Assimilative and co-metabolic degradation of chloral hydrate by bacteria and their bioremediation potential

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

Running title: CH degradation by a bacterial culture

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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 CO₂ 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.
**Key words:** Chloral hydrate; Biodegradation; Soil microcosm; Assimilative; Co-metabolic

**Introduction**

Chloral hydrate (CH) is synthesized by the chlorination of ethanol and is usually used as an intermediate in the production of insecticides and herbicides such as methoxychlor, naled, trichlorfon, dichlorvos, and trichloroacetic acid (TCA) (1). Therefore, environments near factories that produce CH and the abovementioned pesticides may be contaminated. Xu et al. (2) reported that, in China, soil contamination by CH resulted from the use of contaminated river water for field irrigation. In addition, CH is formed when drinking water is disinfected by chlorine (3, 4) and is the third most prevalent disinfection by-product in drinking water after trihalomethanes and haloacetic acids (3).

In the United States, median CH concentrations in finished water have been reported to range from $1.0 \times 10^{-5}$ to $1.5 \times 10^{-5}$ mM, whereas the maximum concentrations in each report ranged from $1.3 \times 10^{-4}$ to $2.8 \times 10^{-4}$ mM (5, 6). Surveys conducted in 1995 and 1997 showed that the CH levels in Canadian drinking water supplies ranged from $7.3 \times 10^{-6}$ to $2.3 \times 10^{-5}$ mM in winter and from $2.2 \times 10^{-5}$ to $5.1 \times 10^{-5}$ mM in the summer, with a maximum level of $1.4 \times 10^{-4}$ mM observed in winter (7, 8).

The high water solubility and low volatility of CH preclude significant exposure by inhalation from the water solution (9). However, according to the World Health Organization (WHO) guidelines, CH should be limited in drinking water because of its...
adverse effect on health; the current maximum contaminant level is set at $6.1 \times 10^{-5}$ mM (10). Additionally, CH has been reported as a potent genotoxic and carcinogenic compound (4). Therefore, efforts should be made to minimize further CH release into the environment and to clean contaminated soil and water.

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

**Materials and methods**

**Mineral salts**

The cultivation media used in our experiments contained mineral salts (MS) in the following quantities: 2.7 g KH$_2$PO$_4$, 2.7 g Na$_2$HPO$_4$, 1.0 g NH$_4$NO$_3$, 0.11 g MgSO$_4$$\cdot$7H$_2$O, 0.6 g Ca(NO$_3$)$_2$$\cdot$4H$_2$O, 0.2 g ZnSO$_4$$\cdot$7H$_2$O, 0.1 g FeSO$_4$$\cdot$7H$_2$O, 0.03 g
MnSO₄·5H₂O, 7.8 mg CuSO₄·5H₂O, 4.5 mg NiSO₄·6H₂O, 4.96 mg Co(NO₃)₂·6H₂O,
0.23 mg H₃BO₃, 0.18 mg Na₂SeO₄, 0.32 mg Na₂WO₄·2H₂O, and 0.25 mg
Na₂MoO₄·2H₂O (all in 1 l of deionized water). The pH was adjusted to 7.0 with 1 M
NaOH. Those chemicals were obtained from Wako Pure Chemicals Ltd., (Osaka,
Japan).

**Isolation of CH-degrading bacteria**

CH-degrading bacteria were isolated by the enrichment culture method using CH
from three samples collected at different sites, namely river sand, paddy field soil, and
lotus field soil in Tsukuba, a region in Japan that has no CH pollution history. The
enrichment culture was carried in two different media: MS medium supplemented with
0.3 mM CH as the sole carbon and energy source, and MS medium supplemented with
0.08 g l⁻¹ Luria-Bertani (LB) broth (Nacalai Tesque, Inc., Kyoto, Japan) and 0.3 mM
CH. The 100-ml enrichment cultures were incubated at 30°C in 200-ml Erlenmeyer
flasks closed with silicone stoppers and shaken at 150 rpm. Their CH concentrations
were regularly monitored by gas chromatography (GC) (GC-2010, Shimadzu, Kyoto,
Japan) as described below. When CH was degraded to levels below the detection limit
(<0.01 mM) in the culture supernatant, 1% transfers to fresh enrichment medium were
performed, totaling five serial transfers. Finally, these cultures were diluted 10-fold and
spread on solidified MS medium containing 0.3 mM CH or on MS medium including
0.08 g l⁻¹ LB agar and 0.3 mM CH to obtain pure isolates. Then, taxonomic
classification targeted to the single colonies was carried out by PCR-restriction
fragment length polymorphism (PCR-RFLP) analysis.

**CH degradation by isolated strains**

The CH degradation experiments with growing cells were performed as described
below. The LF54 strain (approximately $2 \times 10^6$ cells ml$^{-1}$) was suspended in a 100-ml
Erlenmeyer flask containing 25 ml of MS medium and 0.3 mM of CH in the liquid
phase using a syringe. The CH dechlorination activity was monitored in comparison to
respective controls without cells, and the cultures were incubated at 30°C and shaken at
150 rpm. Samples were taken at regular intervals to monitor the CH concentration,
degradation product, amount of chloride, and total protein concentrations.

To investigate the upper limit concentration of CH degraded, an initial inoculum of $2 \times 10^6$ cells ml$^{-1}$ of the LF54 or RS20 (MS medium including 0.08 g l$^{-1}$ LB) strain was
cultured at different CH concentrations (0.3, 0.6, 3, or 6 mM) as described above. The
CH concentration was measured by GC.

To study the kinetics of strains LF54 and RS20, the following first order reaction
kinetic equation was used:

$$-\ln\left(\frac{C_t}{C_0}\right) = k_1 \chi t$$

where $C_t$ and $C_0$ are the CH concentration (mM) at time $t$ (day) and time $t = 0$,
respectively; $k_1$ (l/mg·day) is the pseudo-first-order specific rate reaction constant, and
\( \chi_t \) is the biomass at time \( t \) (day) \((13)\). We converted the protein measurements to biomass by assuming that the cells are 55% protein by mass (dry weight) as in *Escherichia coli* according to Neidhardt et al. (22).

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

The analysis of polymorphisms associated with the 16S rRNA gene fragments was performed as described previously \((14)\). Briefly, the 16S rRNA gene fragments were amplified by PCR in a thermocycler (BIO-RAD Laboratories Inc., Hercules, California, 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 primers used for PCR amplification of the 16S rRNA gene were 27f \( (5' - AGAGTTTGATCCTGGCTCAG - 3') \) and 1494r \( (5' - TGACTGACTGAGGYTACCTTGTTAC - 3') \). The *Ex Taq* polymerase (TAKARA Bio. Inc., Shiga, Japan) was used in this study. The amplified fragments were digested with the *HaeIII*, *RsaI*, and *HpaII* restriction enzymes (Toyobo, Co., Osaka, Japan) at 37°C for 2 h; next, the digestion products were separated on 2% agarose (Nacalai Tesque, Inc., Kyoto, Japan) gels (100 V, 30 min) and visualized with ethidium bromide staining. A 100-bp DNA ladder (Bioneer, Daejon, Korea) was included as a size marker.

**Identification of the LF54 and RS20 strains**

Phylogenetic analysis was carried out with genomic DNA from the LF54 and RS20 strains; the DNA was extracted using the method described by Mizuguchi *et al.* \((15)\). The primers used for PCR amplification of the 16S rRNA gene were 27f and 1494r, as
described above. The purified PCR product was used directly in 16S rRNA gene sequencing reactions with the primers 27f, 357f (5'- CCTACGGGAGGCAGCAG -3'), 518r (5'- GTATTACCGCGGTGCTGG -3'), 907r (5'- CCGTCAATTCTTTGAGTTT -3'), 1387r (5'- GGGCGGWGTGTACAAGGC -3'), and 1494r with a BigDye™ Terminator cycle sequence kit (Applied Biosystems, Perkin-Elmer, Foster City, CA, USA). The complete sequence was assembled and edited with DNASIS® Pro (Hitachi Software Engineering Co., Tokyo, Japan). The assembled sequence was used to query the GenBank database using the BLAST algorithm to identify the sequences most closely related to those of the isolated strains. A phylogenic tree was generated using the NJPlot function in ClustalX (16).

**Measurement of CO₂**

We carried out a resting cell experiment to reliably measure the end product (CO₂). The LF54 or RS20 strains were grown to mid-logarithmic phase in LB medium, washed twice with chloride-free MS medium, and resuspended in a 69-ml serum bottle containing 15 ml of MS medium and 9 × 10⁸ cells ml⁻¹. The bottle was sealed with butyl stoppers, which were crimped with aluminum caps, and the gas in the serum bottle was replaced with pure air (<0.1 vol. ppm of CO, <0.1 vol. ppm of CO₂ and <0.1 vol. ppm of total hydrocarbon) (Grade 1) (Saisan Co., Saitama, Japan) by a needle. After the addition of 1 mM CH in the liquid phase using a syringe, the cultures were incubated at 30°C and shaken at 150 rpm. In the CO₂ analysis, the air in the serum bottle was
replaced with pure air (Grade 1) (Saisan Co., Saitama, Japan); the culture medium was
acidified and replaced with pure air, and the CO₂ was trapped with NaOH (17). The
amount of CO₂ from the culture was deducted from the amount of CO₂ from the control
without bacteria. CO₂ was quantified by a Shimadzu total organic carbon analyzer
(TOC-VCSH). Three serum bottles were consumed each time.

Soil microcosm experiment

Trial polluted soil microcosm experiments were performed to measure the ability of
the LF54 and RS20 isolates to degrade CH in environmental samples. The experiments
were carried out as follows: 10 g of sandy soil (7.3 g of total organic carbon kg⁻¹ and 0.9
g of total nitrogen kg⁻¹) which has no CH pollution history and 1 ml of distilled water
containing the bacteria (1 × 10⁶ cells g⁻¹ of dry soil) were added to 69-ml serum bottles,
which were sealed with butyl stoppers crimped with aluminum caps; then, CH was
added to a final concentration of 0.3 mmol kg⁻¹ of soil. The negative control was
uninoculated. The cultures were incubated at 20°C in the dark without shaking.

Analytical methods

The disappearance of CH and degradation products TCAol and DCAol was
monitored by capillary GC as follows. Samples (1 ml) were extracted with 0.1 ml of
t-butyl methyl ether (Wako, Osaka, Japan) containing 0.3 mM 1,2,3-trichloropropane
(Wako, Osaka, Japan) as an internal standard. The extracts were analyzed by loading
1.0 µl samples into a 30 m × 0.25 mm DB-1 column (J & W Scientific Co., Florida,
USA) using nitrogen as the carrier gas. The column was installed in a model 2010 gas chromatograph (Shimadzu) equipped with a flame ionization detector. The oven temperature was programmed as follows: 7 min (isothermal) at 35°C, followed by a temperature increase at a rate of 20°C min⁻¹ to 250°C. The injector and detector temperatures were 270°C. Chloride release was measured colorimetrically according to the method of Bergmann and Sanik. The chloride was mixed with a reagent consisting of mercury (II) thiocyanate and iron (III) nitrate, the red color appeared which was measured at 463 nm (18).

Cell densities were established by measuring the total cellular protein content. The culture was mixed well by vortexing; then, 1 ml of culture samples were harvested by centrifugation, washed, resuspended in 500 µl of 20 mM Tris-HCl buffer (pH 7.5), and sonicated with an ultrasonic processor (model UP50H; Stahnsdorf, Germany). The total protein content was measured in 10 µl sub-samples by the Bradford method (19) using serum albumin as the standard. All assays were performed in triplicate.

The morphology of the strain was examined through microscope (model BHA, Olympus Optical Co., Tokyo, Japan).

Accession numbers

The 16S rRNA gene sequences of strains LF54 and RS20 were deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession numbers AB525406 and AB525407, respectively. Samples of the strains LF54 and RS20 were
provided to the Japan Collection of Microorganisms (JCM) of RIKEN BioResource Center (RBC) under the culture collection accession numbers JCM 17188 and JCM 17187, respectively.

**Results**

**Characterization of isolates**

Soil-free cultures of microbes showing CH-degrading activities were obtained by the aerobic enrichment of soil samples that were repeatedly transferred to fresh medium. Serial 1:10 dilutions to extinction finally yielded pure cultures; from those, different colony morphologies were observed. Of these pure cultures, 53 colonies were purified from MS agar medium containing 0.3 mM CH, and 87 colonies were purified from supplemented MS agar medium including 0.08 g l\(^{-1}\) LB containing 0.3 mM CH. These purified colonies were tested for their abilities to use CH as their sole carbon and energy sources and for co-metabolism. Next, the similarity of the strains was compared using the PCR-RFLP method as described in the Materials and Methods section. From the DNA-banding profiles, the colonies were classified into six groups; a representative strain was chosen from each group based on the best degradation activity in each group, and these representative strains were used for further studies. These strains were designated RS13, RS15, and RS20 from river sand; PF34 from paddy field soil; and LF51 and LF54 from lotus field soil.

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

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

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

The pseudo-first-order specific rate reaction constant of the representative six strains were LF54, \(k_1 = 0.75\); PF34, \(k_1 = 0.49\); RS20, \(k_1 = 0.45\); RS13, \(k_1 = 0.18\); RS15, \(k_1 = \)
0.19 and LF51, \( k_1 = 0.28 \). Therefore in the following study, the LF54 and RS20 strains displaying the fastest rates of CH removal were used as the representative CH assimilative and co-metabolic strains, respectively.

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

Strain LF54 was identified as a gram-negative, rod-shaped, motile bacterium, and its cells were able to form flocs when grown in liquid medium supplemented with CH. This ability may be due to the production of an extracellular matrix, making accurate determinations of the cell numbers difficult by the optical density method, particularly at low cell densities. Strain RS20 was identified to be a gram-positive obligate aerobe that formed rods during exponential growth and cocci in the stationary phase. However, strain RS20 did not belong to *Rhodococcus* based on 16S rRNA gene sequencing and phylogenetic tree analysis (Fig. 3).

Analyses of 16S rRNA gene sequencing and taxonomy revealed that the LF54 and RS20 strains belong to the *Pseudomonas* and *Arthrobacter* genera, respectively. The closest relatives to the LF54 strain are *Pseudomonas monteilii* and *Pseudomonas fulva* (99% sequence identity to each). Both have been placed in the *P. putida* group (20), and this was confirmed in this experiment (Fig. 3). Therefore, LF54 was indicated to be a *P. putida* strain. Strain RS20 is closely related to *Arthrobacter* sp. BS11 (Fig. 3); although it had been separated from the dechlorinating bacteria *Desulfotobacterium*, *Dehalobacter*, *Dehalococcoides*, *Clostridium*, *Methylosinus*, and *Methylocystis* in the
phylogenetic tree, the similarity was high compared to *Pseudomonas putida* F1, which was reported to perform dehalogenation (21). Based on these results, we identified and named strain LF54 *Pseudomonas* sp. LF54, whereas strain RS20 was identified and named *Arthrobacter* sp. RS20.

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

From the resting cell assay, CO2 was detected as the major end product (Fig. 4A). CH (1 mM) was degraded to levels below the detection limit (<0.01 mM) in 40 h, and CO2, TCAol, and DCAol accounted for 62%, 29% and 9%, respectively, of the initial carbon added. After approximately 10 more hours, almost all of carbon was transformed to CO2 (Fig. 4A). However, in strain RS20, 75% of the initial carbon added was transformed to TCAol in 40 h, and almost all of the CH was transformed to TCAol in 50 h; however, CO2 and DCAol were not observed (Fig. 4B).

**Bioaugmentation potential in the soil microcosm**

To assess the potential of the LF54 and RS20 strains for CH biodegradation in soil, the microcosms were inoculated with $1 \times 10^7$ cells g$^{-1}$ of soil with CH. Both LF54 and RS20 strains degraded an initial concentration of 0.3 mmol of CH kg$^{-1}$ of soil to undetectable levels (<0.01 mM) in 5 days (Fig. 5A, B). TCAol and DCAol were detected during the degradation described above, and a very small amount of TCA was also detected. In the control, CH decreased by 15% in 5 days, and very small amounts of TCA and TCAol were detected (Fig. 5C).
In all of the degradation experiments described above, the pseudo-first-order specific rate constants \( (k_1) (\text{l/mg} \cdot \text{day}) \) of the LF54 and RS20 strains were calculated as follows:
in liquid culture (obtained from Fig. 1A and C), \( k_1 = 0.75 \) and \( k_1 = 0.45 \), respectively,
and in soil microcosms (obtained from Fig. 5A and B), \( k_1 = 0.55 \) and \( k_1 = 0.5 \), respectively.

**Discussion**

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

From the protein analysis in Fig. 1B, the biomass yield of strain LF54 grown in the presence of CH was estimated. Approximately 3.9 mg l\(^{-1}\) of protein was additionally produced per 0.3 mM (49.5 mg l\(^{-1}\)) of CH consumed in 10 days, this corresponds to a yield of 0.12 mg of cells per mg of CH \((Y_{x/s})\) (1.4 g of biomass per mol of available electrons). The specific growth rate \((\mu_{\text{max}})\) was determined to be 0.005 h\(^{-1}\). Furthermore, the growth yield calculated based on the available electrons (2.2 g of biomass per mol of available electrons) is a typical value for aerobic growth on different substrates (23). These values were less than those obtained from growth on chlorinated hydrocarbons...
and methyl tert-butyl ether reported by Yu et al. (23) and Hanson et al. (24). The low
growth rate and low yield found in this study could be explained by the possible
inhibitory feature of CH on bacterial growth, resulting in low-efficiency energy
production (25). In addition, CH may act as an uncoupler of ATP synthesis, or
metabolic intermediates may be toxic in the LF54 strain, as reported by Salanitro et al.
(26).

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

Based on the results described above, we postulated the degradation pathway of CH
by strain LF54 as depicted in Fig. 6. We speculate that CH is first transformed into
TCAol by dehydrogenase; then, TCAol, DCAol and MCAol release one chlorine at
each step and are transformed into CO₂ by dehalogenase. This speculation is tentative,
and further study on enzymes involved in this pathway will be required to support it.

Although TCA was not detected in liquid culture with a single strain (Figs. 1 and 4),
a low concentration of TCA was detected in the soil microcosm (Fig. 5A, B and C).
TCAol was most likely oxidized and transformed to TCA by indigenous
microorganisms in the soil. Although TCA becomes a secondary contaminant, it may be
possible to degrade the TCA produced by co-culturing with TCA-degrading bacteria
such as *Xanthobacter autotrophicus*, which was reported to be a haloacetate-degrading bacterium (27). Even in the control of the microcosm, TCAol and TCA were detected (Fig. 5C), suggesting that other soil microorganisms that could metabolize CH may exist.

Strains LF54 and RS20 were expected to use in CH contaminated water and soil environment; we, therefore, surveyed their kinetics and their products. A comparative evaluation of the degradation rate of CH in the liquid culture described above and in the microcosm was carried out using the first order reaction kinetic equation. In every degradation experiment, the degradation rate of the LF54 strain \((k_1 = 0.75 \text{ in liquid; } k_1 = 0.55 \text{ in soil})\) was faster than that of the RS20 strain \((k_1 = 0.45 \text{ in liquid; } k_1 = 0.5 \text{ in soil})\).

This result may not be unexpected because the RS20 strain converts CH into TCAol, which is probably toxic to growth, whereas the LF54 strain assimilates CH and continues to grow. When the \(k_1\) of strain LF54 was compared in liquid culture and in the soil microcosm, it was lower in the soil microcosm. This result may be caused by interactions with other microorganisms [e.g., predation (28)]; such interactions did not occur in liquid culture because a single strain was used.

From Fig. 2A and B, the CH degradation upper limit of the LF54 and RS20 strains was between 3 and 6 mM. In finished water, the highest concentration of CH was \(2.8 \times 10^{-4} \text{ mM}\), suggesting that the LF54 and RS20 strains could remediate the contaminated finished water. The LF54 strain might also be a promising candidate for
the bioaugmentation of CH-contaminated soil.

Acknowledgments

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References


19. **Bradford, M.:** A rapid and sensitive method for the quantitation of microgram


FIG. 1 CH degradation and cellular protein production by the LF54 and RS20 strains. Symbols: CH (closed circles); TCAol (open squares); DCAol (closed triangles); chloride (open circles); protein concentration (closed squares). In A and B, CH degradation was performed using the LF54 strain in MS medium. In C and D, the RS20 strain was used in MS medium supplemented with LB.

FIG. 2 Degradation of the increasing CH concentrations by the LF54 (A) and RS20 (B) strains. The strains were incubated with CH at initial concentrations of 0.3 (closed circles), 0.6 (closed squares), 3 (open triangles), and 6 (closed triangles) mM. A representative abiotic control (0.3 mM CH) is also shown (open circles).

FIG. 3 Phylogenetic positions of the LF54 and RS20 strains among related chlorinated aliphatic hydrocarbon-degrading strains based on 16S rRNA gene sequence comparisons. The accession numbers of reference organisms have been indicates in parentheses beside the name of organisms. The numbers are bootstrap values for branches based on 100 replicates. The bar shows two nucleotide substitutions per 100 nucleotides.

FIG. 4 Time course of CH degradation products by LF54 (A) and RS20 (B). CH and CO₂ are C2- and C1-compounds, respectively; one molecule of CH forms two
molecules of CO$_2$. In this experiment, to investigate the total mass balance of carbon during the degradation, the detected amount (molar) of C2 compounds (CH, TCAol and DCAol) was doubled, i.e., 1 mM of CH was presented as 2 mM in the figure. In each time, CO$_2$ concentration was presented as the average of three bottles.

**FIG. 5** CH degradation in a soil microcosm inoculated with the LF54 (A) and RS20 (B) strains. Soil samples were inoculated with $1 \times 10^7$ cells g$^{-1}$ of soil and 0.3 mmol CH kg$^{-1}$ of soil. The control (C) was not inoculated. CH (open circles), TCAol (open squares), DCAol (open triangles), and TCA (closed circles).

**FIG. 6** Proposed CH biodegradation pathway in the LF54 and RS20 strains.
FIG. 1
FIG. 2

[Graph showing the percentage of CH remaining over time (d) for different conditions labeled A and B.]

% CH remaining

Time (d)
FIG. 3

Rhodococcus rhodochrous DSM 43241 (FJ468342)

A. sulfonivorans And MW-A1 (FM955888)

Arthrobacter sp. JS443 (EF078488)

A. nicotinovorans Ph13 (AY830986)

RS20

Arthrobacter sp. BS11 (AY452080)

A. aureus (EU086789)

A. ilicis DSM 20138 (NR_026235)

A. bortisolera KCTC 19791 (AB288060)

Dehalococcoides sp. JN18_V108_B (EF059530)

Clostridium bifermentans DPH-1 (EU526032)

Dehalobacter restrictus (U84497)

Desulfotobacterium sp. Y51 (AB049340)

Methyloctis sp. M U81595

Methylosinus trichosporium OB3b (AB008108)

Pseudomonas monteilii PNP-A (FJ377542)

LF54

P. fulva OS-10 (DQ141541)

P. putida F1 (CP000712)

P. putida GI-1 (CP000926)

P. monteilii CIP 104883 (AF064458)

P. veronii CA-4 (AY081814)

P. fluorescens PC37 (DQ178234)

P. aeruginosa HOB1 (EU849119)

Escherichia coli Sari30-10A (FJ418578)
FIG. 4
FIG. 5
FIG. 6

Chloral hydrate → Trichloroethanol → Dichloroethanol → Monochloroethanol → CO₂

RS20 → LF54