore, surveyed their kinetics and their products. A comparative

305 evaluation of the degradation rate of CH in the liquid culture described above and in the

306 microcosm was carried out using the first order reaction kinetic equation. In every

307 degradation experiment, the degradation rate of the LF54 strain ($k_1 = 0.75$ in liquid; $k_1 =$

308 0.55 in soil) was faster than that of the RS20 strain ($k_1 = 0.45$ in liquid; $k_1 = 0.5$ in soil).

309 This result may not be unexpected because the RS20 strain converts CH into TCAol,

310 which is probably toxic to growth, whereas the LF54 strain assimilates CH and

311 continues to grow. When the k_1 of strain LF54 was compared in liquid culture and in the

312 soil microcosm, it was lower in the soil microcosm. This result may be caused by

313 interactions with other microorganisms [e.g., predation (28)]; such interactions did not

314 occur in liquid culture because a single strain was used.

315 From Fig. 2A and B, the CH degradation upper limit of the LF54 and RS20 strains

316 was between 3 and 6 mM. In finished water, the highest concentration of CH was

317 2.8×10^{-4} mM, suggesting that the LF54 and RS20 strains could remediate the

318 contaminated finished water. The LF54 strain might also be a promising candidate for

319 the bioaugmentation of CH-contaminated soil.

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419 **FIG. 1** CH degradation and cellular protein production by the LF54 and RS20 strains.

420 Symbols: CH (closed circles); TCAol (open squares); DCAol (closed triangles);

421 chloride (open circles); protein concentration (closed squares). In A and B, CH

422 degradation was performed using the LF54 strain in MS medium. In C and D, the RS20

423 strain was used in MS medium supplemented with LB.

424

425 **FIG. 2** Degradation of the increasing CH concentrations by the LF54 (**A**) and RS20 (**B**)

426 strains. The strains were incubated with CH at initial concentrations of 0.3 (closed

427 circles), 0.6 (closed squares), 3 (open triangles), and 6 (closed triangles) mM. A

428 representative abiotic control (0.3 mM CH) is also shown (open circles).

429

430 **FIG. 3** Phylogenetic positions of the LF54 and RS20 strains among related chlorinated

431 aliphatic hydrocarbon-degrading strains based on 16S rRNA gene sequence

432 comparisons. The accession numbers of reference organisms have been indicates in

433 parentheses beside the name of organisms. The numbers are bootstrap values for

434 branches based on 100 replicates. The bar shows two nucleotide substitutions per 100

435 nucleotides.

436

437 **FIG. 4** Time course of CH degradation products by LF54 (**A**) and RS20 (**B**). CH and

438 CO_2 are C2- and C1-compounds, respectively; one molecule of CH forms two

439 molecules of CO_2. In this experiment, to investigate the total mass balance of carbon

440 during the degradation, the detected amount (molar) of C2 compounds (CH, TCAol and

441 DCAol) was doubled, i.e., 1 mM of CH was presented as 2 mM in the figure. In each

442 time, CO_2 concentration was presented as the average of three bottles.

443

444 **FIG. 5** CH degradation in a soil microcosm inoculated with the LF54 (**A**) and RS20 (**B**)

445 strains. Soil samples were inoculated with 1×10^7 cells g^{-1} of soil and 0.3 mmol CH

446 kg^{-1} of soil. The control (**C**) was not inoculated. CH (open circles), TCAol (open

447 squares), DCAol (open triangles), and TCA (closed circles).

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449 **FIG. 6** Proposed CH biodegradation pathway in the LF54 and RS20 strains.

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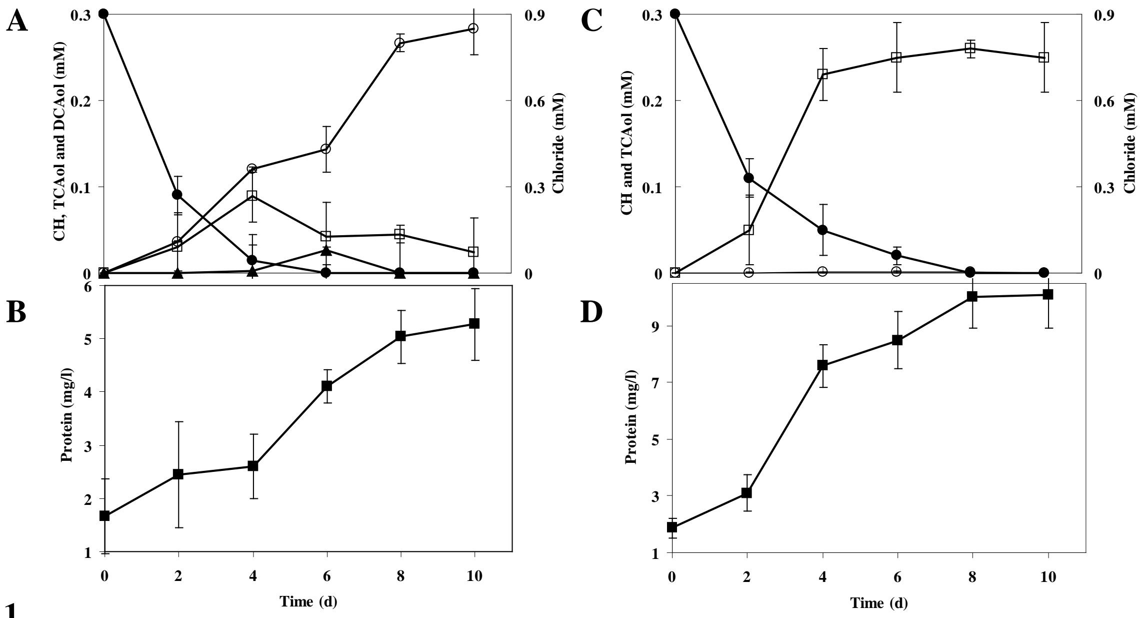

FIG. 1

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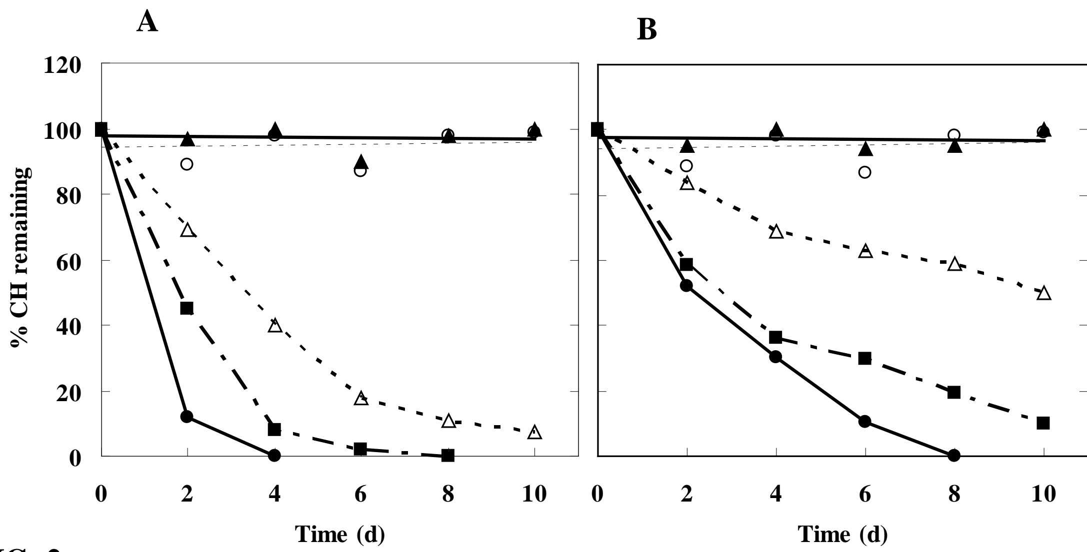

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36 **FIG. 2**

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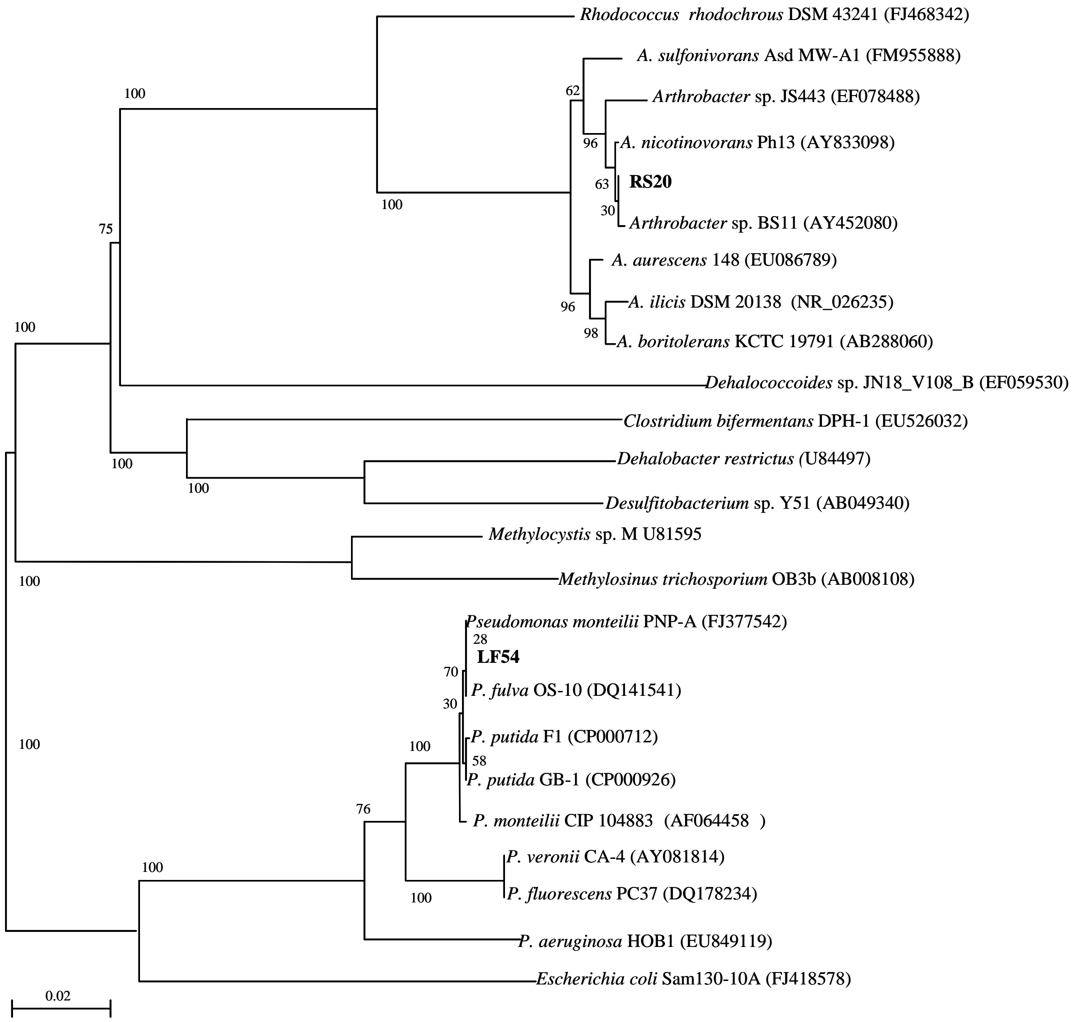

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62 **FIG. 3**

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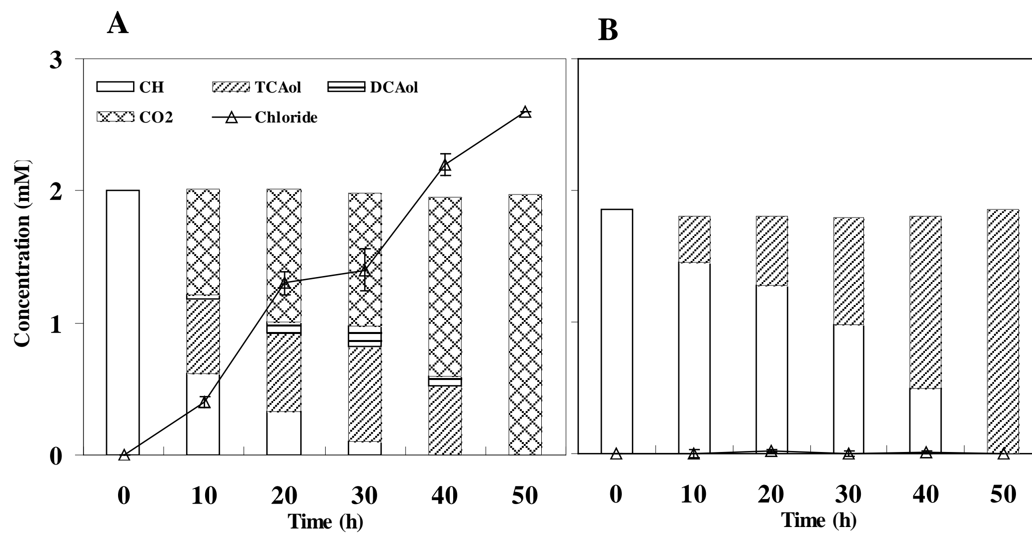

81 **FIG. 4**

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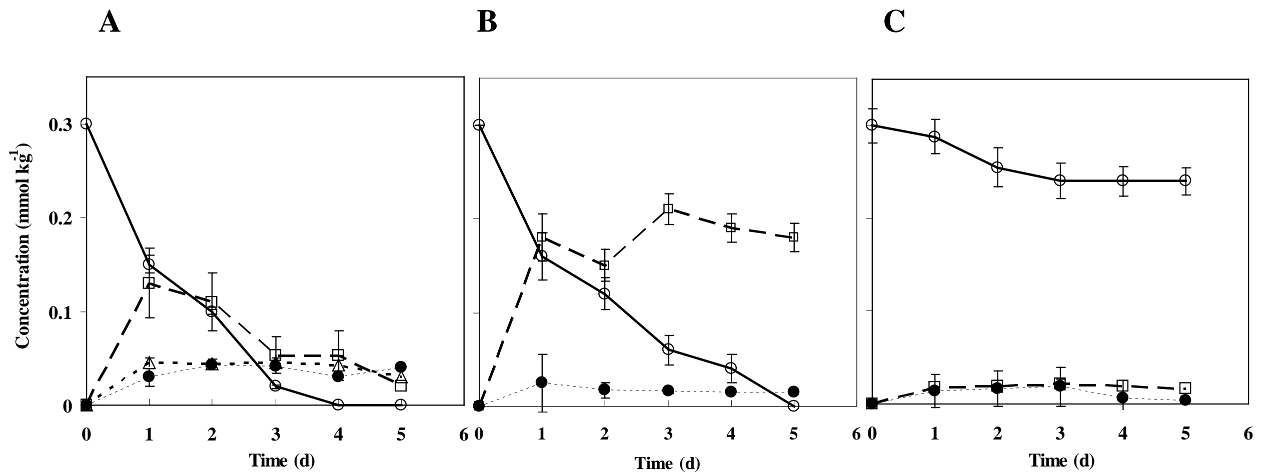

102 **FIG. 5**

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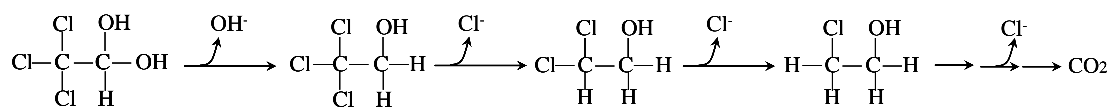

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122 **FIG. 6**

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