

# We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

185,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index  
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?  
Contact [book.department@intechopen.com](mailto:book.department@intechopen.com)

Numbers displayed above are based on latest data collected.  
For more information visit [www.intechopen.com](http://www.intechopen.com)



---

# Dechlorination of Chloral Hydrate by *Pseudomonas putida* LF54 which Possesses Biofilm Adhesin Protein LapA

---

Wanjun Zhang, Huhe, Yuanbai Pan, Yunxiang Cheng and Hiroo Uchiyama

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/60861>

---

## Abstract

Because of the lack of enzymes in critical steps of catabolic pathways, low-molecular-weight halogenated compounds are often recalcitrant to biodegradation. In our previous study, we isolated *Pseudomonas* sp. LF54 (LF54), the first bacterium that has been shown to use chloral hydrate (CH) as sole carbon source by an assimilation pathway in which dechlorination is the critical step. In this study, we identified a transposon (Tn) mutant that can render LF54 defective in CH dechlorination. The molecular characterization of Tn mutants revealed that the transposon insertion sites map to *lapA*. Sequence analyses verified the existence of *lapA* in LF54. Additionally, induced expression of *lapA* in the conditional *lapA* mutant of LF54 further verified that defective *lapA* expression renders LF54 defective in dechlorination. Recent studies have revealed that the largest cell-surface-associated protein LapA, a biofilm adhesin, is able to initiate biofilm formation. This function was also verified in the induced conditional *lapA* mutant and in LF54. Furthermore, we also found out that the defective *lapA* mutant rendered the variation of bacterial motility. LapA, the largest biofilm adhesin protein of *P. putida*, which influences CH dechlorination and flagella motility, is a novel discovery not previously reported.

**Keywords:** Chloral hydrate, dechlorination, *lapA*, biofilm, motility

## 1. Introduction

Chloral hydrate (CH) is synthesized by the chlorination of ethanol. As a sedative and hypnotic drug, CH is used in human and veterinary medicine. Its chemical formula is  $C_2H_3Cl_3O_2$ . The

anhydrous chemical, chloral, is used as an intermediate in the production of insecticides and herbicides such as dichlorvos, naled, methoxychlor, trichlorfon, and trichloroacetic acid [1].

Drinking water is the major exposure route of CH to the general public, as CH is the third by-product formed when drinking water is disinfected with chlorine [2, 3]. Therefore, environments near factories that produce CH and the above-mentioned pesticides might be contaminated. CH is irritating to the skin and mucous membranes. It often causes some symptoms even at the recommended clinical dose [1]. According to the World Health Organization (WHO) guidelines, CH has adverse effect on health and should be limited in drinking water [4]. In addition, CH was reported as a potent genotoxic and carcinogenic compound [3]. Therefore, more efforts should be made to minimize further CH release and contamination in the environment.

Studies of CH were concentrated on animal metabolism, as CH was used as a sedative and hypnotic drug. In animal, the major metabolites are trichloroethanol (TCAol), TCAol glucuronide, trichloroacetic acid (TCA), and a small amount of dichloroacetic acid (DCA) [5-8]. The major excretion pathway of CH metabolite is elimination in the urine. With regard to the bacterial degradation of CH, several cometabolism processes have been reported; methanotrophic-degrading bacteria *Methylocystis* sp. M and *Methylosinus trichosporium* OB3b can transform trichloroethylene into CH, which is further degraded to TCAol and TCA [9, 10]. In our previous study, we successfully isolated *Pseudomonas* sp. LF54 (LF54), the first bacterium reported to date that can use CH as the sole carbon source via an assimilation pathway [11]. This strain transforms CH to TCAol, which is then dechlorinated to 2, 2-dichloroethanol (DCAol), and CO<sub>2</sub> was detected as the end product (Figure 1).

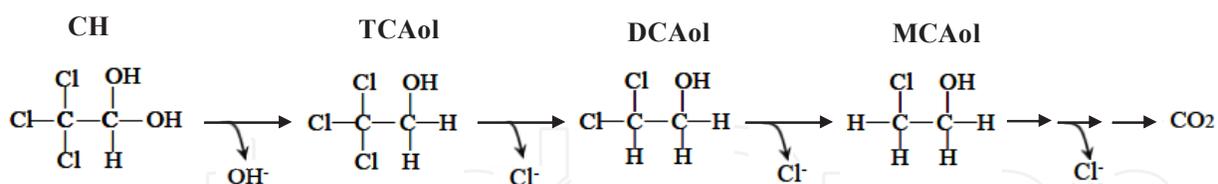


Figure 1. Proposed chloral hydrate biodegradation pathway in *Pseudomonas putida* LF54.

In general, halohydrin dehalogenation is intramolecular substitution that enzymes convert vicinal halohydrins to an epoxide, a proton and a halide ion [12-14]. To detoxify the compounds, halohydrin dehalogenases remove halogen substituents and put them to enter central metabolism. In view of halohydrin dehalogenases properties, the cleavage carbon-halogen bonds started with cloning and expression of three different genes that encode these enzymes [14]. However, the dechlorination in LF54 is reduction that chlorine (Cl) was substituted by hydrogen (H). This is a novel pathway of halohydrin dehalogenation has not been reported to date. Therefore, the further study of LF54 would advantage CH dechlorination and bioreme-

diation. The aim of this Chapter is to identify genes related to CH degradation and to investigate mechanism of CH degradation in *P. putida* LF54 with the method of molecular biology.

CH, chloral hydrate; TCAol, Trichloroethanol; DCAol, Dichloroethanol; MCAol, Monochloroethanol.

## 2. Identification of genes related to CH degradation

DNA transposition is an important biological phenomenon and also offers versatile tools for genetic analysis of a variety of biological processes. It is applied in creating insertional knockout mutations, providing genetic or physical landmarks for adjacent DNAs, generating the fusions of gene–operon to reporter functions, and locating primer binding sites for the analysis of DNA sequence [15]. Tn5 is one of the simplest and best characterized transposons; there are only Tn5 transposase, transposon DNA flanked by Tn5 inverted repeats, and target DNA are required in vitro transposition [16]. Then electroporation of transposon into a bacterial cell, the reaction proceeds and results in insertion of the transposon DNA at random into the genome to create gene “knockouts” which can block metabolic process.

The transposon used in this chapter was generated with EZ-Tn5™ <KAN-2> Tnp Transposome™ Kit carrying a kanamycin resistance marker. The transposon can be electroporated into living cells where the transposase is activated by Mg<sup>2+</sup> in the host’s cellular environment, resulting in random insertion of transposon into the host genomic DNA. The transposon-specific primers can be used for bidirectional DNA sequencing from genomic DNA without cloning or locating the transposon insertion sites in target genomic DNA.

In this chapter, in order to identify genes related to CH degradation, a transposon mutant library was generated and Tn mutants (CH degradation-defective) were screened by two screening steps. Transposon insertion sites were sequenced bidirectionally using special sequencing primers from the two sides of the transposon and located using BlastN programs.

### 2.1. Transposon mutant (Tn mutant) library

LF54 cells were made competent utilizing a microcentrifuge-based procedure [17]. The cells grown in LB medium were distributed into microcentrifuge and harvested by centrifugation at room temperature for 2 min at 16,000 × g. The cell pellet was washed twice at room temperature and resuspended in 100 µl 300 mM sucrose, which contained average 10<sup>9</sup>–10<sup>10</sup> viable bacteria.

For electroporation, 10 ng of pSUP104 [18] and 20 ng Transposome was mixed with 100 µl electrocompetent cells, and the mixture transferred to 2 mm gap width electroporation cuvette. After applying a pulse (settings: 25 µF; 200 Ω; 2.5 kV on a Bio-Rad GenepulserXcell™, Bio-Rad) [17], the mixture was transferred into 1 mL of room temperature SOC and shaken for 1 h at 30°C; 100 µl of the pSUP104 mixture was plated on Tc plates, and each 100 µl of Transposome mixture was plated on Kana plates. The plates were incubated at 30°C within 24 h.

Controls included cells that were pulsed with added H<sub>2</sub>O and were planted on both Tc and Kana plates. Mutagenized cells (Tn mutants) were selected by plating on Kana plate. The strains were stored in 20% glycerin LB media at -80°C.

## 2.2. Tn mutants screening strategy

Tn mutants (CH dechlorination defective) were screened by two screening steps. The cells were harvested by centrifugation, and washed twice with chloride-free MS medium and were resuspended in a 25 mL serum bottle containing 5 mL of MS medium in proper cells concentration as indicated below. After addition of 1mM CH, the cultures were incubated at 30°C and shaken at 150 rpm for 18 h.

### 1. First screening

The first screening was performed by multistep colorimetric method, as CH degradation is a multistep reaction (Figure 1). The concentration of resuspended cells in this step is  $4 \times 10^9$  cells mL<sup>-1</sup>. In weak alkali conditions, 3-methyl-1-phenyl-5-pyrazolone reacts with CH and a brownish red compound is formed and in proportion to CH in 480 nm [19] measured by Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The chloridion was mixed with a reagent consisting of Hg<sup>2+</sup>, SCN<sup>-</sup>, and Fe<sup>3+</sup>; a new yellow complex was formed which was measured at 463 nm [20].

### 2. Second screening

The second screening was performed by GC [11]. The concentration of resuspended cells is  $2.5 \times 10^{10}$  cells mL<sup>-1</sup>. The samples were 10 times concentrated by extraction with t-butyl methyl ether (Wako, Osaka, Japan), and 1,2,3-trichloropropane (Wako) was used as an internal standard. The oven temperature was programmed as follows: 35°C (isothermal) sustained for 7 min then increased to 250°C at a rate of 20°C min<sup>-1</sup>. The injector and detector temperatures were 270°C.

## 2.3. Transposon location

Transposon insertion sites were sequenced bidirectionally using sequencing primers, KAN-2 FP-1 and KAN-2 RP-1 (Table 1), specific for the ends of the inserted transposon [21]. The DNA sequencing reactions (two microgram of bacterial genomic DNA and 5 pmoles of primer) were performed using a BigDye™ Terminator Cycle Sequence Kit (Applied Biosystems, Perkin-Elmer, Foster City, CA, USA). The conditions of sequencing reactions are not shown[21]. Sequencing reactions were purified by ethanol precipitation, and resuspended in 10 µl of formamide (Wako, Osaka, Japan). After denaturing at 95°C for 2 min, all products were analyzed with an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems).

The sequences were assembled and the genomic transposition sites were located using BlastN programs maintained at National Center for Biotechnology Information website (<http://blast.ncbi.nlm.nih.gov/>).

	Relevant characteristics	source of reference
<b>Stains</b>		
LF54	Wild type <i>Pseudomonas</i> sp. LF54	11
PpY101	Wild type <i>P.putida</i> PpY101	22
F1	Wild type <i>P.putida</i> F1	23
Tn-mus01	LF54 lapA inactivated by transposon, F	This study
Tn-mus03	LF54 lapA inactivated by transposon, F	This study
Tn-mus04	LF54 lapA inactivated by transposon, F	This study
Tn-mus11	LF54 lapA inactivated by transposon, F	This study
Tn-mus13	LF54 lapA inactivated by transposon, F	This study
Tn-mus14	LF54 lapA inactivated by transposon, F	This study
Tn-mus15	LF54 lapA inactivated by transposon, F	This study
Tn control	Tn mutant which can degraded CH as	This study
LF54-lapA	Conditional lapA mutant in LF54 :lapA	This study
E.coli S17-1 $\lambda$ pir	Used for conjugation of plasmids into L 30	
E.coli DH5 $\alpha$	Plamid extraction as control.	Takara
<b>Plamids</b>		
Transposon	EZ-Tn5 <sup>TM</sup> <KAN-2>Tnp Transposom	EPICENTRE Technolog
pSC200	Vector used for single cross-over knock	28,29
pSC200-lapA	lapA fragment inserted into pSC200 fo	This study
pSUP104	the broad-host-range vector Tc <sup>r</sup> , Cm <sup>r</sup>	
pGEM-T	plamid, Amp <sup>r</sup>	
<b>Primers</b>		
EZTN-F	5'- TCTTGCTCGAGGCCGCG-3'	This study
EZTN-R	5'-TTGCATGCC TGCAGGTCG -3'	This study
KAN-2 FP-1 Forward Prii	5'-ACCTACAACAAAGCTCTCATCA	EPICENTRE Technolog
KAN-2 RP-1 Reverse Prii	5'-GCAATGT AACATCAGAGATTTT	EPICENTRE Technolog
lapA-F1	5?- GGAATTCCATATGATGAGCAGC	This study
lapA-R1	5?- GGGTACCTTATCGAGTACGCC	This study
lapA-upstream-F	5'-TATCCAGCAGGGGATCGTCA-3'	This study
lapA-R2	5?- TTATCGAGTACGCCCCGAAA-3'	This study
pSC200-F	5'-CGATAGGGCGTCTGCATCC-3'	This study
lapA-R3	5?-TGACGCTACCACTTGTCTGC-3'	This study

**Table 1.** Bacterial Strains, Plasmids, and Primers Used in the Study

## 2.4. Results

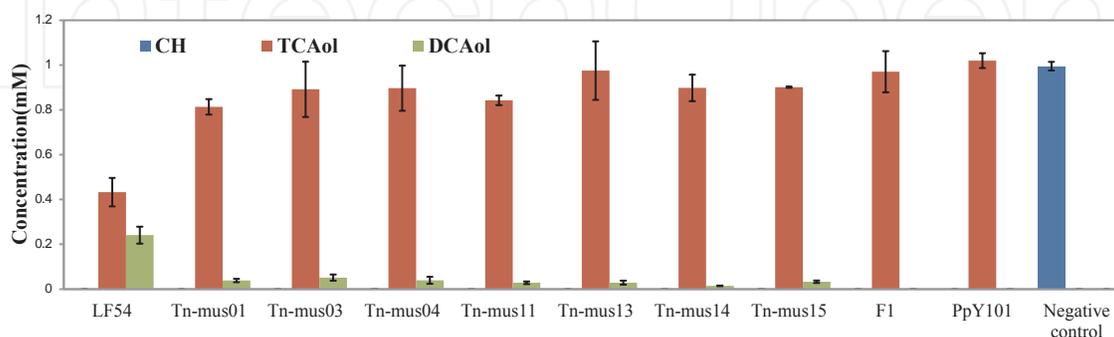
The transformation efficiency is shown in Table 2. The efficiency of pSUP104 is  $1.92 \times 10^{-6}$ ; and the efficiency of transposon is  $7.61 \times 10^{-5}$ . The efficiency of the transposome is almost the same as the plasmid. A library of mutants was generated using Tn mutants in LF54 and stored at  $-80^{\circ}\text{C}$ .

Host	DNA	Efficiency (CFU $\mu\text{g}^{-1}$ DNA)
LF54	H <sub>2</sub> O (none)	0
	pSUP104 (9.5 kbp)	$1.92 \times 10^6$
	Transposome	$7.61 \times 10^5$

**Table 2.** Electroporation Efficiency

The first screening for the degradation ability defect was performed by multistep colorimetric method. CH solution (MS medium) as the negative control and LF54 as the positive control were monitored. The results are not shown, as the data are excessive. Disappearance of CH was detected among Tn mutant library; we did not find Tn mutant, in which the process of CH transformed into TCAol was inhibited. According to chloridion release, 96 Tn mutants were picked up (the data are not shown).

The second screening was confirmed using GC; there were 7 Tn mutants that obviously produced less DCAol than LF54 (Figure 2). These strains were designated Tn-mus01, Tn-mus03, Tn-mus04, Tn-mus11, Tn-mus13, Tn-mus14, and Tn-mus15. *P. putida* F1 [22] and PpY101 [23] as the negative control was monitored. All the strains can transform the whole CH into TCAol after 18 h. For strain LF54, approximately 25% TCAol was dechlorinated into DCAol and part of them was converted into CO<sub>2</sub> as the end product [11]. F1 and PpY101 transformed CH into TCAol, but did not subsequently dechlorinate it. However, the DCAol levels in Tn mutants were obviously lower than those in LF54 and closer to F1 and PpY101.



**Figure 2.** Chloral hydrate (CH) degradation by transposon mutants. The samples were incubated at  $30^{\circ}\text{C}$  and shaken at 150 rpm for 18 h. The initial concentration of CH was 1 mM. CH solution, *P. putida* F1 and PpY101 as the negative control was monitored.

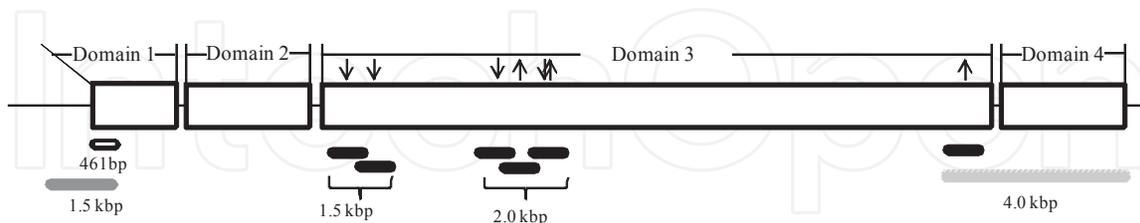
Genomic DNA from 7 Tn mutants that showed inhibited CH dechlorination was purified and directly sequenced. Using primers from each end of the transposon, nearly a kilobase of sequence was assembled from each Tn mutant. All 7 transposon insertion site-flanking sequences (KC686681–KC686687) were mapped to *lapA* in *P. putida* KT2440 [24, 25]; the sequence identities were 91–96% (Table 3).

	Tn-mus01	Tn-mus03	Tn-mus04	Tn-mus11	Tn-mus13	Tn-mus14	Tn-mus15
<b>Aminoacid sites</b>	3157-3471	3392-3717	2929-3242	1663-2003	7334-7651	3388-3658	1252-1573
<b>Sequence identities</b>	92%	94%	94%	94%	91%	95%	94%

**Table 3.** Sequence Identities of Transposon Mutants to *lapA* in *Pseudomonas putida* KT2440

### 3. Assay of *lapA* in LF54

Recently, some studies have revealed that the largest cell-surface-associated protein LapA, a biofilm adhesin, is able to initiate biofilm formation and achieve stable, “irreversible” binding to a large variety of surfaces in *P. fluorescens* and *P. putida* [24, 26, 27]. However, no study of *lapA* in connection with the biodegradation of low-molecular-weight chlorinated compounds has been reported to date. The *lap* genes were conserved among environmental pseudomonads such as *P. putida* and *P. fluorescens*, but absent from pathogenic pseudomonads such as *P. syringae* and *P. aeruginosa* [24, 27]. In *P. putida* KT2440 (Figure 3), LapA protein is one of the largest bacterial proteins (8,682 amino acids), with an estimated molecular weight of 888 kD. It contains an N-terminal transmembrane region (domain 1), an extensive repetitive region consisting of 9 repeats of 100 amino acids (domain 2) and 29 repeats of 218–225 amino acids (domain 3), and several conserved motifs and domain at the C-terminus of the protein (domain 4).



**Figure 3.** LapA protein in *P. putida* KT2440 and *P. putida* LF54. In *P. putida* KT2440, LapA contains an N-terminal transmembrane region (domain 1), an extensive repetitive region consisting of 9 repeats of 100 amino acids (domain 2) and 29 repeats of 218–225 amino acids (domain 3), and several conserved motifs at the C-terminus (domain 4). ↓ and ↑ indicate transposon insertion sites and directions. ↓ : Forward direction, ↑ : Backward direction. The bold line indicates *lapA* sequenced in the LF54 chromosome. The dark gray line indicates an upstream fragment including a part of the 5-region of *lapA*; the sequence identity was 89%. The black line indicates the transposon insertion sites flanking the sequence; the sequence identities were 91–96%. The light grey line indicates the 5end sequence of the NODE\_189 contig; the sequence identity was 93%. The hollow line indicates the 5-region of *lapA* (461 bp), which was used for constructing the conditional *lapA* mutant.

In this chapter, in order to verify the existence of *lapA* in LF54, we sequence part of *lapA* in LF54. Because the entire *lapA* sequence of LF54 was not acquired from the whole genome sequence analysis, strains defective in *lapA* were assessed to define further the role of *lapA* in CH dechlorination. This strategy created LF54-*lapA*, in which the expression of *lapA* depended on the rhamnose concentration in the medium.

### 3.1. Existence of *lapA* in LF54

The complete genome sequence of LF54 was performed by Genome Analyzer Iix (Illumina, San Diego, CA) provided by Hokkaido System Science Co., Ltd. The genomic DNA sequence was queried the database using genomic blast programs maintained at National Center for Biotechnology Information website ([http://www.ncbi.nlm.nih.gov/sutils/genom\\_tree.cgi](http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi)). The upstream fragment includes part of 5' region of *lapA*, amplified by PCR with primers *lapA*-upstream-F, and *lapA*-R2 (Table 1). Primers were synthesized based on the nucleotide sequence from the *Pseudomonas* genome database (<http://www.pseudomonas.com>). The purified fragment was sequenced using a BigDye™ Terminator Cycle Sequence Kit. The product was analyzed with an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems). The sequence was used to query the GeneBank database.

### 3.2. Conditional *lapA* mutant

To construct a conditional *lapA* mutant in LF54 (LF54-*lapA*), the 5-region fragment (461 bp) and the upstream fragment including part of the 5-region of *lapA* amplified by PCR, using the primers *lapA*-F1, *lapA*-R1, *lapA*-upstream-F, and *lapA*-R2 (Table 1) and LF54 genomic DNA as the template were used. Then, the 5-region fragment (461 bp) was digested with *NdeI/KpnI* and ligated into the multiple-cloning site of pSC200 to yield pSC200-*lapA* [28, 29].

The plasmid pSC200-*lapA* was introduced into the mobilizer strain *E. coli* S17-1  $\lambda$  pir and was then transferred to LF54 by conjugation [30]. Single crossover events were selected on Gm plates to obtain the conditional *lapA* mutant (LF54-*lapA*). The correct insertions were verified by colony PCR with the primers of pSC200-F and *lapA*-R3 (Table 1). DNA fragments were confirmed with electrophoresis on 1.2% agarose gel.

The protein expression of *lapA* was induced by 0.1% (wt/vol) rhamnose in the medium. CH degradation ability of conditional *lapA* mutant was affirmed by GC method mentioned.

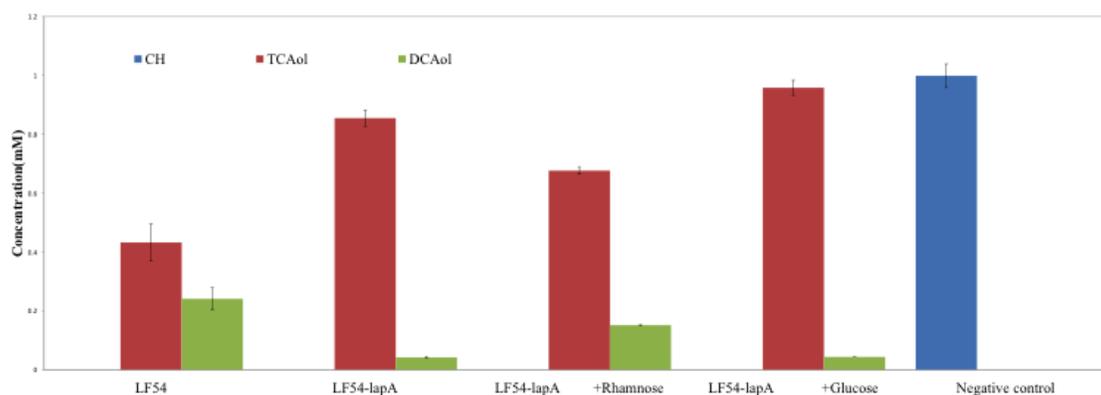
### 3.3. Results

In this study, the complete genome sequence of LF54 was performed by Genome Analyzer Iix containing 222 contigs. Total contig length is 5,632,841 bp (the data are not shown). The complete genome sequence of LF54 was blasted with 34 *Pseudomonas* genomes databases by Genomic blast program maintained at NCBI website. The almost complete genome sequence of LF54 shows high similarity to *P. putida* strains, the coverage of 86% to *P. putida* F1 (total score =  $9.643e^{+06}$ ), and 85% to *P. putida* KT2440 (total score =  $9.132e^{+06}$ ).

As domains 2 and 3 are an extensive repetitive region, at this stage of the complete genome sequence of LF54 it has not been possible to assemble the complete *lapA* gene. Among the 7

CH dechlorination defective Tn mutants, Tn-mus13 mapped to the end of LapA domain 3, and was also located at 5' end of the NODE\_189 contig (294,102 bp, AOUR00000000). Therefore, NODE\_189 included a part of domain 3 and the entire domain 4 (Figure 3, light gray line); the sequence identity was 93% to *lapA* in *P. putida* KT2440. In addition, the sequence identity of the upstream fragment including a part of the 5' region of *lapA* (KC686680) was 89% (Figure 3, dark gray line). Combining with sequence flanking the transposon insertions (corresponding to domain 3, Figure 3, black line), the structure of the protein appears to be very similar to its *P. putida* counterpart.

CH degradation ability of LF54-*lapA* was detected by GC analysis (Figure 4). LF54-*lapA* with glucose was used as the negative control, and LF54 was the positive control. LF54-*lapA* can completely transform CH into TCAol after 18 h, irrespective of the presence of rhamnose or glucose. CH dechlorination was inhibited in LF54-*lapA* in the absence of rhamnose, and recovered by rhamnose induction. The sample without rhamnose induction was similar to the glucose control and to the 7 Tn mutants that showed inhibited CH dechlorination.



**Figure 4.** Induction expression of conditional *lapA* mutant (LF54-*lapA*). The samples were incubated at 30°C and shaken at 150 rpm for 18 h. The initial concentration of CH was 1 mM. CH solution (MS medium) as the negative control was monitored.

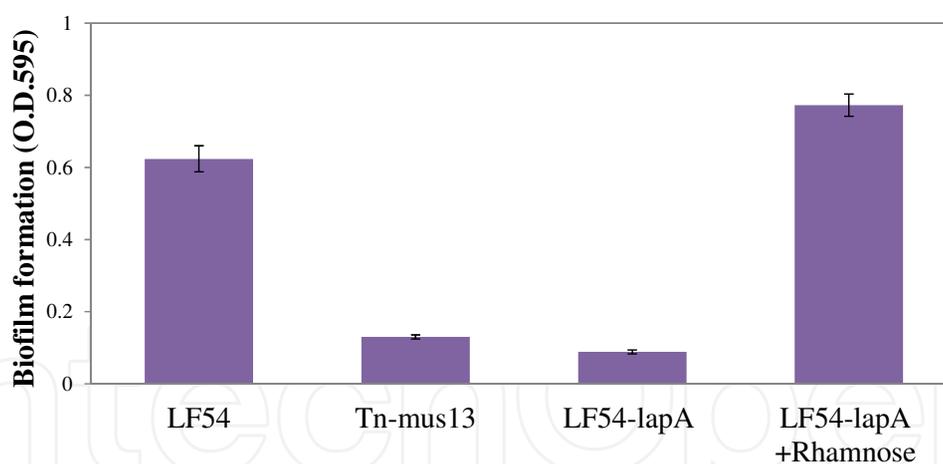
#### 4. Relationship between CH degradation and biofilm formation

In the past few years, bioremediation mechanisms by *Pseudomonas* for numerous halogenated pollutants have been described and many studies have reported the growth of planktonic cultures under controlled laboratory conditions [31-34]. However, bacteria are mostly found in multicellular communities known as biofilms in the environment, and not in the planktonic state [35, 36]. Biofilm formation in *Pseudomonas* has been proposed to involve a series of regulated steps [37, 26]. Firstly, utilizing flagellar-mediated motility, bacteria swim toward a certain surface and initiate reversible (or transient) attachment. Then, the attached bacteria become irreversibly attached to the surface to form small microcolonies. Finally, the microcolonies develop into a mature biofilm with an architecture separated by fluid-filled channels.

Recent studies have revealed that LapA is able to initiate biofilm formation and achieve stability [24, 26, 27]. However, no study of *lapA* in connection with the biodegradation of CH has been reported to date. We evaluated whether this function is conserved in LF54.

The cultures were started at an optical density of 600 nm ( $OD_{600}$ ) of 0.01 in glass tubes. After 20 h of growth, the tubes were washed with distilled water and stained with 0.1% crystal violet (Sigma, St. Louis, MO, USA). Following the staining, the tubes were washed twice with distilled water. The tubes were subsequently dried and the crystal violet was dissolved in 96% ethanol for quantification by spectrometry at 595 nm [38].

Biofilm formation in LF54-*lapA* and the Tn-mus13 strain was significantly lower than that in the wild-type strain (shown in Figure 5). The ability to initiate biofilm formation in LF54-*lapA* was restored when *lapA* was induced by rhamnose. These results indicate the relationship between biofilm formation and CH degradation.



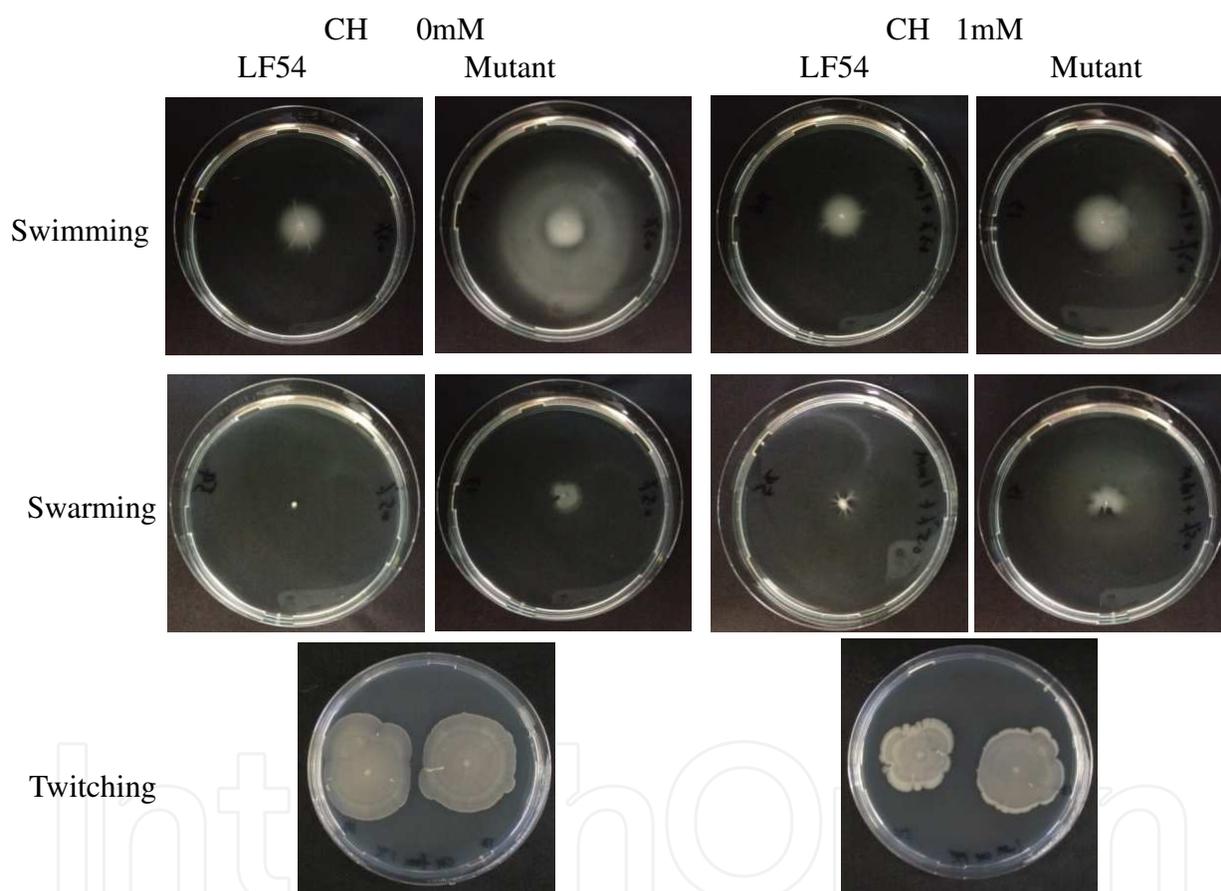
**Figure 5.** Biofilm formations. The start concentration is 0.01  $OD_{600}$ ; the culture was grown in LB medium and shaken at 150 rpm for 20 h.

## 5. LapA influences bacterial motility

Biofilm research has renewed our interest in bacterial motility [39]. Flagellar and twitching motility are necessary for biofilm development [40]. Biofilm formation of LF54 was verified, and in this chapter, these motilities were observed to verify the influences of LapA.

Twitching motility [41] was measured by stab-inoculating 1.0% agar LB plates with a single bacterial colony. Plates were incubated at 37°C for 2–3 days. Flagellar motility [38] including swimming and swarming motility was measured as the diameter of zone travelled by bacteria point-inoculated into 0.3% and 0.5% agar LB plates, respectively. The plates were incubated for 12 h at 30°C. CH was added in a series concentration: 0.1, 0.3, 0.5, 1 mM.

Swimming and swarming motility of mutants was faster than LF54 (shown in Figure 6). Although, twitching motility of mutants was also faster than LF54, the difference was not much. Within limits the movement become slowly with the raise of concentration of the CH.



**Figure 6.** Swimming and swarming motility of mutant was faster than LF54. Twitching motility (pilus movement) of mutant was a little faster than LF54.

Chemotaxis is the movement of organisms toward or away from a chemical. In this study, the defective *lapA* in mutants of LF54 results in the increasing external pressure to cell. That is, the normal environment for the defective *lapA* mutant, it will amount to over nutrition, the mutant escaped rapidly like the negative chemotaxis. In the CH occasion, the motility appears to like the positive chemotaxis, and the speed was lower than the normal condition. Therefore, we supposed that the other reason of motility change maybe is the biofilm adhesion of mutant

changed. The defective adhesion of mutant appears to makes flagellar and twitching motility easily than LF54 and results in faster motility.

## 6. Conclusion

So, for the first time we demonstrate dechlorination of CH by *Pseudomonas putida* LF54 which possesses Biofilm Adhesin Protein LapA. Although the *lapA* gene is conserved in *P. putida* strains [9], *P. putida* F1 and PpY101 were not able to dechlorinate CH (Figure 3). Therefore, the LapA protein may have a unique function in LF54; conversely, dechlorination process may involve other factors. The latter possibility is more appealing to us because LapA does not share any similarity with enzymes possessing dehalogenation functions [17]. And we also found out that the defective *lapA* mutant rendered the variation of bacterial motility. For the future, an interesting challenge will be to verify the interactions between LapA and the CH dechlorination enzyme and to examine whether biofilm formation influences CH dechlorination; and to verify the relationship among the CH dechlorination and LapA and bacterial motility.

Nucleotide sequence accession numbers. The nucleotide sequence data of *P. putida* LF54 have been submitted to NCBI GenBank under accession numbers KC686680 to KC686687 and AOUR00000000. The version described in this paper is the first version, AOUR01000000.

## Acknowledgements

This work was supported by the National Science Foundation for Distinguished Young Scholars of China(31402118), the National Natural Science Foundation of China (31460029), State Key Laboratory of Grassland Agro-ecosystems (SKLGAE201407), the Agricultural Science and Technology Innovation Program (2013GAAS04-04) from Gansu Academy of Agricultural Sciences, a grant-in-aid for scientific research (no. 21310050, to H.U.) and by the CREST program of the Japan Science and Technology Corporation (JST).

We thank Hideaki Nojiri for providing the *P. putida* F1 strain. We thank Leo Eberl for providing the pSC200 plasmid.

## Author details

Wanjun Zhang<sup>1</sup>, Huhe<sup>2</sup>, Yuanbai Pan<sup>3</sup>, Yunxiang Cheng<sup>4</sup> and Hiroo Uchiyama<sup>3</sup>

\*Address all correspondence to: zhangwanjun0922@yahoo.co.jp

1 College of Environment and Resources, Dalian Nationalities University, Jinzhou New District, Dalian, Liaoning Province, China

2 Institute of Soil and Fertilizer and Save Water Agricultural, Gansu Academy of Agricultural Sciences, Lanzhou, Gansu, China.

3 Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan

4 State Key Laboratory of Grassland Agro-ecosystems, College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou, China

## References

- [1] World Health Organization (WHO). 2000. Chloral hydrate. Concise international chemical assessment document, No25. World Health Organization, Geneva.
- [2] Dabrowska A, Nawrocki J. 2009. Controversies about the occurrence of chloral hydrate in drinking water. *Water Res.* 43:2201-2208.
- [3] Meric S. 2007. Toxicity of disinfectants and disinfection by-products: what did we learn to explore more? p 231-249. In A. Nikolaou, L. Rizzo, H. Selcuk (eds.), *Control of Disinfection By-Products in Drinking Water Systems*. Nova Science Publishers, Inc., New York, NY.
- [4] World Health Organization (WHO). 2004. *Guidelines for Drinking Water Quality*. World Health Organization, Geneva. Volume 1 – Recommendations, third edition.
- [5] Beland FA, Schmitt TC, Fullerton NF, Young JF. 1998. Metabolism of chloral hydrate in mice and rats after single and multiple doses. *J Toxicol Environ Health A.* 54:209-226.
- [6] Allen BC, Fisher JW. 1993. Pharmacokinetic modeling of trichloroethylene and trichloroacetic acid in humans. *Risk Anal.* 13:71-86.
- [7] Ketcha MM, Stevens DK, Warren DA, Bishop CT, Brashear WT. 1996. Conversion of trichloroacetic acid to dichloroacetic acid in biological samples. *J Anal Toxicol.* 20:236-241.
- [8] Abbas R, Seckel CS, Kidney JK, Fisher JW. 1996. Pharmacokinetic analysis of chloral hydrate and its metabolism in B6C3F1 mice. *Drug Metab Dispos.* 24:1340-1346.
- [9] Nakajima T, Uchiyama H, Yagi O, Nakahara T. 1992. Novel metabolite of trichloroethylene in a methanotrophic bacterium, *Methylocystis sp. M*, and hypothetical degradation pathway. *Biosci Biotech Biochem.* 56:486-489.
- [10] Oldenhuis R, Vink RL, Janssen DB, Witholt B. 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. *Appl Environ Microbiol.* 55:2819-2826.

- [11] Huhe, Nomura N, Nakajima T, Uchiyama H. 2011. Assimilative and co-metabolic degradation of chloral hydrate by bacteria and their bioremediation potential. *J Biosci Bioeng.* 111:448-453.
- [12] Lutje Spelberg JH, van Hylckama Vlieg JET, Bosma T, Kellogg RM, Janssen DB. 1999. A tandem enzyme reaction to produce optically active halohydrins, epoxides and diols. *Tetr Assymm.* 10:2863-2870.
- [13] de Jong RM, Tiesinga JJ, Rozeboom HJ, Kalk KH, Tang L, Janssen DB, Dijkstra BW. 2003. Structure and mechanism of a bacterial haloalcohol dehalogenase: a new variation of the short-chain dehydrogenase/reductase fold without an NAD(P)H binding site. *EMBO J.* 22:4933-4944.
- [14] van Hylckama Vlieg JET, Tang L, Lutje Spelberg JH, Smilda T, Poelarends GJ, Bosma T, van Merode AE, Fraaije MW, Janssen DB. 2001. Halohydrin dehalogenases are structurally and mechanistically related to short-chain dehydrogenases/reductases. *J Bacteriol.* 183:5058-5066.
- [15] Goryshin IY, Jendrisak J, Hoffman LM, Meis R, Reznikoff WS. 2000. Insertional transposon mutagenesis by electroporation of released Tn5 transposition complexes. *Nat Biotechnol.* 18:97-100.
- [16] Fernandes PJ, Powell JA, Archer JA. 2001. Construction of *Rhodococcus* random mutagenesis libraries using Tn5 transposition complexes. *Microbiology.* 147:2529-2536
- [17] Choi KH, Kumar A, Schweizer HP. 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. *J Microbiol Methods.* 64:391-397.
- [18] Kurabayashi T, Iwasaki K, Uchiyama H, Nakamura K, Tanaka H, Yagi O. 1997. Characteristics of *Escherichia coli* HB101 and *Pseudomonas putida* PpY101 harboring a recombinant plasmid with tandem insertion of the mercury resistance operon. *Biosci Biotechnol Biochem.* 61:1187-1189.
- [19] Water quality. 2000. Determination of trichloro-aldehyde.3-methyl-1-phenyl-5-pyrazolone spectrophotometric method. HJ/T 50-1999. [http://english.mep.gov.cn/standards\\_reports/standards/water\\_environment/method\\_standard2/](http://english.mep.gov.cn/standards_reports/standards/water_environment/method_standard2/).
- [20] Bergmann JG, Sanik J. 1957. Determination of trace amounts of chlorine in naphtha. *Anal Chem.* 29:241-243.
- [21] Hoffman L, Jendrisak J. 1999. Use of EZ::TN™ Transposomes™ for genetic analysis and direct sequencing of bacterial genomic DNA. EPICENTRE Technologies. [http://www.arb-ls.com/products/epicentre\\_forum/6\\_3.pdf](http://www.arb-ls.com/products/epicentre_forum/6_3.pdf).
- [22] Spain JC, Gibson DT. 1988. Oxidation of substituted phenols by *Pseudomonas putida* F1 and *Pseudomonas* sp. strain JS6. *Appl Environ Microbiol.* 54:1399-1404.

- [23] Kurabayashi T, Iwasaki K, Uchiyama H, Nakamura K, Tanaka H, Yagi O. 1997. Characteristics of *Escherichia coli* HB101 and *Pseudomonas putida* PpY101 harboring a recombinant plasmid with tandem insertion of the mercury resistance operon. *Biosci Biotechnol Biochem.* 61:1187-1189.
- [24] Hinsa SM, Espinosa-Urgel M, Ramos JL, O'Toole GA. 2003. Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. *Mol Microbiol.* 49:905-918.
- [25] Nelson KE, Weinel C, Paulsen IT, Dodson RJ, Hilbert H, Martins dos Santos VA, Fouts DE, Gill SR, Pop M, Holmes M, Brinkac L, Beanan M, DeBoy RT, Daugherty S, Kolonay J, Madupu R, Nelson W, White O, Peterson J, Khouri H, Hance I, Chris Lee P, Holtzapple E, Scanlan D, Tran K, Moazzez A, Utterback T, Rizzo M, Lee K, Kosack D, Moestl D, Wedler H, Lauber J, Stjepandic D, Hoheisel J, Straetz M, Heim S, Kiewitz C, Eisen JA, Timmis KN, Dusterhöft A, Tümmler B, Fraser CM. 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ Microbiol.* 4:799-808.
- [26] Gjermansen M, Nilsson M, Yang L, Tolker-Nielsen T. 2010. Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms: genetic elements and molecular mechanisms. *Mol Microbiol.* 75:815-826.
- [27] Ivanov IE, Boyd CD, Newell PD, Schwartz ME, Turnbull L, Johnson MS, Whitchurch CB, O'Toole GA, Camesano TA. 2012. Atomic force and super-resolution microscopy support a role for LapA as a cell-surface biofilm adhesin of *Pseudomonas fluorescens*. *Res Microbiol.* 163:685-691.
- [28] Ortega XP, Cardona ST, Brown AR, Loutet SA, Flannagan RS, Campopiano DJ, Govan JR, Valvano MA. 2007. A putative gene cluster for aminoarabinose biosynthesis is essential for *Burkholderia cenocepacia* viability. *J Bacteriol.* 189:3639-3644.
- [29] Werneburg M, Zerbe K, Juhas M, Bigler L, Stalder U, Kaech A, Ziegler U, Obrecht D, Eberl L, Robinson JA. 2012. Inhibition of lipopolysaccharide transport to the outer membrane in *Pseudomonas aeruginosa* by peptidomimetic antibiotics. *Chembiochem.* 13:1767-1775.
- [30] de Lorenzo V, Timmis KN. 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Methods Enzymol.* 235:386-405.
- [31] Arif MI, Samin G, van Leeuwen JG, Oppentocht J, Janssen DB. 2012. Novel dehalogenase mechanism for 2,3-dichloro-1-propanol utilization in *Pseudomonas putida* strain MC4. *Appl Environ Microbiol.* 78:6128-6136.
- [32] Goswami M, Recio E, Campoy S, Martín JF, Coque JJ. 2007. Environmental significance of O-demethylation of chloroanisoles by soil bacterial isolates as a mechanism

- that improves the overall biodegradation of chlorophenols. *Environ Microbiol.* 9:2512-2521.
- [33] Travkin VM, Solyanikova IP, Rietjens IM, Vervoort J, van Berkel WJ, Golovleva LA. 2003. Degradation of 3,4-dichloro- and 3,4-difluoroaniline by *Pseudomonas fluorescens* 26-K. *J Environ Sci Health B.* 38:121-132.
- [34] Garbi C, Casasús L, Martínez-Alvarez R, Ignacio Robla J, Martín M. 2006. Biodegradation of oxadiazon by a soil isolated *Pseudomonas fluorescens* strain CG5: Implementation in an herbicide removal reactor and modelling. *Water Res.* 40:1217-1223.
- [35] Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. 1995. Microbial Biofilms. *Ann Rev Microbiol.* 49:711-745.
- [36] Davey ME, O'Toole GA. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol Biol Rev.* 64:847-867.
- [37] O'Toole G, Kaplan HB. 2000. Kolter R. Biofilm formation as microbial development. *Ann Rev Microbiol.* 54:49-79.
- [38] O'Toole GA, Kolter R. 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol.* 28:449-461.
- [39] Daniel B. Kearns. 2010. A field guide to bacterial swarming motility. *Nat Rev Microbiol.* 8:634-644.
- [40] O'Toole GA, Kolter R. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol.* 30:295-304.
- [41] Whitchurch CB, Hobbs M, Livingston SP, Krishnapillai V, Mattick JS. 1991. Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. *Gene.* 101:33-44.