Aerobic Dechlorination of Dichloromethane Using Biostimulation Agent BD-C in Continuous and Batch Cultures of Xanthobacter autotrophicus GJ10

Akifumi Hosoda¹*, Yuta Isomura¹, Syungo Takeo¹, Takuho Onai¹, Kazutaka Takeuchi¹, Minoru Toda² and Hiroto Tamura¹

¹ Department of Environmental Bioscience, Meijo University, 1-501 Shiogamaguchi, Tenpaku-ku, Nagoya 468-8502, JAPAN
² Miyoshi Oil & Fat Co., 4-66-1 Horikiri, Katsushika-ku, Tokyo 124-8510, JAPAN

Abstract: It is important to construct microbiological treatment systems for organic solvent-contaminated water. We developed a continuous culture supplemented with a biostimulation agent named BD-C, which is formulated from canola oil, and Xanthobacter autotrophicus strain GJ10 for an aerobic dichloromethane (DCM)-dechlorinating microorganism. The continuous culture was a chemostat constructed using a 1 L screw-capped bottle containing artificial wastewater medium with 2.0 mM DCM and 1.0% (v/v) BD-C. The expression of genes for DCM metabolism in the dechlorinating aerobe was monitored and analyzed by reverse transcription–quantitative PCR. Strain GJ10 was able to dechlorinate approximately 74% of the DCM in medium supplemented with BD-C during 12 days of incubation. The DCM dechlorination rate was calculated to be 0.11 mM/day. The ΔΔCt method showed that expression of haloalkane dehalogenase increased 5.4 times in the presence of BD-C. Based on batch culture growth tests conducted with mineral salt medium containing three DCM concentrations (0.07, 0.20, 0.43 and 0.65 mM) with BD-C, the apparent maximum specific consumption rate (vmax) and the saturation constant (Ks) determined for DCM degradation in this test were 19.0 nmol/h/CFU and 0.44 mM, respectively. In conclusion, BD-C enhanced the aerobic degradation of DCM by strain GJ10.

Key words: dichloromethane, aerobic dechlorination, haloalkane dehalogenase, Xanthobacter autotrophicus, biostimulation

1 Introduction
In recent years, the processing of drainage contaminated with organic solvents has used energy-consuming systems such as incineration or chemical degradation by catalysts. However, on-site processing and treatment of contaminants is desirable. Thus, the construction of biological systems using microbes for the processing of drainage contaminated with chlorinated solvents is important. Dichloromethane (DCM) is a representative chlorinated compound that has been used as a washing agent for cleaning oils from metals or as a solvent for various chemical reactions. Its use in industry is determined by its physical and chemical properties, including its low boiling temperature (40°C), high solubility in water (approximately 20 g/L, i.e., 235 mM), relative inertness, and low freezing temperature (−97°C)¹. The degradation products of DCM via formaldehyde catalyzed by glutathione S-transferase are suspected to be carcinogenic in animals²,³; it is also argued that exposure to DCM in Japan causes cholangiocarcinoma⁴ and that it is carcinogenic in human liver and kidney⁵. Therefore, the increased attention of researchers has been drawn to the problem of persistent, biologically hazardous compounds accumulating in the environment as a result of intensive industrial production and use. Some studies of microbial bioremediation and DCM dechlorination have been performed, using aerobic and anaerobic bacterial strains capable of DCM dechlorination using various enzyme systems that have been isolated from the environment⁶. Methylotrophic bacteria are well known as DCM degraders under aerobic conditions⁷.

Aerobic DCM-dechlorinating bacteria convert DCM to CO₂ via formate using dichloromethane dehalogenase (DcmA, EC4.5.1.3)⁸, cytochrome P450 monoxygenase (P450/MO, EC1.14.14.1), or haloalkane dehalogenase (DhlA, EC3.8.2.12)¹,⁶, and the spectrum of C1- or C2-halogenated compounds that can be degraded by DhlA has...
been characterized\(^8\)\(^9\). The *Xanthobacter autotrophicus* strain GJ10 used in this study dechlorinates mainly haloalkenes such as 1,2-dichloroethene and 1,2-dibromoethene using DhIA catalysis\(^10\). *X. autotrophicus* strain GJ10 is able to utilize some chlorinated short-chain hydrocarbons and carboxylic acid as the sole carbon and energy source for growth, and some genes involved in the assimilation of these compounds have been isolated and characterized\(^1\)\(^2\)\(^3\)\(^4\)\(^5\). DhIA, which has a significant \(K_m\) value for 1,2-dichloroethene (\(\log K_m = -0.28\)), 1,2-dibromoethene (\(\log K_m = -2.0\)), and DCM (\(\log K_m = -2.0\)), is composed of 310 amino acids residues and has a molecular mass of 35 kDa\(^10\)\(^15\). The mechanism of dechlorination by DhIA has been well studied by enzymatic and X-ray crystal structure analysis\(^8\)\(^16\). X-ray crystallography shows that the active site of the enzyme is located between the main domain and the cap domain in an internal, predominantly hydrophobic, cavity that can be reached from the solvent through a tunnel\(^10\). DhIA gene clusters from *X. autotrophicus* GJ10 were also isolated, sequenced, successfully expressed in *Escherichia coli*\(^17\) and clarified their organization. Although bacteria that have the DhIA pathways were not able to grow on DCM as the sole carbon source\(^16\), an isolate recently obtained from a wastewater treatment plant can degrade DCM as the sole carbon source\(^19\).

We reported in a case study that a microbial biostimulation agent named BD-1 formulated from canola oil, fatty acids, and detergents, effectively enhances anaerobic bacterial dechlorination of trichloroethene (TCE)\(^19\). The mechanism of anaerobic dechlorination of TCE in the presence of BD-1 is the direct provision of carbon and energy sources to fermentative bacteria for the production of hydrogen, which acts as an electron donor during the dechlorination of organochlorines. However, it remains unclear whether biostimulation agents with high total organic carbon (TOC) such as BD-1 induce the activation of aerobic dechlorination of chlorinated hazardous industrial chemicals. In this study, we investigated the conditions for enhancement of the aerobic bacterial dechlorination of hazardous DCM-contaminated wastewater. To investigate aerobic DCM dechlorination, we applied a biostimulation agent named BD-C, which is a modified BD-1 by adding with corn steep liquor which provided with vitamins to increase the performance of microbial activity, to DCM-contaminated water cultured with aerobic bacteria, and determined the enhancement of DCM dechlorination by *X. autotrophicus* GJ10 in the presence of BD-C was assessed by analysis of the expression of genes involved in DCM metabolism. Furthermore, the kinetics of DCM dechlorination were determined by batch cultures test.

## 2 Materials and methods

### 2.1 Chemicals

DCM was purchased from Wako Pure Chemical Industries (Osaka, Japan) and used as the standard for gas chromatography (GC) analysis. The microbial biostimulation agent BD-C was supplied by Miyoshi Oil & Fat Co. Ltd (Tokyo, Japan). The BD-C was prepared using 10% (v/v) of BD-1, which was developed in a former study, with the addition of 0.1% (v/v) corn steep liquor\(^20\).

### 2.2 Bacterial strains and growth for preculture

*X. autotrophicus* GJ10 (ATCC43050) was used for aerobic DCM degradation. Strain GJ10 was inoculated to nutrient broth (NB) (3.0 g/L beef extract, 5.0 g/L peptone) and incubated aerobically at 27°C until OD\(_{680}\) reached 0.5.

### 2.3 Experimental setup in continuous culture

We used continuous cultures to apply practical degradation of DCM-contaminated wastewater by strain GJ10. A glass medium bottle (2 L volume) containing 1.8 L of artificial wastewater (AW) medium (1.36 g/L KH\(_2\)PO\(_4\); 2.13: Na\(_2\)HPO\(_4\); 0.50: (NH\(_4\))\(_2\)SO\(_4\); 0.20: MgSO\(_4\); 7H\(_2\)O; pH 7.0) containing 2.00 mM DCM and 1.0% (v/v) BD-C, two continuous flow-through reactors (1 L each), and tanks for effluent (1 L each) were connected with PharmEx® BPT tubing (3.15 mm inner diameter (id), 5.2 mm outer diameter; Eyela, Tokyo, Japan). Each piece of tubing was connected to three glass tubes to which were attached a phenol resin cap with a Teflon-coated silicon stopper. A branching glass tube was used for sampling. The tube length between the AW medium bottle and the reactors was 120 cm, and between the reactors and effluent tanks was 90 cm (Fig. 1). The volume of AW medium in the reactor was 400 mL. The tested strain GJ10 was precultured in 5 mL of NB medium (4.0 g/L yeast extract, 5.0: polypeptide, 50: glucose, 0.55 KH\(_2\)PO\(_4\); 0.43 NaCl, 0.13: CaCl\(_2\); 0.13: MgSO\(_4\); 7H\(_2\)O; 0.03: FeCl\(_3\); 6H\(_2\)O; 0.03: MnSO\(_4\); H\(_2\)O; pH 7.0) in a test tube and 4 mL of cells was inoculated to the reactor. Control reactors without bacteria were included in each DCM degradation experiment. The flow rate of fresh AW medium was set by the hydraulic retention time (HRT), at 144 hour or 96 hour. Strain GJ10 was grown by medium agitation with a stirrer in the reactor at 30°C for 12 days. Samplings to assess DCM dechlorination, acetate and formate concentration, chloride ion concentration, and to obtain cells for gene expression analysis were conducted after 0, 1, 3, 5, 7, and 12 days of incubation.

### 2.4 Analytical procedures

To determine DCM concentration, a 3.0 mL aliquot was collected from cultures after a specified period and immediately extracted with an equal volume of hexane. The resulting 10 μL aliquot of the prepared sample was subjected to capillary GC equipped with an electron capture detector.
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The GC operated with a stainless steel capillary column (Rtx-624, 60 m × 0.32 mm id × 1.8 μm, GL Science Inc., Tokyo, Japan) and was programmed as follows: injector temperature, 200°C; detector temperature, 220°C; injection system split ratio, 1:5; and argon carrier flow rate, 136.9 kPa. The GC temperature program ran as follows: 50°C for 7 min, 50°C rising to 80°C at a rate of 5°C min⁻¹, 80°C rising to 150°C at a rate of 7°C min⁻¹, and 150°C held for 1 min. Quantification was based on external calibration curves. Volatile fatty acids (VFAs) were analyzed by sampling a further 0.5 mL volume and washing with an equal volume of chloroform to remove impurities. Analytes were collected from the water layer. VFAs were quantified by high-pressure liquid chromatography (HPLC) using a Prominence system equipped with an electrical conductivity detector, CDD10AVP (Shimadzu). The HPLC system was operated with a Shim-pack SCR column (300 mm × 7.9 mm id; Shim-pack SCR-102H). A 100 μL aliquot of analyte was loaded and eluted isocratically using 5 mM p-toluenesulfonate (pH 2.7) at a flow rate of 0.8 mL min⁻¹. The oven temperature was 40°C. Chloride ion concentration in the cultures was determined by chemical-suppressed ion analyzer (IA-300, TOA DKK, Tokyo, Japan) in an anion exchange column (PCI-205), and the analyses were performed isocratically with 1.8 mM Na₂CO₃/1.7 mM NaHCO₃ as the mobile phase and 10 mM sulfuric acid for chemical eluent suppression, at a flow rate of 1.0 mL/min. Samples were prepared from 0.5 mL of 10- or 100-times diluted culture and passed through a 0.22 μm PVDF filter (Millipore, Billerica, MA, USA).

2.5 RNA isolation and synthesis of cDNA

RNA extraction was performed in triplicate. Total RNA was extracted from cells centrifuged from 2 mL of batch or continuous culture using a High Pure RNA isolation kit (Roche GmbH, Mannheim, Germany) according to the manufacturer’s instructions with a slight modification. The concentration and purity of the extracted RNA were measured by UV absorption (Bio Spec mini DNA/RNA/protein analyzer, Shimadzu). The RNA samples were frozen immediately at −80°C until use.

cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription (RT) kit according to the manufacturer’s protocols (Life Technologies, Carlsbad, CA, USA). Total RNA was treated with DNase to remove any contaminating DNA and to ensure that only RNA was being amplified. Each 20 μL reaction contained 100 ng of total RNA, 2.0 μL of 10 × RT random primers, 0.8 μL of 25× dNTP mix (100 mM), 2.0 μL of 10 × RT buffer, 1.0 μL of RNase inhibitor, 1.0 μL of MultiScribe™ Reverse Transcriptase, and nuclease-free H₂O up to a final volume of 20 μL. RT was performed in a Takara PCR Thermal Cycler Dice (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. A negative control containing all reagents except for the RNA template, and a DNase control for each RNA template were also prepared.
2.6 Quantitative PCR (qPCR)

To confirm the amplification efficiency of the primers used in the experiments, qPCR for enumerating the target genes was conducted on a StepOne plus™ Real-Time PCR system (Life Technologies). A 20 µL reaction mixture was used, and each reaction mixture contained 10 µL Fast SYBRgreen Master Mix (Life Technologies), 7.6 µL sterile H₂O, 1.6 µL DNA template, and 0.4 µM each of the forward and reverse primers. Triplicates of nontemplate controls, containing sterile H₂O, were included in each run. To amplify the dhlA gene encoding haloalkane dehalogenase, the TetR-type regulator of dhlA (designated as TetR) and fdh (formate dehydrogenase), and the primer pairs DhlA-F1/R1, TetR-F/R, and FDH-F2/R2, respectively, were used. The 16S rRNA gene was amplified using the 16SRt-F/R primer pair as internal standard. Details on primer sequences, amplicon characteristics, and amplification efficiency are summarized in Table 1. The annealing temperature (60°C) of all reactions was checked for specificity by lack of smearing. The amplification efficiency of each target was around 100%. The specificity of all amplicons was also confirmed by DNA sequencing. The fold change in the target gene normalized to 16S rRNA genes was calculated for each sample using the △ΔCₗ method²¹.

2.7 Determination of the kinetic parameters

In order to estimate the maximum specific consumption rate (vₘₐₓ) and saturation constant (Kₛ) batch cultures were grown using AW medium with 0.07, 0.20, 0.43, and 0.65 mM DCM with 1.0% (v/v) BD-C and without BD-C (using NB medium). The vₘₐₓ and Kₛ were calculated with the Monod equation (eq.1).

v = vₘₐₓ × S / (Kₛ + S)  
(eq.1)

where v is the specific consumption rate, S is the DCM concentration, and Kₛ is the saturation constant. The specific consumption rate for DCM (v) was calculated based on the reduction rate of DCM (mM/h) and cell growth (CFU). The cell growth was determined by the dilution plate method where 0.1 mL of culture was diluted from 10⁻⁴ to 10⁻¹ and inoculated onto an agar plate (3.0 g/L: yeast extract, 5.0: polypeptone, and 15: agar, pH 7.0, and autoclaved for 15 min at 121°C incubated at 27°C for 48 h. The DCM concentration was analyzed according to the procedure outlined in Section 2.4.

3 Results and discussion

3.1 Continuous culture for dechlorination of DCM

The effect of BD-C on DCM dechlorination was not significantly improved at the used concentrations in 0.1% to 1.0% (v/v) range of BD-C (data not shown). Thus, the concentration was defined to 1.0% (v/v) as following experiments. In the 144-hour HRT experiment, the initial DCM concentration of 1.36 mM decreased to 1.09 mM after 5 days of incubation, which means that 19.9% of initial DCM (0.27 ± 0.06 mM) was dechlorinated at a rate of 0.054 ± 0.01 mM/day. After 12 days of continued incubation, 75.1% of dechlorination (1.02 ± 0.02 mM) was confirmed at a rate of 0.080 ± 0.008 mM/day (Fig. 2a). No formate accumulation concomitant with DCM dechlorination was observed during the incubation (Fig. 2b). However, acetate accumulated up to about 8 mM in the control reactor, suggesting that fatty acid derived from BD-C might be oxidized chemically or aerobically. According to no production of chloride ion observed in the control reactor, the fact that the 2.90 ± 0.10 mM of chloride ion produced in the test reactor with strain GJ10 demonstrated that DCM dechlorination in the test reactor was due to a biological reaction (Fig. 2c). The OD₆₆₀ₙₗ value increased from 0.01 to 0.53 because of the growth of strain GJ10. Considering the utilization of VFA (acetate or formate) as growth substrate by the genus Xanthobacter²³, strain GJ10 seemed to assimilate the formate for its growth after DCM dechlorination. However, the DCM concentration also decreased in the control experiment. As no chloride ion production was observed in the control reactor, we considered that the decrease in DCM was the result of vaporization (Fig. 2a and 2c). To enhance the effectiveness of DCM dechlorination in this

Table 1 Target gene primers for quantitative PCR, and amplicon characteristics.

| Gene       | Accession number | Primer sequences (forward/reverse) | Amplicon Tm(°C) | Fragment size (bp) | Amplification efficiency (%) | References          |
|------------|------------------|-----------------------------------|-----------------|-------------------|-------------------------------|---------------------|
| DhlA       | M26950           | RT-dhlA-F1/R1 5'- CGGCAGAGAAATCCACATG -3' | 77.0 ± 0.06     | 78                | 108.6 ± 0.13                  | This study          |
|            |                  | RT-dhlA-R1 5'- CTACGTTCTGAGCGCT -3' |                 |                   |                               |                     |
| TetR-type  | M26950           | RT-TetR F 5'- TTGGTTTGAGTAGTCCG -3' | 80.6 ± 0.06     | 133               | 93.1 ± 0.79                   | This study          |
|            |                  | RT-TetR R 5'- GACTGTTAGTGGTGCACATC -3' |                |                    |                               |                     |
| FDH        | CP000781         | RT-FDH F2 5'- GATCGTGAGCTTTCGGTG -3' | 82.8 ± 0.12     | 80                | 103.6 ± 0.68                  | This study          |
|            |                  | RT-FDH R2 5'- GATCGTTAGTGGTGCACATC -3' |                |                    |                               |                     |
| 16S rRNA   | NC_007779        | 16SR-F 5'- ACTCCTAGGCGAGCG -3' | 81.2 ± 0.08      | 197               | 114.1 ± 1.0                   | Hosoda et al. 2013  |
|            |                  | 16SR-R 5'- ATTACCGCGCTGTCG -3' |                |                    |                               |                     |
reactor, we changed the experimental condition to a 96-hour HRT and incubated the reactor containing 400 mL of AW with 2.00 mM DCM and 1.0 \(^\text{v/v}\) BD-C for 12 days. In this case that only HRT was altered, the initial DCM concentration of 1.70 mM decreased to 1.04 mM after 5 days of incubation, which means that 31.8 \(\%\) of the initial DCM (0.46 \(\pm\) 0.10 mM) was dechlorinated at a rate of 0.110 \(\pm\) 0.03 mM/day. As in the 144-hour HRT experiment, 73.0 \(\%\) of the dechlorination (1.26 \(\pm\) 0.17 mM) was confirmed at a rate of 0.105 \(\pm\) 0.01 mM/day after 12 days of continued incubation (Fig. 3a). The residual DCM concentration in the reactor was 0.46 \(\pm\) 0.05 mM (39.1 ppm). The OD\(_{660nm}\) value increased from 0.07 to 0.40 because of the growth of strain GJ10. Although no formate accumulation...
was observed, similar to the 144-hour HRT experiment, the utilization of acetate derived from BD-C was revealed by the growth of GJ10 (Fig. 3b). In the 96-hour HRT experiment, the $2.29 \pm 0.03$ mM of chloride ion produced in the test reactor inoculated with strain GJ10 demonstrated that DCM dechlorination in the test reactor was due to a biological reaction (Fig. 3c). These results indicated that strain GJ10 was able to utilize DCM as an energy source but assimilation for growth required DCM dechlorination. We also observed that in the absence of BD-C, strain GJ10 did not grow or dechlorinate DCM and there was no significant expression of $dhlA$ (data not shown). The results obtained with the two different HRT conditions showed that 96-hour HRT and the addition of 1.0% (v/v) BD-C was most suitable for effective aerobic dechlorination (approximately 75% of DCM at a rate of 0.11 mM/day) by strain GJ10 in this continuous culture. However, BD-C addition was able to shift strain GJ10 to a DCM-dechlorinating metabolism. Troz et al. reported that the metabolism of halogenated C1 and C2 compounds during the growth of $X. autotrophicus$ strain GJ10 was influenced by 1,2-DCE dechlorination; moreover, they found that DCM dechlorination by strain GJ10 was not influenced by other organic compounds$^{17}$. We consider that the chemostat reactor supplemented with BD-C was suitable and practical for high concentration of DCM dechlorination.

3.2 qPCR analysis of haloalkane dehalogenase and related genes for dechlorination

Quantitative PCR for genes previously reported to be involved in DCM metabolism was conducted to estimate the metabolic flux of DCM dechlorination in this continuous culture of $X. autotrophicus$ strain GJ10. $dhlA$ (haloalkane dehalogenase) and TetR-type gene (transcriptional regulator for $dhlA$) were selected as the target genes representative of DCM dechlorination in strain GJ10$^{17}$. In addition, because strain GJ10 seemed to assimilate formate in the chemostat culture, formate dehydrogenase, which is well known in formate-assimilating bacteria and is downstream of the dechlorination of C1- or C2-halogenated compounds, was also chosen as a qPCR target$^{2, 21}$. The quantity and quality of RNA extracted from each sample of cells were in the range of 2.5–8.1 ng /μL and $A_{260/280} = 1.8–2.4$. Because 12 days of incubation was required for more than 80% DCM dechlorination in the chemostat culture under the 96-hour HRT condition, we analyzed the DCM metabolizing genes from the cDNA of strain GJ10 in this culture (Fig. 8). The results showed that $dhlA$ gene expression was increased 4.3 times after 4 days of incubation, concomitant with DCM dechlorination, and 5.4 times after 12 days of incubation (Fig. 4a). Although the significant decrease of $dhlA$ gene expression in the 5 and 7-day incubation seemed to be inactivation of tested strain, chloride ion concentration was continuously increased. Considering that the chloride ion was produced stoichiometrically with the dechlorination of DCM in Fig. 3, the continuous release of chloride ions during the decrease of $dhlA$ gene expression was considered that spontaneous chloride ion dechlorination was detected in the resulting compounds dechlorinated by DhlA which catalyze elimination of one chloride ion$^{1}$. Similarly, the expression of the TetR-type gene was also 144-fold to 193-fold higher, in parallel with $dhlA$ expression (Fig. 4b). Since the TetR-type genes are known as a family of microbial transcriptional regulators that act as repressors or activators, the TetR-type gene may also prob-

![Fig. 4](image_url) Fold change of gene expression of $dhlA$ (a), TetR-type gene (b), and $fdh$ (c) in 96-hour HRT continuous culture. The expression of target genes normalized to 16S rRNA gene was calculated for each sample using the $\Delta\Delta Ct$ method.
ably regulate \(dhlA\) of strain GJ10\(^{23}\). Furthermore, the expression of \(fdh\), which catalyzes formate assimilation downstream of DCM dechlorination, was increased a maximal 445-fold after 3 days of incubation and reduced slightly to 345-fold after 12 days. Considering the accumulation and degradation of formate in Fig. 3b, the pattern of \(dhlA\) expression observed over 5 days of incubation indicated that formate derived from DCM was utilized as a growth substrate by strain GJ10\(^{23}\). In contrast, in the 144-hour HRT experiment, \(dhlA\) expression was decreased (maximum 2.4-fold), and the maximum increase in expression of the TetR-type gene and \(fdh\) was 23-fold and 43-fold, respectively (Fig. 5). Although DCM dechlorination and chloride ion production were observed in this study (Fig. 2), expression of target genes was not significant (Fig. 5). Besides, significant expression of target genes was not observed without BD-C addition (Fig. 6). This indicates that the expression level of RNA was different compared with the 96-hour HRT experiment because of an unexpected event in the cells. Thus, further investigation of cell condition and RNA expression that occurred in this study is needed. Taken together, the results of expression analysis for DCM metabolism genes by qPCR revealed that the expression of \(dhlA\), its transcriptional regulator, TetR-type gene, and \(fdh\), which is downstream of DCM metabolism,

![Fig. 5](image1.png)

**Fig. 5** Fold change in gene expression of \(dhlA\) (a), TetR-type gene (b), and \(fdh\) (c) in 144-hour HRT continuous culture. The expression of target genes normalized to 16S rRNA gene was calculated for each sample using the \(\Delta\Delta C_T\) method.

![Fig. 6](image2.png)

**Fig. 6** Fold change in gene expression of \(dhlA\) (a), TetR-type gene (b), and \(fdh\) (c) without BD-C continuous culture. The expression of target genes normalized to 16S rRNA gene was calculated for each sample using the \(\Delta\Delta C_T\) method.
were significantly increased with BD-C addition. Since the acetate assumed to be derived from BD-C was detected 30-fold lower than DCM and degraded within 24 hours, it could be negligible for DhlA activity of strain GJ10. However, further studies on the effect of acetate on DhlA activity might be needed. We also consider that DCM dechlorination by strain GJ10 was due to the appropriate location of DCM in the amino acids residues of the DhlA active-site cavity, and that acetate derived from BD-C addition in this study promoted gene expression and the formation of the DhlA active-site cavity. The mechanisms of gene regulation by some TetR proteins have been reported to be mediated by their repression or activation of gene expression by DNA binding. The observation that the expression pattern of the TetR-type gene of strain GJ10 increased to 5.2 × 10^7 CFU/mL with 0.1 v/v glycerol, and water, supplemented with 0.1% (v/v) corn steep liquor containing small amounts of sugars, amino acids, and vitamins to enhance the performance of biostimulation. Because these results indicate that DCM dechlorination by strain GJ10 required a suitable acetate concentration. Thus, an extensive analysis of the correlation between BD-C and DCM dechlorination by strain GJ10 is needed. In this study, we investigated how the addition of BD-C to aerobic microbial DCM dechlorination using the v value and the Monod equation (Fig. 7). The \( K_s \) value obtained in this study was about 1.5 times lower than that reported for Xanthobacter sp. strain TM-1 (\( K_s = 0.61 \) mM)\(^{18} \), which suggests that the addition of BD-C was effective for the enzyme reaction. Despite the fact that there was slightly lower growth with BD-C than those with NB medium, cells grown with BD-C had a similar affinity for DCM, as evidenced by almost the same \( K_s \) value as that of cells grown in NB medium (data not shown). These results indicate that affinity for DCM in strain GJ10 was increased by the addition of 1.0% (v/v) BD-C. Considering the residual DCM concentration after the 12-day chemostat experiments (0.46 mM: 96-hour HRT, 0.34 mM: 144-hour HRT) and \( K_s \) values obtained in the growth tests, it is assumed that the complete DCM dechlorination will be achieved through the additional 5 to 7-day incubation in the present study. The VFA analysis showed that acetate detected at day 0 would be artifact derived from BD-C degradation by autoclaving. Therefore, the efficacy of BD-C for enhancing aerobic DCM dechlorination by strain GJ10 primarily consisted of the contribution of appropriate acetate. In the chemostat culture, expression analysis of DCM metabolism genes also showed that the addition of BD-C induced \( dhla \) and its transcriptional regulator gene, and indicated that components of BD-C such as acetate acted as DhlA substrate analog or cosubstrate for catalyzing DCM dechlorination. The kinetic parameters and qPCR analyses suggest that the DCM dechlorination mechanism involves the induction of \( dhla \) and related gene expression by acetate derived from BD-C, which allowed strain GJ10 to dechlorinate DCM (Fig. 8). Recent treatments for DCM removal from water, such as air stripping, adsorption, and pervaporation, have a process efficiency of about 99%, 90%, and

### 3.3 Effect of biostimulation agent BD-C for aerobic dechlorination of DCM

We previously developed a biostimulation agent, BD-1, and conducted a microcosm study and case study for reductive dechlorination of TCE\(^{19} \). In our previous study, the effect of BD-1 was revealed to be indirect activation of dechlorinating bacteria that use VFAs or hydrogen as an electron donor during the dechlorination of chlorinated compounds, via the direct stimulation of fermentative or hydrogen-producing bacteria that can assimilate VFAs and produce hydrogen by BD-1 degradation\(^{20} \). BD-C is a modification of biostimulation agent BD-1, which is composed of canola oil (melting point ≤ 0°C), polyol (10) oleyl ether as a nonionic surfactant, glycerol, and water, supplemented with 0.1% (v/v) corn steep liquor containing small amounts of sugars, amino acids, and vitamins\(^{20} \) to enhance the performance of biostimulation. Because these results indicate that DCM dechlorination by strain GJ10 required a suitable acetate concentration. Thus, an extensive analysis of the correlation between BD-C and DCM dechlorination by strain GJ10 is needed. In this study, we investigated how the addition of BD-C to aerobic microbial DCM dechlorination using the v value and the Monod equation (Fig. 7). The \( K_s \) value obtained in this study was about 1.5 times lower than that reported for Xanthobacter sp. strain TM-1 (\( K_s = 0.61 \) mM)\(^{18} \), which suggests that the addition of BD-C was effective for the enzyme reaction. Despite the fact that there was slightly lower growth with BD-C than those with NB medium, cells grown with BD-C had a similar affinity for DCM, as evidenced by almost the same \( K_s \) value as that of cells grown in NB medium (data not shown). These results indicate that affinity for DCM in strain GJ10 was increased by the addition of 1.0% (v/v) BD-C. Considering the residual DCM concentration after the 12-day chemostat experiments (0.46 mM: 96-hour HRT, 0.34 mM: 144-hour HRT) and \( K_s \) values obtained in the growth tests, it is assumed that the complete DCM dechlorination will be achieved through the additional 5 to 7-day incubation in the present study. The VFA analysis showed that acetate detected at day 0 would be artifact derived from BD-C degradation by autoclaving. Therefore, the efficacy of BD-C for enhancing aerobic DCM dechlorination by strain GJ10 primarily consisted of the contribution of appropriate acetate. In the chemostat culture, expression analysis of DCM metabolism genes also showed that the addition of BD-C induced \( dhla \) and its transcriptional regulator gene, and indicated that components of BD-C such as acetate acted as DhlA substrate analog or cosubstrate for catalyzing DCM dechlorination. The kinetic parameters and qPCR analyses suggest that the DCM dechlorination mechanism involves the induction of \( dhla \) and related gene expression by acetate derived from BD-C, which allowed strain GJ10 to dechlorinate DCM (Fig. 8). Recent treatments for DCM removal from water, such as air stripping, adsorption, and pervaporation, have a process efficiency of about 99%, 90%, and

![Fig. 7](image_url)
Aerobic dechlorination of dichloromethane using biostimulation agent BD-C

![Diagram of DCM dechlorination activation with BD-C in DhlA pathway.](image)

80%, respectively\(^{25}\). However, because strain GJ10 stimulated by BD-C could dechlorinate a high DCM concentration, this process is efficient compared with aerobic degradation of low concentrations (less than 10 ppm of TCE or 20 ppm of vinyl chloride monomer) of chlorinated compounds by other bacteria\(^{26}\). We reported that BD-1 indirectly activates bacteria of the genus *Dehalobacter*, which use VFAs or hydrogen as electron donors during the reductive dechlorination of organochlorines, via BD-1’s direct stimulation of other fermentative or hydrogen-producing bacteria that can assimilate VFAs and produce hydrogen by BD-1 degradation\(^{19}\). In the case of strain GJ10, BD-C may also be assimilated into VFAs, coupled with DCM dechlorination. Therefore, in this study, the DCM dechlorination activity of strain GJ10 in the presence of BD-C was enhanced by VFAs (e.g., acetate) derived from BD-C degradation, which directly activated the DCM dechlorinating enzyme, although the detailed dechlorination mechanism—whether VFAs act as an electron donor or acceptor—still remains unclear. The practical use of supplementation with 1.0 \%(v/v) BD-C as a biostimulation agent of microbial aerobic DCM dechlorination is very useful and important for the treatment of waste containing high concentrations of chlorinated organic solvent.

4 Conclusions

In this study, the aerobic DCM dechlorination by *X. autotrophicus* strain GJ10 was investigated using a chemostat culture with biostimulation agent BD-C, a modified BD-1, which contributed to microbial reductive dechlorination of TCE. In the chemostat culture, approximately 74\% dechlorination of DCM was achieved after 12 days of incubation. However, significant DCM dechlorination was not observed in the chemostat without BD-C. The analysis of expression of genes for DCM metabolism (*dhlA*, TetR-type gene, and *fdh*) revealed that expression of these genes was significantly increased by BD-C addition and that strain GJ10 was able to dechlorinate DCM. Therefore, the essential role of the added BD-C in this study was in contributing to supporting the growth of strain GJ10 and enhancing its ability to dechlorinate DCM. Although further detailed investigation is needed, acetate derived from BD-C may contribute to enhancing the transcription of *dhlA* and the TetR-type gene. In conclusion, BD-C becomes a practical biostimulation agent to enhance the efficacy of aerobic DCM dechlorination by strain GJ10 to similar levels to reductive dechlorination of halogenated compounds.

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