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## Genetic engineering for trichloropropane degradation

Samin, Ghufrana

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**Genetic Engineering  
For Trichloropropane  
Degradation**

**Ghufrana Samin**

The research described in this thesis was conducted in the Groningen Biomolecular Sciences and Biotechnology Institute (Department of Biochemistry, University of Groningen, The Netherlands).

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**Dedicated to my parents**



# Chapter 1

## **General introduction: Transformation and biodegradation of 1,2,3-trichloropropane (TCP)**

Ghufrana Samin and Dick B. Janssen

Accepted for publication.

## Abstract

**Purpose.** 1,2,3-Trichloropropane (TCP) is a persistent groundwater pollutant and a suspected human carcinogen. It is also an industrial chemical waste that has been formed in large amounts during epichlorohydrin manufacture. In view of the spread of TCP via groundwater and its toxicity, there is a need for cheap and efficient technologies for the cleanup of TCP-contaminated sites. In-situ or on-site bioremediation of TCP is an option if biodegradation can be achieved and stimulated. This paper presents an overview of methods for the remediation of TCP-contaminated water with an emphasis on the possibilities of biodegradation.

**Conclusions.** Although TCP is a xenobiotic chlorinated compound of high chemical stability, a number of abiotic and biotic conversions have been demonstrated, including abiotic oxidative conversion in the presence of a strong oxidant and reductive conversion by zero-valent zinc. Biotransformations that have been observed include reductive dechlorination, monooxygenase-mediated cometabolism, and enzymatic hydrolysis. No natural organisms are known that can use TCP as a carbon source for growth under aerobic conditions, but anaerobically TCP may serve as electron acceptor. The application of biodegradation is hindered by low degradation rates and incomplete mineralization. Protein engineering and genetic modification can be used to obtain microorganisms with enhanced TCP degradation potential.

## Introduction

1,2,3-Trichloropropane (TCP, CAS No. 96-18-4) is a non-natural, biodegradation-recalcitrant and toxic compound that occurs in groundwater and soil, mainly as a result of improper disposal of TCP-contaminated chemical waste (1). TCP is formed as a by-product during the synthesis of various chemicals, most notably in the classical synthetic route to epichlorohydrin and was present in commercial preparations of the soil fumigant 1,3-dichloropropene (also known under the trade name D-D), which is now abandoned (62). TCP is also applied as an intermediate in the production of various other chemicals. For example, fluorination of TCP is used to produce the cross-linking agent hexafluoropropylene, which is applied for making elastomers. TCP is also used in the chemical industry as a solvent for oils and fats, waxes, and resins. Other past uses of this compound include in paint thinner and varnish remover, and as a degreasing agent. The Toxic Substances Control Act inventory of the US Environmental Protection Agency (US EPA) estimated the usage of TCP in 2002 as  $1\text{-}5\cdot 10^6$  kg (65). The Federal Facilities Restoration and Reuse Office (FFRRO) of the US EPA has listed TCP as an Emerging Contaminant in December 2010 for which physical, toxicological and environmental data were summarized in a fact sheet (17) and a review of the toxicology of TCP was written (66). The European Union Chemicals Agency (ECHA) has listed 1,2,3-trichloropropane as a chemical of very high concern because of its carcinogenic, mutagenic and reproductive effects (13).

Contamination of soil and groundwater by TCP has occurred both as point source pollution, due to improper disposal of wastes or accidental spillage, and as diffuse contamination, due to its presence in the nematicide 1,3-dichloropropene. TCP has been detected in hundreds of surface water and drinking water sources, e.g. in the United States, at levels of 0.1-100  $\mu\text{g/l}$  (1, 2, 6, 46). In a Dutch

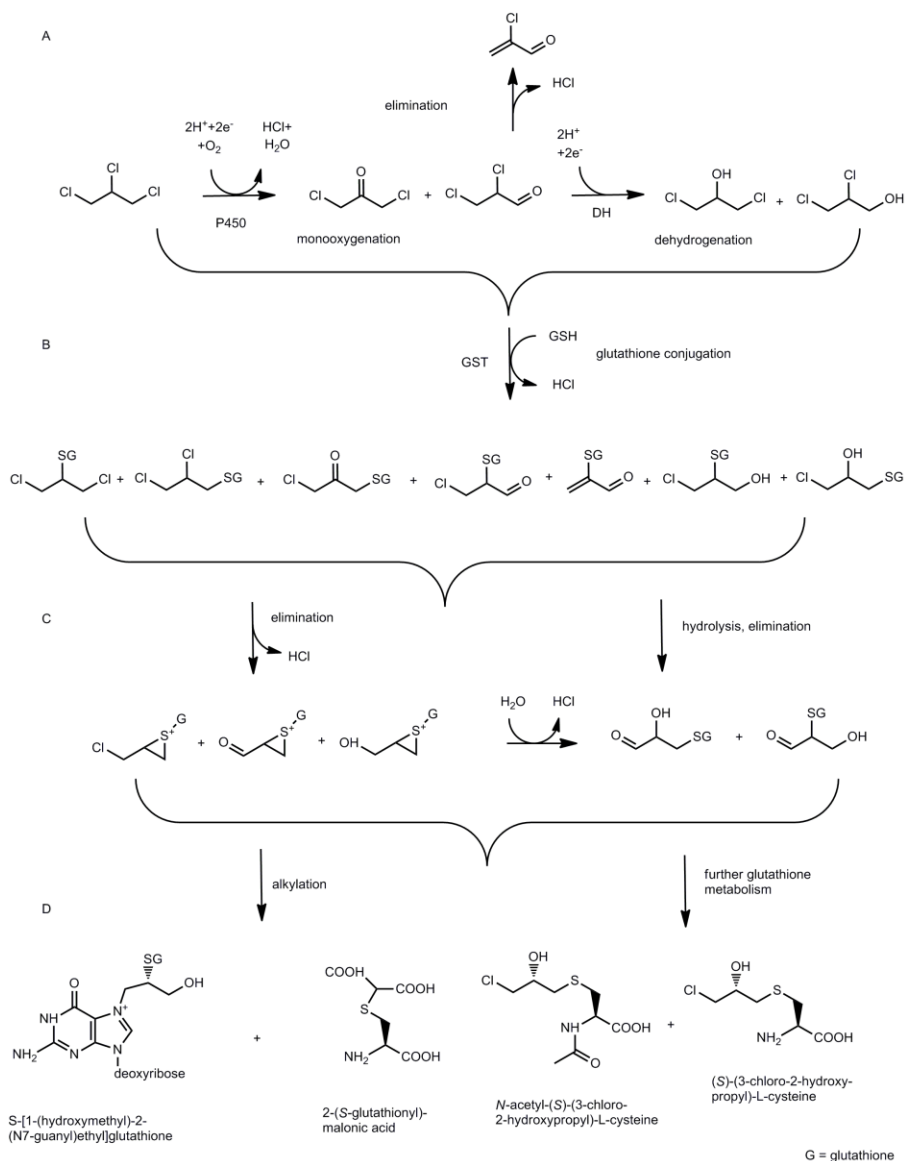
monitoring program, TCP was detected in surface water of the Rhine, Meuse, Westerscheldt, and in the Northern Delta Area (39). Groundwater samples from the Netherlands were found to contain TCP as well as 1,2-dichloropropane due to the application of impure nematicides, especially in potato fields (33). TCP was also detected in the river Nitra, Slovakia (35) and along with a range of other volatile organohalogenes in water at an industrial site in the Osaka area, Japan (75). These examples illustrate that TCP is a very widespread contaminant.

TCP is a suspected human carcinogen based on evidence of tumor formation in studies with rats and mice (23, 66). Because of its toxicity, the presence of TCP in groundwater can pose a serious risk to human health and ecosystem quality, and the National Toxicology Program (44, 45) of the US Department of Health has listed TCP as reasonably anticipated to be a human carcinogen (67). Toxicological properties of TCP are included in the ECHA Registered Substances Database (14) and the NIH Hazardous Substances Data Bank (22).

Remediation of TCP-contaminated sites is difficult due to its persistent nature and its physiochemical properties, which cause spreading with flowing groundwater (57). Biodegradation has been observed both under anaerobic and aerobic conditions, but appears to be slow and mostly due to cometabolism, with little evidence for TCP supporting growth or adaptation of bacteria. Based on laboratory studies there are indications that TCP may serve as an electron acceptor under anaerobic conditions. In this review, we discuss abiotic and biotic transformations of TCP, as well as the possibilities of enzymatic dehalogenation of TCP and genetic construction of TCP-degrading bacteria.

**TCP as an environmental chemical.** 1,2,3-Trichloropropane (TCP), also known as allyl trichloride, trichlorohydrin or glycerol trichlorohydrin, is a clear and colorless liquid, with a strong odor similar to that of chloroform or trichloroethylene. It is soluble in ethanol, ether, and chloroform, and only slightly soluble in water. Like other chlorinated hydrocarbons, it reacts with some metals, strong basic agents, and oxidizing agents. It is sensitive to prolonged exposure to light and heat. TCP is flammable, and when heated to decomposition, it yields toxic fumes of hydrogen chloride gas (67).

**Metabolism and toxicity of TCP.** The toxicological properties of TCP were recently reviewed by the US EPA (2009) and earlier by the WHO (2003) (66, 73). The major pathways for metabolism of 1,2,3-trichloropropane in higher organism starts with oxidative transformation by microsomal cytochrome P-450 or substitution by glutathione transferase (38, 72). P450-monooxygenase-mediated conversion leads to formation of 2,3-dichloropropanal and 1,3-dichloroacetone, which can be reduced to 2,3-dichloropropanol and 1,3-dichloropropanol, presumably by dehydrogenase activity (Fig. 1). 2,3-Dichloropropanal also decomposes non-enzymatically to 2-chloroacrolein. Glutathione conjugation of TCP may produce glutathione adducts that can undergo intermolecular substitution to form a highly reactive episulfonium ion, but glutathione can also prevent alkylation of proteins by the electrophilic monooxygenase products 2,3-dichloropropanal and 1,3-dichloroacetone (72).



**Fig. 1.** Metabolic pathways of 1,2,3-trichloropropane (32, 38, 73, 66). Reactions: A, Initial mono-oxygenase (P450)-mediated conversion and dehydrogenation leading to dichloro-metabolites; B, glutathione conjugation can occur on TCP and oxidized derivatives; C, glutathione adducts can be converted to highly toxic species, such as episulfonium ions; D, the final products include DNA adducts, as well as glutathione derivatives that are secreted.

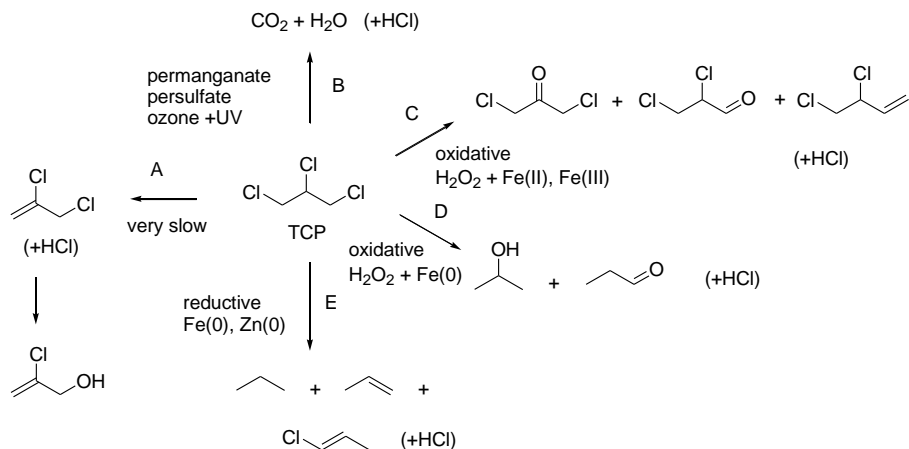
Metabolism of glutathione conjugates formed from TCP and its oxidation products also gives rise to various glutathione, cysteine and N-acetylcysteine conjugates. Thus, TCP itself is not mutagenic, but the products (such as 2-chloroacrolein and episulfonium ions) are strong alkylating agents, explaining the toxicity and mutagenicity of TCP. Using radiolabeled TCP, formation of DNA adducts after exposure to TCP has indeed been demonstrated in mice (32).

Animal studies have shown that long-term exposure to TCP may cause kidney failure, reduced body weight and tumors (1, 14, 23, 66). The guidelines for carcinogen risk assessment and IRAC monographs reported that TCP is expected to be carcinogenic to humans, based on animal studies, as well as resemblance of metabolism between human and rodent microsomes, *in vitro* mutagenicity, and the ability to form DNA adducts (23, 66, 74).

**Environmental fate of TCP.** Properties that determine the behavior of TCP in the environment include: its high density (1.39 g/ml); the modest water solubility (1.75 g/L at 20°C; for trichloroethylene the value is 1.4 g/l at 20°C); the low octanol-water partitioning coefficient ( $\log K_{ow}=2.0-2.5$ , quite similar to the value for trichloroethylene); and the low Henry coefficient ( $H=3.2-3.4 \cdot 10^{-4}$  atm-m<sup>3</sup>/mol at 25°C). The high density causes TCP, when dumped as a liquid on soil or in ponds, to sink to lower levels, e.g. into groundwater or into accessible subsurface structures like rock fissures. The low octanol-water partitioning coefficient implies substantial distribution via groundwater flows, even when organic carbon is present. Furthermore, TCP has a tendency to evaporate from surface water to air (but less than trichloroethylene,  $H=10 \cdot 10^{-4}$  atm-m<sup>3</sup>/mol), where, on exposure to sunlight, it is subjected to photo-degradation by reaction with hydroxyl radicals with a half-life of about 15 days.

The volatility of TCP, the possibility of washout by precipitation, and its resistance to degradation in water, may result in cycling of TCP between environmental compartments (1, 66). Bioaccumulation and biomagnification are expected to be of minor importance in view of the modest lipophilicity of TCP.

TCP can stay in groundwater for a prolonged period of time, in part due to its low organic carbon partitioning coefficient, but especially because rates of abiotic and biotic degradation in groundwater are low. Abiotic hydrolysis of TCP under basic and neutral abiotic conditions has been studied at different temperatures and pH values and in the presence of different ions such as sulfide and carbonate (59). TCP appears to be highly stable under a variety of conditions, with an expected half-life of hydrolysis under environmental conditions (25°C, pH=7) in the order of hundreds of years (48). At high temperatures 2,3-dichloro-1-propene was detected as a product that could be converted to 2-chloro-2-propen-1-ol (Fig. 2). Other non-stimulated abiotic reactions in water under environmental conditions have not been characterized.



**Fig. 2.** Abiotic transformations of TCP under non-stimulated conditions (A), photochemical conversion or catalytic conversion in the presence of radical-generating oxidants (B), conversion in the presence of Fenton reagent (C, D), and conversion under stimulated anaerobic (reductive) conditions (E). Non-catalyzed conversion under neutral conditions is very slow. See text for details.

**Remediation of TCP-contaminated sites: abiotic transformations.** The physical and chemical properties of TCP obviously strongly influence possibilities for remediation of soil and groundwater (58). On-site methods for treating TCP-polluted groundwater include pump and treat and vacuum extraction. The latter is used for different volatile organohalogenes, but it is not a very favorable approach because of the low Henry's law constant of TCP (7). Extracted water can be treated by absorption, using activated carbon, by chemical oxidation or by vacuum extraction. For example, full-scale remediation of TCP-contaminated groundwater is under investigation at the Tyson superfund site near Philadelphia, Pennsylvania, using vacuum extraction of soil, extraction of groundwater, and treatment of extracted water and vapor with activated carbon for the removal of TCP (52).

Chemical oxidation is performed with oxidizing agents such as ozone, permanganate or hydrogen peroxide. They cause decomposition of TCP into carbon dioxide, water and chloride ions (12) (Fig. 2). With a mild oxidant such as permanganate, transformation of TCP is slow whereas in the presence of strong oxidants such as hydroxyl radicals, generated photochemically, and sulphate radicals, catalytically generated from persulfate, transformation is much faster (7).

Chemical TCP oxidation may also be initiated by the Fenton reagent (H<sub>2</sub>O<sub>2</sub> with an iron catalyst). Khan et al. (2009) studied the effect of iron type (Fe<sup>+2</sup>, Fe<sup>+3</sup> and Fe<sup>0</sup>) on the removal of TCP by H<sub>2</sub>O<sub>2</sub>, and found that Fe<sup>2+</sup> was most effective in reducing TCP and increasing its biodegradability (26). Degradation products were 1,3-dichloroacetone and 2,3-dichloro-1-propanal if Fe<sup>2+</sup> and Fe<sup>3+</sup> were used, and isopropanol and propionaldehyde if Fe<sup>0</sup> was used, confirming extensive oxidative conversion. Such oxidative transformation studies have been done in batch reactors. Chemical oxidation can also be used for in-situ treatment of TCP contamination. In case TCP is present in the form of a dense non-aqueous-phase liquid (DNAPL), oxidants can be introduced into the subsurface to achieve contaminant oxidation.

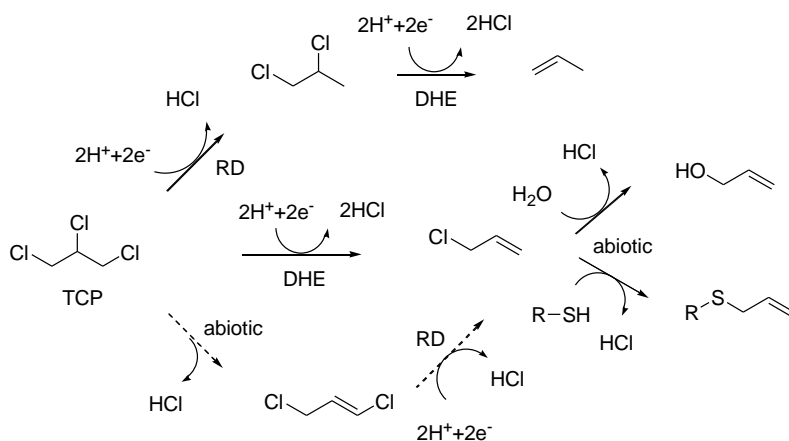
Chlorinated solvents such as trichloroethene, carbon tetrachloride and TCP may also be removed by abiotic reduction with zero-valent iron. Thus, distribution of TCP via flowing groundwater can be prevented by using a zero-valent iron ( $\text{Fe}^0$ ) barrier. The predominant removal mechanisms are sorption and reductive abiotic transformation. Klausen et al. (2003) investigated the effects of carbonate, silica, chloride and organic matter on the removal of various organohalides by granular iron using column studies (27). The results indicated that differences in groundwater chemistry have a strong effect on the activity and longevity of the granular iron, which will influence the design of reactive barriers. Compounds enhancing metal corrosion (carbonate, chloride) may improve reactivity, whereas compounds such as  $\text{FeCO}_3$  and  $\text{Na}_2\text{SiO}_3$  can reduce the activity, especially upon prolonged treatment, through deactivation of the metal surface. Propane, propylene, and trace amounts of 1-chloro-2-propene were detected as TCP transformation products (Fig. 2), indicating a role for reductive dechlorination and elimination of HCl in the removal of TCP.

Reductive transformation of TCP was also found with zero-valent zinc, which exhibited a reactivity that was more than an order of magnitude higher than that of iron (58, 59). Groundwater components that influenced zinc surface properties through corrosion or formation of an inactive layer of  $\text{ZnO}$  or  $\text{Zn}(\text{OH})_2$  had a large influence on the removal kinetics. No degradation products other than propene were detected, suggesting that dechlorination is extensive.

**Anaerobic biodegradation of TCP.** Few studies have been done aimed at establishing the possibilities for biodegradation and bioremediation of TCP. Growth-supporting biodegradation of halogenated compounds is generally based on one of the following processes: 1) chemotrophy with an oxidizable electron donor (hydrogen, lactate) and use of the halogenated compounds as a physiological electron acceptor (anaerobic conditions); 2) use of the halogenated compound as a carbon and energy source with an external electron acceptor (oxygen, nitrate); 3) fermentative metabolism, in which the halogenated compound serves both as electron donor and (indirectly) as electron acceptor. Biotransformation processes not linked to growth may also be important. Such cometabolic transformations are due to the broad substrate spectrum of many microbial enzymes, the general reactivity of cofactors, or the formation of reactive intermediates in the catalytic cycle of some enzymes. Examples are reductive dechlorination by cobalamin cofactors of anaerobic bacteria, and oxidative transformation by broad-specificity metal-containing monooxygenases of aerobic bacteria. Obviously, a biodegradation process that stimulates growth of the active organisms is preferable in a bioremediation situation since it allows adaptation at the population level, leading to an increase of the amount of active biomass during the treatment process.

Reductive biotic transformation of TCP has been demonstrated under anaerobic conditions (19, 36, 53) (Fig. 3) and sequential aerobic-anaerobic conditions (37). Reductive dechlorination of both TCP and chloroethanes was observed with an enrichment culture that dechlorinated 1,2-dichloropropane, and propene and 1,2-dichloropropane were detected as products (36). Experiments in which various halogenated aliphatic compounds were incubated with anaerobic sediments indicated zero-order conversion kinetics for TCP and dichloromethane, whereas most other organohalogens were transformed according to first order kinetics (53).

Recently, two strains (BL-DC-8 and BL-DC-9) of an anaerobic Gram-negative bacterium were isolated from contaminated groundwater at a Superfund site located near Baton Rouge, and characterized as belonging to the new species *Dehalogenimonas lykanthroporepellens* (40, 76). These bacteria utilize TCP as an electron acceptor under anaerobic conditions but not chlorinated alkenes. Hydrogen was the electron donor. For both strains, allyl chloride was detected as the main initial dechlorination product (Fig. 3). However, allyl chloride is unstable and is hydrolyzed abiotically to allyl alcohol, whereas in the presence of cysteine or sulphide, allyl chloride was transformed to allyl mercaptan, S-allyl mercaptocysteine and allyl sulphides. The mechanism of the reductive dechlorination reaction is not completely clear, as the enzymes responsible for TCP dechlorination have not yet been isolated and characterized. In freshwater environments, transformation of TCP into allyl chloride followed by the formation of allyl alcohol could be toxic to fish and aquatic life (16).



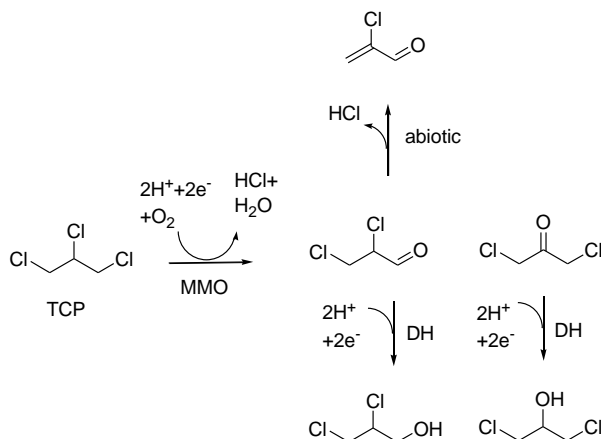
**Fig. 3.** Anaerobic biotransformations of TCP. Both reductive dehalogenation (RD) and dihaloelimination reactions (DHE) are observed. Formation of allylchloride may occur by dihaloelimination (76) or possibly via 1,3-dichloropropene (broken arrows).

Bioremediation of contaminated groundwater through in-situ reductive dechlorination can be performed by injecting a compound such as hydrogen, lactic acid or another oxidizable organic substrate that is used by microorganisms to produce hydrogen, which induces reductive dechlorination and serves as electron donor (64). At a site in California 99.9% reduction of TCP contamination has been found over a period of 1,000 days. However, biotic dechlorination through hydrogen-releasing compounds may be applicable only at low concentrations, such as less than 1 mg TCP/l (7).

**Aerobic cometabolic conversion.** Various halogenated aliphatic hydrocarbons can be transformed in a cometabolic manner by broad-specificity monooxygenase involved in hydrocarbon degradation, such as methane monooxygenase (19, 47). The soluble methane monooxygenase produced by cells of the methanotrophic bacterium *Methylosinus trichosporium* OB3b can convert TCP, giving rise to dichloropropanols after subsequent reduction (5, Fig. 4). However, TCP is a poor substrate for the enzyme as compared to other pollutants such as trichloroethylene.



The conversions are analogous to those catalyzed by cytochrome P450 in mammalian systems (Fig. 1). The major drawback of such cometabolic conversions is product toxicity. In case of TCP conversion by methane monooxygenase, the insertion of oxygen preferentially occurs on the terminal carbon atom, which yields chlorinated carbonyl compounds that may undergo elimination to produce 2-chloroacrolein, a very reactive compound.



**Fig. 4.** Conversions of 1,2,3-trichloropropane initiated by methane monooxygenase (MMO) produced by *M. trichosporium* OB3b cells. Reduction to alcohols is caused by alcohol dehydrogenase activity (DH).

The aerobic conversion of TCP reported by Leahy et al. (2003) using a mixture of hydrocarbon-degrading bacteria is probably based on similar reactions, but the products were not identified (34). Aromatic hydrocarbon-degrading bacteria produce monooxygenases that are capable of chlorinated hydrocarbon degradation through similar oxidative reactions as the methane monooxygenase of methanotrophs. For example, the toluene monooxygenase of a *pseudomonas* was described to convert chlorinated hydrocarbons (43).

**Recalcitrant behavior of TCP towards growth-supporting aerobic biotransformation.** Microbial transformation of TCP to  $\text{CO}_2$ ,  $\text{H}_2\text{O}$  and  $\text{HCl}$  by oxidative metabolism with oxygen as an electron acceptor and by reduction to lesser chlorinated propanes and  $\text{HCl}$  is thermodynamically possible (11). However, no aerobic organisms, enrichment cultures, or bioreactors have been described that demonstrate the use of TCP as a growth-supporting oxidizable substrate. Various attempts to enrich TCP-degrading microorganisms from environmental samples, including from sites with a long history of TCP or epichlorohydrin pollution, or to obtain TCP degradation in continuous-flow columns inoculated with samples from contaminated sites, have failed (3). This indicates that TCP is indeed a very recalcitrant compound and nature has not yet evolved aerobic organism that are adapted to it. The fact that the thermodynamic calculations indicate that aerobic oxidation of TCP is energetically favorable suggests biochemical hurdles instead of another fundamental reason as the cause of the apparent recalcitrance of TCP.

An example of such a biochemical hurdle is toxicity of intermediates. In case of halogenated aliphatic compounds, several reactive intermediates occur

along the metabolic pathways, requiring optimization of fluxes to prevent accumulation of such reactive intermediates to toxic levels (70). It also may be due to formation of dead-end side products that are toxic. Formation of such reactive intermediates will act against evolutionary selection of more efficient initial enzymes for TCP metabolism. The recalcitrant nature of a non-natural compound might also be due to presence of structural elements that cannot be recognized and converted by microbial enzymes, which evolved for the conversion of natural compounds (56).

When inspecting the possible pathways for productive aerobic metabolism of TCP, hydrolysis of a carbon-halogen bond as the first step seems the most attractive reaction, because it does not involve reactive intermediates and leads to 1,3-dichloro-2-propanol 2,3-dichloro-1-propanol. These compounds are known to be biodegradable and pure cultures capable of using dichloropropanols for growth under aerobic conditions are known (15, 21, 68, 78).

Hydrolysis of carbon-halogen bonds in chlorinated compounds is carried out by a diversity of microbial enzymes called dehalogenases. These belong to different phylogenetic classes, of which the haloalkane dehalogenases that are members of the  $\alpha/\beta$ -hydrolase fold superfamily of proteins are the best characterized (24, 28). Another prominent class is the HAD-superfamily of haloacid dehalogenases and phosphatases, with dehalogenases that act on 2-chloroacetate and 2-chloropropionate. Haloalkane dehalogenases are known to convert compounds such as 1,2-dichloroethane, 1,2-dibromoethane, 1,3-dichloropropane, 1,2-dichloropropene, and (slowly) hexachlorocyclohexane (25, 28, 55). The conversion of TCP by a haloalkane dehalogenase was first described by Yokota et al. (1986) using an enzyme from *Corynebacterium* strain m15-3 (77), but the activity was very low ( $k_{\text{cat}}/K_{\text{m}} = 36 \text{ s}^{-1}\text{M}^{-1}$ ) (4). Sequence analysis and structural studies identified the protein (which is commonly called DhaA) as a member of the  $\alpha/\beta$ -hydrolase fold family. Another dehalogenase that has low activity with TCP is LinB, an enzyme originally discovered in bacteria that degrade hexachlorocyclohexane (41).

The first DhaA gene sequence was described by Kulakova et al. (1997) in the 1-chlorobutane degrader *Rhodococcus rhodochromus* NCIMB13064 (29). Poelarends et al. (2000) found that the same gene is geographically widely distributed by using PCR analysis and dehalogenase gene sequencing of different bacteria enriched with other haloalkanes, including 1,3-dichloropropene (54). Comparison of the genetic organization in different organisms revealed that the haloalkane dehalogenase gene likely originates from *Rhodococcus* strains, where it is present in an operon together with an alcohol dehydrogenase and an aldehyde dehydrogenase gene, as well as a regulatory gene that influences gene expression. The latter may act as a repressor in the absence of a halocarbon substrate (like 1-chlorobutane). When the dehalogenase gene regions from a 1,2-dibromoethane degrading *Mycobacterium* and a 1,3-dichloropropene dehalogenating *Pseudomonas* were examined, it appeared that the repressor gene was absent or inactivated by mutations to allow production of the enzyme in the presence of these new, non-inducing substrates (54, 55). In the absence of a functional regulatory gene, inactivation of the repressor causing constitutive expression of a dehalogenase appears a way to allow genetic adaptation and biodegradation.

Lack of microbial growth on TCP and lack of adaptation in column or enrichment experiments is most likely due to the very rare occurrence of a haloalkane dehalogenase gene with a suitable activity in a host organism that is

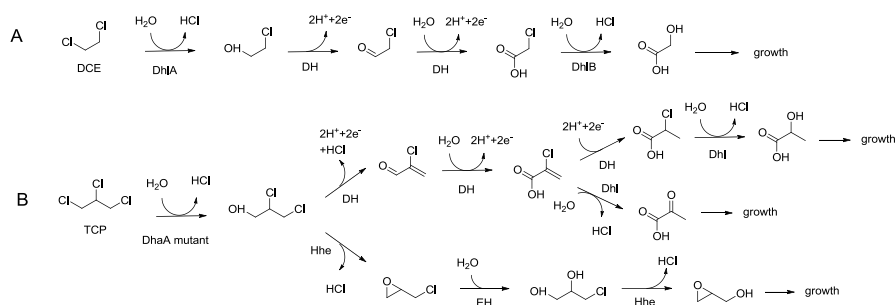
capable of dichloropropanol conversion. Mutations in the haloalkane dehalogenase that would lead to an enhanced substrate range that includes TCP would be unlikely to propagate in an organism that does not grow on the hydrolysis product and thereby provide a selective growth advantage. When DNA sequence databases, both of completed bacterial genomes and environmental sequences, are searched for genes that encode the DhaA-type haloalkane dehalogenase, or the haloalcohol dehalogenases known to be involved in 2,3-dichloro-1-propanol metabolism (except in organisms isolated on these compounds), no hits are found. These genes seem very rare, and can only be recovered by appropriate enrichment culture techniques starting with polluted environmental samples.

The evolution of bacteria that have the capacity to degrade TCP aerobically is thus restricted by the selectivity of haloalkane dehalogenases, and the rare occurrence of bacteria growing on dichloropropanols (Fig. 5). Consequently, attempts were made to obtain organisms capable of TCP detoxification by a combination of protein engineering and heterologous gene expression (3, 4).

**Engineering enzymes and organisms for TCP conversion.** Different reports on the engineering of haloalkane dehalogenase variants with enhanced activity towards TCP have been published. By using error prone PCR and DNA shuffling, Bosma et al. (2002) generated a DhaA mutant (e.g. a variant called DhaAM2 with the mutations C176Y and Y273F) that had three times higher catalytic efficiency ( $k_{cat}/K_m = 280 \text{ s}^{-1}\text{M}^{-1}$ ) than wild-type enzyme (3). Similarly, Gray et al. (2001) performed in vitro evolution studies which also yielded a mutant with a substitution at position 176 and a mutation close to the N-terminus that showed higher activity with TCP as compared to wild-type, and further mutations enhanced the stability of the enzymes (18).

The strategy to construct a recombinant TCP-degrading strain was based on the use of an improved haloalkane dehalogenase into an organism that grows on the product of hydrolytic dehalogenation, which is 2,3-dichloro-1-propanol. For this, a host was used that could degrade both 2,3-dichloropropanol and 1,3-dichloropropanol: *Agrobacterium radiobacter* AD1 (68). First, the wild-type haloalkane dehalogenase gene for DhaA from *Rhodococcus* was placed under control of a strong constitutive promoter and cloned on a broad host range plasmid (pLAFR3) that was introduced into strain AD1 (4). Growth of the resulting strain was not significant, but after incubation for 25 days 0.7 mM of TCP was converted and a small increase of biomass was observed. The strain did utilize 1,2,3-tribromopropane and 1,2-dibromo-3-chloropropane as sole carbon source, showing for the first time growth on a trihalopropane.

Growth on TCP could be obtained when a DhaA-type dehalogenase with improved activity for TCP was used. The *dhaAM2* gene for the improved dehalogenase was constitutively expressed in strain AD1. The resulting strain, *A. radiobacter* AD1(pTB3-M2), was able to utilize TCP as carbon and energy source under aerobic conditions. After 10 days, 3.6 mM TCP was converted by a culture initially inoculated to an  $OD_{450}$  of 0.14. Due to production of hydrochloric acid, the pH dropped to 6.0 (3).



**Fig. 5.** Comparison of catabolic pathways for 1,2-dichloroethane (DCE) and TCP. DCE bioremediation has been established at full scale, using bacterial cultures that use DCE as carbon source for growth according to the pathway that is shown (A). It starts with hydrolytic dehalogenation catalyzed by a haloalkane dehalogenase (DhaA). TCP is much more recalcitrant, but productive catabolic pathways can be envisaged (B). The upper routes could proceed from 2-chloroacrylic acid either via dehydrogenation (DH) (30) or dechlorination (DHl) (42). The lower route is thought to proceed in the strain constructed by Bosma et al. (2002) in *A. radiobacter* AD1 expressing a mutants haloalkane dehalogenase (DhaAM2) and involves dehalogenases (Hhe) and epoxide hydrolase (EH).

The construction of a recombinant strain using an improved haloalkane dehalogenase that was expressed under a strong constitutive promoter in a host that degrades a dichloropropanol, is an important step towards obtaining an organism that is suitable for TCP bioremediation under aerobic conditions. However, the system has still limitations and drawbacks (3): 1) although the initial dehalogenase is significantly improved for TCP conversion (ca. 5-fold as compared to wild-type) the activity of DhaAM2 is still too low to rapidly transform TCP. Consequently, the estimated doubling time of the constructed strain was 90 h, which, for comparison, is much slower than the ca. 10 h measured for the 1,2-dichloroethane-degrader *Xanthobacter autotrophicus* strain that is used for full-scale groundwater bioremediation; 2) degradation of TCP was incomplete due to the enantioselective conversion of only the (*R*)-2,3-dichloropropanol by the host *A. radiobacter* AD1. The DhaAM2 dehalogenase produced a racemic mixture of (*R*)- and (*S*)-2,3-dichloropropanol from TCP; 3) the modified dehalogenase gene for DhaAM2 was introduced into strain AD1 using the cloning vector pLAFR3, which is a transmissible plasmid. Such a plasmid may be modified or lost under stress conditions, or it may be transferred to other bacteria; 4) application of specialized bacteria in bioremediation operations will likely make use of open systems, such as an immobilized-cells bioreactor from which organisms may detach and end up in effluent water. This may lead to spread of resistance genes (in this case tetracycline) if the engineered organism contains additional antibiotic resistance markers. To remedy these limitations, further improvements are under investigation.

## Prospects

The catabolic potential of naturally occurring organisms towards organic compounds is the result of long evolution processes, whereas the time in which organisms have been tempted to evolve new enzymes, pathways and regulatory mechanisms that allow conversion of xenobiotic industrial chemicals is quite short.

The industrial synthesis of compounds such as trichloropropane only started in the first half of the 20<sup>th</sup> century. Nevertheless, the presence of these synthetic compounds in the biosphere has already triggered the evolution of new metabolic activities, as illustrated by various examples (24, 25, 50).

An important example of bacteria capable of TCP degradation are the strictly anaerobic strains BL-DC-8 and BL-DC-9 of *D. lykanthropopellens*, isolated from contaminated groundwater in the USA (Yan et al. 2009). The net dihaloelimination reaction catalyzed by these organisms implies transfer of electrons to TCP, with chloride release. This suggests the possibility of reductive dehalogenation coupled to electron transfer from hydrogen or another electron donor to TCP (dehalorespiration (60)). Since this process could possibly stimulate growth, as indicated by an increase in cell numbers (76) genetic- or population-level adaptation of cultures to TCP under anaerobic conditions can be envisaged. This may yield faster growing cultures than those currently described (maximum specific growth rate 0.15-0.17 day<sup>-1</sup>). It would also be highly interesting to identify the genes, proteins and cofactors involved in anaerobic conversion of TCP to allyl chloride and to establish their possible association with energy metabolism. The biochemical basis of dihaloelimination reactions is currently not well understood, although they may be important for different chlorinated substrates (10, 60). For in situ bioremediation, anaerobic transformation may be more attractive than aerobic processes, due to the difficulty of homogeneous oxygen supply and its preferred use for other oxidative processes if TCP is a low-level contaminant.

Anaerobic degradation of TCP was described to produce next to allyl chloride also small amounts of further conjugation products (diallyl sulfide, allyl mercaptan), probably due to abiotic reactions with sulfide (76). The chemically labile carbon-halogen bonds in allyl chloride, as well as its sensitivity to cleavage by hydrolytic dehalogenases, suggest that more rapid biodegradation of allyl chloride with reduced formation of sulfur conjugates can be achieved when adapted mixed cultures are used. Thus, further studies on the anaerobic metabolism of TCP and allyl chloride, in combination with appropriate enrichment and adaptation strategies, may well lead to more rapid anaerobic degradation as compared to what is currently possible.

Regarding aerobic degradation of TCP, genetic engineering can contribute to the acquisition of new bioremediation organisms, as illustrated by Bosma et al. (2002). To further enhance the biodegradation of TCP, use of a better haloalkane dehalogenase is desirable. By using rational design and directed evolution, the activity of DhaA against TCP was recently improved by Pavlova et al. (2010). Tunnel residues leading to the active site of DhaA were selected as target spots for mutagenesis based on the notion that substrate binding and/or product release may limit the rate of catalysis. The best variants that were obtained carried three new mutations as compared to variant DhaAM2, and had 36-times higher activity ( $k_{cat}$ ) than the natural enzyme towards TCP (Table 1). In the degradation pathway of 1,2-dichloroethane (DCE) by *X. autotrophicus* GJ10, the first step is catalyzed by DhIA, which is a phylogenetically related haloalkane dehalogenase. Since this organism was successfully used for groundwater cleanup at full scale (61), it is interesting to compare the catalytic rates of the initial haloalkane dehalogenases (Table 1). The differences in Table 1 are important since kinetic properties and expression levels of the dehalogenases have a major impact on the kinetic properties of chloroalkane degradation (substrate affinity, growth rate) by the host organism (69). Even though the activity of DhaA31 is significantly improved by

directed evolution, the  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_m$  values of DhaA31 for TCP are still lower than the corresponding values of Dh1A for DCE (Table 1). Thus, an engineered organism expressing the evolved DhaA31 will still have a lower affinity for TCP than strain GJ10 for DCE. It is well possible that further variants of haloalkane dehalogenases that convert 1,2,3-trichloropropane even better can be obtained. Strategies for laboratory evolution of new enzyme activities are still improving, and recently we were able to obtain complementary 1,2,3-trichloropropane dehalogenating mutants that produce almost enantiopure (*R*)- or (*S*)-2,3-dichloro-1-propanol. Although dehalogenase enantioselectivity may be unimportant for groundwater and soil bioremediation, it holds great promise for converting TCP waste to economically valuable chiral building blocks for use in the fine chemicals and pharmaceutical industries (71).

Improved conversion of 2,3-dichloropropanol by a better host is under investigation with new isolates that were obtained from a site contaminated with epichlorohydrin and chloropropanols due to leakage of waste from epichlorohydrin manufacture. This organism, a strain of *Pseudomonas putida*, uses a pathway for 2,3-dichloropropanol degradation that is different from the route detected in *Agrobacterium* strains (21, 68) and lacks enantioselectivity. However, none of the current dichloropropanol degraders has been selected on the basis of its potential to form a biofilm on a solid support under groundwater flow conditions, and in competition with other bacteria. Furthermore, substrate supply will likely be low, which also may impose physiological requirements on the host organism.

**Table 1.** Kinetic parameters of haloalkane dehalogenase variants with TCP and 1,2-dichloroethane (DCE). DhaA and variants thereof indicate the *Rhodococcus* enzyme that was subjected to directed evolution for enhanced TCP conversion. Dh1A indicated the *X. autotrophicus* dehalogenase that was applied in a whole-cell cleanup process for 1,2-dichloroethane removal.

Variant	Substrate	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_m$ (mM)	$k_{\text{cat}}/K_m$ ( $\text{M}^{-1}\cdot\text{s}^{-1}$ )	Ref
DhaA wild-type	TCP	$0.035 \pm 0.002$	$0.98 \pm 0.17$	36	3
DhaAM2	TCP	0.28 <sup>a</sup>	1.0 <sup>a</sup>	280	3
DhaA27	TCP	$1.02 \pm 0.031$	$1.09 \pm 0.10$	930	51
DhaA31	TCP	$1.26 \pm 0.031$	$1.2 \pm 0.15$	1060	51
Dh1A wild-type	DCE	$3.3 \pm 0.5$	$0.53 \pm 0.2$	6200	31

<sup>a</sup>Margin of error not given

The use of plasmid-based systems, as in the *A. radiobacter* AD1(pTB3-M2) recombinant (3) is undesirable for the construction of bioremediation organisms, especially when in situ remediation is targeted (8, 63). A recombinant organism applied in situ should be capable of establishing itself an environment where the conditions cannot be controlled (9). This may cause stress, leading to plasmid loss or lysis, as well as to spread of recombinant DNA. The presence of antibiotic-resistance based selection markers and the use of transmissible plasmids can be avoided by employing chromosomal integration, for which efficient transposon-based systems were developed. For example, a modified Tn5 transposon system can be used to integrate a foreign gene into the chromosome, leading to stable

integration (8). Such cloning vectors have been used successfully to construct strains for environmental applications (49).

If an efficient pathway can be assembled or evolved in the laboratory, in a robust host organism that can maintain itself under practical conditions, the prospects of successful application of such a genetically engineered organism for bioremediation are good. The limited success that has been achieved so far in this area is mainly due to the fact that few recombinant organisms have been engineered to degrade compounds which are really recalcitrant and where the poor degradability is due to biochemical factors instead of low solubility, limiting oxygen supply, poor bioavailability etc. On the other hand, evolution of dehalogenases also occurs in natural environments (24), and it is well possible that at some day, due to continued evolutionary pressure, TCP becomes a degradable compound and that TCP-degrading organisms can be obtained by classical enrichment.

## Conclusions

The toxicity and environmental behavior of TCP has stimulated research into techniques for removal of TCP from polluted sites. However, cleanup of TCP-contaminated water and soil is difficult due to its physiochemical properties and persistent nature. Biodegradation could be an attractive approach if suitable cultures become available.

Both aerobic and anaerobic processes have been investigated, but further work is needed to obtain cultures and processes with sufficient activity for testing under practical conditions and scale-up. Until then, water treatment can be done by chemical methods such as oxidative degradation using a strong oxidant and a catalyst or UV light to generate radicals. Reductive dechlorination by zero-valent iron and especially zinc are also suitable options, also for in situ application as barriers to prevent spreading via groundwater flow. Soil venting, stripping and activated carbon absorption may be used for removing TCP contaminants from soil and water. For in situ treatment, reductive dechlorination may be the best option, especially if it can be coupled to growth-supporting dehaloelimination.

Recent developments in molecular biology and protein engineering have led to the construction of genetically engineered strains that allow slow but complete biodegradation of TCP under aerobic conditions. If these strains can be further evolved to exhibit degradation rates that compare favorably or are similar to those of 1,2-dichloroethane degradation by *X. autotrophicus* GJ10, which is used at full scale for groundwater cleanup (61), it is likely that a full-scale TCP bioremediation is feasible. The physico-chemical properties of the two compounds are very similar. The construction of such strains is dependent on dehalogenase with high activity, robust host strains that resist uncontrollable conditions, and the possibility to obtain growth-supporting metabolic pathway that completely mineralizes TCP.

## Outline of the thesis

A recombinant strain of *Agrobacterium radiobacter* was reported earlier to degrade 1,2,3-trichloropropane (TCP) and use it as sole carbon source for growth. However, this system has several drawbacks: 1) slow growth on TCP and incomplete degradation; 2) instability of the dehalogenase gene that is encoded on a plasmid; 3) the presence of an antibiotic resistance marker; 4) incapability of the organisms

to establish it and to degrade TCP in a continuous flow bioreactor. The aim of the work presented in this thesis is to construct and characterize a better TCP degrading strain with the following characteristics: 1) fast degradation of TCP through use of a better initial dehalogenase and a non-enantioselective host; 2) stable chromosomal integration of the recombinant dehalogenase gene, avoiding the use of a plasmid-based expression system; 3) absence of antibiotic resistance markers. In addition, demonstrating the removal of TCP from water by a genetically engineered strain in a packed-bed bioreactor is an important goal. For the construction of such a genetically modified strain, we required an improved dehalogenase with better activity against TCP and a better DCP-degrading organism. In this work, we used the improved dehalogenase variants DhaA27 and DhaA31 obtained by Pavlova et al. through structure-based directed evolution. Furthermore, an improved host, *Pseudomonas putida* MC4, was isolated in our laboratory from polluted soil as a bacterium able to degrade 2,3-dichloropropanol.

Chapter 1 gives an overview of the toxicity, environmental properties, and degradation of TCP. It is described that different transformations are known, but biodegradation under aerobic conditions through growth-supporting processes is unknown for TCP. Furthermore, TCP is recognized by regulatory agencies as an emerging contaminant, and a number of important toxicological and transformation studies have been carried out recently.

Chapter 2 describes the isolation and characterization of the *Pseudomonas putida* strain that is able to degrade DCP under aerobic conditions. TCP serves as a carbon source and electron donor for oxidative metabolism. A novel alcohol dehydrogenase (DppA) is described that belongs to the class of quinoxinoproteins but also exhibits dehalogenase activity. This type of dehalogenating enzyme has not been reported before.

In Chapter 3, an improved haloalkane dehalogenase variant (DhaA31) is integrated into the chromosome of the DCP-degrading strain MC4 using a Tn5-derived transposition system. The accompanying antibiotic resistance gene is subsequently removed from the chromosome. The MC4-derived strains constructed by this way were indeed able to completely degrade TCP, using it as sole carbon source.

Chapter 4 reports the unusual localization behavior of the recombinant haloalkane dehalogenase (DhaA31) in *E. coli* and *P. putida* MC4. During the construction of the MC4-derivative, we noticed that the improved dehalogenase variant (DhaA31) is transported to the periplasm although it does not contain any known signal peptide and was expected to be a cytoplasmic protein. Moreover, DhaA31 was used as a fusion partner for the transport of two unrelated cytoplasmic proteins to the periplasm.

Chapter 5 presents the application of the new genetically engineered TCP-degrading strains to clean up TCP-contaminated water. Cells are immobilized in a packed bed reactor, and for the first time continuous removal of TCP under aerobic conditions is achieved. The degradation efficiency is investigated at various TCP concentrations and residence times.

In Chapter 6 the results are briefly summarized and conclusions are given. The possibilities and limitations of groundwater cleanup with genetically engineered bacteria are discussed, including steps to be taken to apply the systems explored in this thesis.



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## Chapter 2

### **A novel dehalogenase mechanism in *Pseudomonas putida* strain MC4 for 2,3-dichloro-1-propanol utilization**

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## Abstract

A *Pseudomonas putida* strain (MC4) that can utilize 2,3-dichloro-1-propanol (DCP) and several aliphatic haloacids and haloalcohols as sole carbon and energy source for growth was isolated from contaminated soil. Degradation of DCP was found to start with oxidation and concomitant dehalogenation catalyzed by a 72 kDa monomeric protein (DppA) that was isolated from cell lysate. The *dppA* gene was cloned from a cosmid library and appeared to encode a protein equipped with a signal peptide and possessing high similarity to quinohemoprotein alcohol dehydrogenases, particularly ADH IIB and ADH IIG from *Pseudomonas putida* HK. This novel dehalogenating dehydrogenase has a broad substrate range, encompassing a number of non-halogenated alcohols and haloalcohols. DppA exhibited a  $k_{cat}$  of  $17 \text{ s}^{-1}$  with DCP as substrate.  $^1\text{H-NMR}$  experiments indicated that DCP oxidation by DppA yielded 2-chloroacrolein, which was subsequently oxidized by the same enzyme to 2-chloroacrylic acid.

## Introduction

Dichloropropanols are widely used in the chemical industry, particularly as intermediates for epichlorohydrin production. The classical epichlorohydrin manufacturing process proceeds via hydrochlorination of allylchloride, which yields both 2,3-dichloro-1-propanol and 1,3-dichloro-2-propanol (32). Because of the increasing availability of glycerol as a side product from biodiesel synthesis, this classical process is being replaced by the use of glycerol as a renewable feedstock for epichlorohydrin manufacture, again via chlorination to the same dichloropropanols or via 1-chloro-2,3-propanediol (6). Epichlorohydrin itself as well as the production intermediates 2,3-dichloropropanol (DCP) and 1,3-dichloropropanol are mutagenic, genotoxic, and carcinogenic, and therefore their release and possibility of human exposure are of significant concern (40). DCP also occurs as a contaminant in cellulose and starch hydrolysates, soy sauce and baked foods (29).

Microorganisms that metabolize dichloropropanols are of interest in view of their role in the removal of these compounds from waste streams and contaminated environments (17), from food and pulp products as well as carbohydrate hydrolysates (60), and because dichloropropanols occur as intermediates in a catabolic pathway for degradation of the emerging priority contaminant 1,2,3-trichloropropane (8). Of the dichloropropanols, 2,3-dichloro-1-propanol is chemically more stable and more difficult to degrade than 1,3-dichloropropanol (17). Furthermore, microorganisms converting dichloropropanols can be used in the preparation of enantiopure building blocks for the pharmaceutical industry (29, 32). Several bacterial strains are known to grow on dichloropropanols (25), such as *Pseudomonas* sp. strain OS-K-29 (32), *Alcaligenes* sp. strain DS-K-S38 (31), *Mycobacterium* sp. strain GP1 (42) *Agrobacterium* sp. strain NHG3 (17, 23) and *Arthrobacter* sp. strain AD2 (57).

During the microbial conversion of *vicinal* haloalcohols, dehalogenation is usually the first step and this reaction can be catalyzed by haloalcohol dehalogenases (27). These enzymes, also called halohydrin dehalogenases, are composed of 2-4 subunits of molecular mass 28-35 kDa and are phylogenetically related to the short-chain dehydrogenase-reductase superfamily (SDR proteins) (55), even though they do not possess a nicotinamide cofactor binding site. They



catalyze the intramolecular displacement of a halogen by the *vicinal* hydroxyl group yielding an epoxide, a halide ion and a proton (58). At least six different halohydrin dehalogenases have been found so far: two enzymes from *Corynebacterium* sp. strain N-1074 (HheA and HheB) (62), and homologs in *Arthrobacter* sp. strain AD2 (HheB<sub>AD2</sub>) (58), *Agrobacterium* sp. strain NHG3 (DehB) (23), *Arthrobacter erithii* H10a (DehA) (5), and *Agrobacterium radiobacter* strain AD1 (HheC) (57). Structures are known and the catalytic mechanism is well understood (14, 15, 24). The halohydrin dehalogenases have a preference for substrates with the halogen group on a terminal (primary) carbon atom, which can be explained by analysis of X-ray structures (14, 24). However, important compounds such as 2-chloro-1-propanol and DCP are not easily converted. The potential importance of DCP as an intermediate in the degradation of 1,2,3-trichloropropane prompted us to search for new ways of DCP metabolism (9).

A well-established mechanism for the conversion of alcohols is oxidative conversion by alcohol dehydrogenases (ADHs). Many ADHs are NAD- or NADP-dependent enzymes (36). Oxidation of alcohols by oxidases which generate hydrogen peroxide is also possible. A special class of alcohol dehydrogenases is formed by the periplasmic quinoprotein ADHs, which contain a quinoid cofactor such as pyrroloquinoline quinone (PQQ), and Ca<sup>2+</sup>. A quinoprotein methanol dehydrogenase has been found to be responsible for 2-chloroethanol oxidation in the 1,2-dichloroethane catabolic pathway (28). PQQ-dependent ADHs have been discovered in a wide variety of bacteria such as *Acetobacter*, *Gluconobacter*, *Pseudomonas* and *Comamonas* strains (1, 2, 4, 22, 50). Some of these enzymes contain heme as a secondary prosthetic group and are known as quinoxemoproteins (4). The periplasmic quinoxemoproteins transfer electrons to the membrane-bound bacterial respiratory chain (4, 36).

In this work, we show that such a quinoxemoprotein alcohol dehydrogenase may act as DCP dehalogenase. We started with the isolation of a DCP-degrading organism from a site (Botlek area, the Netherlands) polluted with epichlorohydrin-production waste. We report the properties of this new DCP-utilizing bacterium, analyze the gene encoding the quinoxemoprotein alcohol dehydrogenase and propose a pathway for DCP metabolism.

## Materials and methods

**Chemicals, reagents and enzymes.** All chemicals were obtained from Alfa Aesar, Sigma-Aldrich, and Acros Organics. Oxidase test discs were obtained from Fluka. Plasmid DNA was isolated with Qiagen plasmid isolation kit. Enzymes used for cloning were either from Roche or New England Biolabs. The PCR master mix for screening was purchased from Promega. PCR primers were obtained from Sigma-Genosys.

**Isolation and characterization of strain MC4.** The organism used in this work, *Pseudomonas putida* strain MC4, was isolated from contaminated soil by enrichment cultivation with DCP as a sole carbon and energy source. Its growth spectrum with different halogenated and non-halogenated compounds was determined by replica plating on minimal media (MMY) agar plates supplemented with the carbon source of choice. The organism will be deposited at DSMZ [to be completed and strain number to be added if accepted or with minor revision].

The 16S rRNA DNA was amplified from the genomic DNA of strain MC4 by PCR with the universal primers 27F and 1492R (35). The final PCR mixture (20  $\mu$ l) was composed of 50 ng genomic DNA, 1x Pfx50 buffer, 0.25  $\mu$ M of each dNTP (Invitrogen), 0.1% (w/v) bovine serum albumin, and 0.25  $\mu$ M forward and reverse primers. After initial denaturation for 11 min at 94°C, the sample was maintained at 80°C and 0.5 U of Pfx50 DNA polymerase (Invitrogen) was added. The PCR mixture was then subjected to 25 cycles of 94°C for 1 min, 55°C for 1 min, and 68°C for 1 min, followed by a final extension step at 68°C for 10 min. Amplification products were purified (Qiaquick PCR purification kit, Qiagen) and cloned into the pZero-2 vector (Invitrogen). Purified plasmids were extracted from 5 ml overnight cultures and the inserts were sequenced. The nucleotide sequence of the partial 16S rRNA genes was deposited with GenBank under accession number JF825523.

**Growth and enzyme purification.** Strain MC4 was grown in a 2.5 l fermenter in MMY medium containing 5 mM of DCP as the sole carbon source. The inoculum was prepared by growing strain MC4 overnight in LB at 30°C. After batch cultivation, cells were collected by centrifugation and washed in MMY medium. This was added to the batch culture to an initial OD<sub>600</sub> of 0.05. The OD<sub>600</sub> and chloride release were monitored with regular intervals. The pH of the growing culture was maintained at 7.0 with 2 M NaOH and the temperature maintained at 30°C. At an OD<sub>600</sub> of 0.45, more substrate was added to a total input of 10 mM. At OD<sub>600</sub> of 0.7, the cells were collected, centrifuged, and washed with 10 mM Tris-SO<sub>4</sub>, pH 8.0. The cell pellet was resuspended in 5 volumes of 10 mM Tris-SO<sub>4</sub>, pH 8.0, and stored at -80°C until further use. Five batches obtained in this manner were combined and sonicated with a Vibra Cell sonifier (Sonics & Materials), for 20 min with 10 s pulse and 30 s cooling intervals. The sonicated lysate was centrifuged at 15,000 rpm for 20 min to remove cell debris. The supernatant was again centrifuged at 40,000 rpm for 2 h to separate the membrane fraction and the cell-free extract.

The cell-free extract was subjected to ammonium sulfate fractionation. Fractions of 55%, 60% and 65% precipitation were pooled together and desalted with a desalting column (EconoPac 10DG, BioRad Laboratories). The pooled ammonium sulfate fractions were applied to a 60 ml DEAE-Sepharose column (GE Healthcare) pre-equilibrated with 10 mM Tris-SO<sub>4</sub>, pH 8.0. A salt gradient of 1 M NaCl in 10 mM Tris-SO<sub>4</sub>, pH 8.0, was used to elute the proteins and 5 ml fractions were collected and checked for activity on DCP. Active fractions were pooled, concentrated with an Amicon filter (Millipore YM30) and the buffer was exchanged to 10 mM phosphate buffer, pH 8.0. The concentrated fraction was further purified on a ceramic HAP column pre-equilibrated with 10 mM potassium phosphate buffer, pH 8.0. The enzyme was eluted with a gradient of 0.01-0.5 M potassium phosphate buffer, pH 8.0, concentrated, and the buffer was exchanged to 10 mM Tris-SO<sub>4</sub>, pH 8.0. Fractions of high purity were pooled and stored for further work.

**Enzyme characterization.** For molecular weight determination of native enzyme, purified dehydrogenase (DppA) was analyzed on a gel filtration column (Superdex 200) calibrated with the following molecular weight markers: catalase (182 kDa), aldolase (158 kDa), ovalbumin (43 kDa), chymotrypsin (25 kDa) and ribonuclease (13.7 kDa). The purity and molecular weight of the protein was determined by

SDS- PAGE (12%) analysis. Heme staining of the SDS-PAGE gels was done by the method of Francis and Becker (18).

To determine heme c concentrations, we measured difference spectra of reduced and oxidized pyridine hemochrome according to a published protocol (54). The absorption coefficient of pyridine hemochrome in the 550 nm – 535 nm difference spectrum is  $26.5 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ . Protein containing oxidized pyridine hemochrome was prepared by adding 20% (v/v) pyridine, 0.2 M NaOH and 3 mM potassium ferricyanide (final concentrations) to a 1 ml enzyme sample containing 0.5 mg of protein. The enzyme solution was then reduced by adding 2 mg sodium dithionite and the absorbance was recorded.

**Enzyme assays.** All enzyme assays were performed at 25°C. The dehydrogenase/dehalogenase (DppA) activity towards DCP in cell free extract was measured by following the reduction of the electron acceptor DCPIP (2,6-dichlorophenolindophenol) with PMS (phenazine methosulfate) as an intermediate electron carrier. The reaction mixture (1 ml) contained 50 mM potassium phosphate, pH 7.4, 35  $\mu\text{M}$  2,6-DCPIP, 5 mM DCP and cell-free extract. The reaction was started by addition of 1.6 mM PMS and the absorbance was monitored at 600 nm. The absorption coefficient of DCPIP at 600 nm is  $21.0 \text{ mM}^{-1}\cdot\text{cm}^{-1}$  (19).

For routine measurements, the activity of the dehydrogenase/dehalogenase enzyme was measured by following the reduction of potassium ferricyanide ( $\text{K}_3[\text{Fe}(\text{CN})_6]$ ) at 420 nm in a 1 ml reaction mixture consisting of 50 mM Tris- $\text{SO}_4$ , pH 8.0, 5 mM DCP and 1 mM potassium ferricyanide. The extinction coefficient of ferricyanide at 420 nm is  $1 \text{ mM}^{-1}\cdot\text{cm}^{-1}$  (25). One unit of enzyme activity was defined as the amount of enzyme catalyzing the reduction of 1  $\mu\text{mol}$  of potassium ferricyanide per min under the conditions described above.

The level of heme c was used to calculate the concentration of DCP dehalogenase (DppA) as it directly reflects the active enzyme present in the solution. The kinetic constants were obtained by fitting the initial rates measured at varying substrate concentrations to the Michaelis-Menten equation.

**DCP conversion and product identification.** For measuring DCP conversion, chloride production, and product formation, a 50 ml reaction mixture was prepared containing 5 mM DCP as substrate, 10 mM potassium ferricyanide ( $\text{K}_3[\text{Fe}(\text{CN})_6]$ ) as artificial electron acceptor, and a suitable amount of enzyme in 50 mM potassium phosphate buffer, pH 8.0. At several times, a 3 ml sample was withdrawn and the reaction was stopped by adding 10  $\mu\text{l}$  of 5 M phosphoric acid. Samples were extracted with diethyl ether (1 ml) containing mesitylene (0.2 ml/L) as internal standard. The separated diethyl ether layer was analyzed on a gas chromatograph containing a HP1 column (30 x 0.25 mm; 0.25  $\mu\text{m}$ ) according to the following method: 50°C for 5 min, temperature increase from 50°C to 200°C in 20 min. The carrier gas was helium.

For chloride measurements, the remaining aqueous layers from diethyl ether extractions were analyzed on an ion chromatograph (DX 120; Dionex, Sunnyvale, CA, USA) equipped with an Alltech A-2 anion column (100 x 4.6 mm; 7  $\mu\text{m}$ ) and an Alltech guard column (50 x 4 mm). A mixture of  $\text{NaHCO}_3$  and  $\text{Na}_2\text{CO}_3$  (3 mM each), pH 10, in deionized water was used as eluent at a flow rate of 1.0 ml/min.

For identification of the expected aldehyde product of the dehydrogenase reaction, 1 ml of the reaction mixture was derivatized by adding dinitrophenyl

hydrazine to 5 mM at pH 3.5 (3). Acetaldehyde and propanal were used to standardize the derivatization procedure. The derivatized products were separated on an LC-MS system (LCQ Fleet ion trap MS, Thermo Scientific, USA) equipped with a C-18 Lichrosorb (Agilent technologies, Santa Clara, USA) reverse-phase column (150 x 3 mm ID; 5  $\mu$ m), an electrospray ionization (ESI) ion source, and a photo-diode array detector set at 365 nm. The flow rate was 0.2 ml/min and the sample (10  $\mu$ l) were analyzed as follows: 3 min at 30% acetonitrile in water, linear gradient to 80% acetonitrile in water in 20 min, 80% acetonitrile for 2 min and re-equilibration with 30% acetonitrile in water in 10 min. The analysis was carried out at negative ion mode with the following settings: source voltage, 5.3 kV; capillary voltage, -1 V; capillary temperature, 300°C; tube lens voltage, -39.98 V. The data were analyzed by Xcalibur software 2.0.

To determine the position of chlorine in the reaction product, we studied the conversion of DCP using NMR spectroscopy by recording 1D proton NMR spectra at 25°C on a Varian Unity Plus 500 MHz spectrometer. The NMR tube (1 ml) contained 5 mM DCP, 20 mM potassium ferricyanide, 100 mM potassium phosphate buffer, pH 8.0 and 20  $\mu$ l of enzyme solution, all in D<sub>2</sub>O. The reaction was started by adding the enzyme, followed by gentle mixing and recording of 1D <sup>1</sup>H NMR spectra for 24 h. Each experiment was performed with 176 scans per transient, an evolution time of 2 s, and an interscan delay of 3 s, giving rise to a net acquisition time of ~15 min per spectrum.

**N-terminal sequencing and primer design.** The purified DppA protein was subjected to N-terminal sequencing by automated Edman degradation (Eurosequence B.V., Groningen). The resulting sequence was used in a BLAST search at NCBI database (<http://blast.ncbi.nlm.nih.gov/>) to identify homologous sequences. The primers prF1 (forward 5' CAA GTC GAC CAG GCG GCA ATC ATC GC 3') and prR1 (reverse 5'ACA TAG AAG AAG CCG TTT TTG GGC GC 3'), which are based on the N-terminus of the protein and a conserved region of the homologous quinoxinone alcohol dehydrogenases, were used for PCR amplification. The amplified DNA was cloned in vector pZero-2 and sequenced. Next, three specific primers based on the amplified sequence were designed, prF2 (forward 5' AGC CAA TGG CTC AGC CAT GGC CGC ACC TAC 3'), prR2 (reverse 5' TCT CCA GGG TCG CCA GGG TAA TCT GCT GGG 3') and prR3 (5' CGT CCA GGG TGC CAA TGA AGA CCT TGC CGT 3'), and used for screening the gene libraries described below.

**Cloning and sequencing of the dichloropropanol dehalogenase gene region.**

General procedures for cloning and DNA isolation and manipulation were performed according to the published protocols (44). Total genomic DNA was isolated and the DNA pellet was resuspended in 1 ml of TE buffer, pH 7.4, and incubated with 100  $\mu$ g/ml RNase A to remove RNA for one h at 37°C before storing at -20°C for further experiments.

The genomic DNA of strain MC4 strain was partially digested with Sau3A to yield fragments of an average size of 15-30 kb. Fragments of appropriate size were cloned into pLAFR3 and packaged in vitro with Packagene Lambda DNA Packaging system (Promega) (46). *E. coli* VCS257 cells were transduced with the packaged mixture and colonies were selected on LB plates containing 12.5  $\mu$ g/ml tetracycline. Next, colonies of the transduced *E. coli* cells were arrayed in microtiter plates containing 100  $\mu$ l of LB medium and incubated for 24 h. After

growth, glycerol (20  $\mu$ l) was added to the wells and the plates were stored at  $-80^{\circ}\text{C}$  until further use. Working cultures were prepared by replicating to LB+tetracycline (25  $\mu\text{g}/\text{ml}$ ) agar plates or to microtiter plates containing 100  $\mu$ l of the same medium.

A PCR-based method (39) was used for screening the gene library. Cells from microtiter plate wells were grown on agar plates, collected and suspended in 25 ml LB medium. An aliquot of 5 ml from such a pool was used for plasmid extraction followed by PCR to check for the presence of the *dppA* gene. Each DNA pool that showed amplification was correlated to its respective microtiter plate, which was further screened by columns and rows. The identified positive clones were grown in LB+tetracycline and stored as glycerol stocks at  $-80^{\circ}\text{C}$ .

*EcoRI* and *HindIII* were used to generate separate sub-libraries in pZero-2. Ligation mixtures were transformed into competent *E. coli* TOP10 cells (Invitrogen) and selected with X-gal and kanamycin on LB plates. White colonies were selected, arrayed into microtiter plates and retested by PCR as mentioned above. This yielded a positive *EcoRI* subclone with a 2.5 kb insert that was sequenced and found to contain an incomplete sequence of the target gene. From a second sublibrary generated with *HindIII* and screened in same way, a further part of the *dppA* gene was sequenced. The complete gene sequence, including the putative ribosome binding site and promoter sequence, was assembled and analyzed by Clone Manager software. The sequence was further scanned for similarities using online BLAST tools at NCBI database (<http://blast.ncbi.nlm.nih.gov/>).

The nucleotide sequence of the DCP dehydrogenase/dehalogenase gene fragment was deposited at GenBank under accession number JN162364.

**Heterologous expression of DppA in *E. coli*.** The deduced amino acid sequence of DppA was analyzed for possible subcellular localization and N-terminal cleavage sites by PSORTb v.2.0 (20) and SignalP V3.0 (7). Based on the predicted peptide cleavage site, a 114 bp forward primer with an *NdeI* site (3' ACA CAG GAA ACA G CATATG AA ACA AAG CAC TAT TGC ACT GGC ACT CTT ACC GTT ACT GTT TAC CCC TGT GAC AAA AGC CCA GGT CGA CCA GGC CGC CAT CAT TGC CAG CAA GCA 5') was designed to replace the 24 amino acid N-terminal sequence of DppA with the 21 amino acid N-terminus of *E. coli* alkaline phosphatase (accession no. AAA24358). This was used for PCR with a reverse primer containing a *KpnI* site (3' AGG CCA CGC CGT AGA CGT AG GGTACC A TTC GAA AGG T 5'). The PCR product was cut with *NdeI/KpnI* and cloned in a pBAD vector to give plasmid pNDL1.

For protein expression, plasmid pNDL1 was cotransformed with plasmid pEC86 that constitutively produces cytochrome c maturation proteins (49) into *E. coli* TOP10 (Invitrogen) and JCB712 (26). Cells were grown in 1 l LB medium containing 50  $\mu\text{g}/\text{ml}$  ampicillin and 175  $\mu\text{g}/\text{ml}$  chloramphenicol under aerobic conditions at  $30^{\circ}\text{C}$  until an  $\text{OD}_{600}$  of 0.5. Cultures were induced with 0.02% L-arabinose and incubated in a rotary shaker at  $17^{\circ}\text{C}$  and 200 rpm for 24 h. Cells were harvested by centrifugation and the periplasmic fractions were obtained with an osmotic shock procedure (20). The periplasmic fraction was incubated with 100  $\mu\text{M}$  PQQ and 1 mM  $\text{CaCl}_2$  at  $30^{\circ}\text{C}$  for 30 min to form the holoprotein. Enzyme activity was measured with the potassium ferricyanide reductase assay mentioned earlier. Heme staining of the periplasmic fraction was also performed to verify incorporation of heme into the active protein.

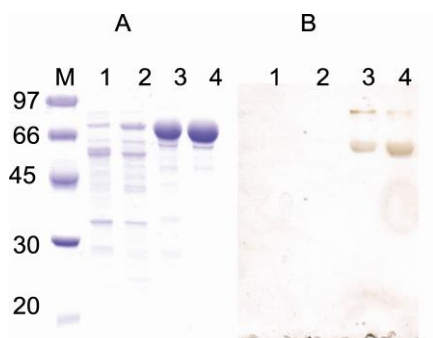
## Results

**Isolation and characterization of strain MC4.** Strain MC4 was isolated from contaminated soil using enrichment cultivation with DCP as sole carbon and energy source. Cells of strain MC4 were Gram-negative, motile and rod shaped. The organism was oxidase positive, catalase positive and it hydrolyzed starch. Strain MC4 was able to grow on sugars (fructose, glucose, galactose, ribose), citrate, acetate, succinate, benzoate, primary alcohols, haloalcohols (3-bromo-2-methyl-1-propanol, (*R*)-3-chloro-1,2-propanediol, 3-chloro-1-propanol, *rac*-2-chloro-1-propanol, 2-chloroallyl alcohol, and *rac*-2,3-dichloro-1-propanol) while it did not utilize *n*-alkanes or 1-chloro-*n*-alkanes as growth substrate. Some haloacids like 2-bromoacrylic acid, 2-chloroacrylic acid, *rac*-2-chloropropionic acid, *rac*-2,3-dichloropropionic acid and 4-chlorobutyric acid were also good growth substrates for strain MC4. Growth on DCP was not fast (ca. 0.013 h<sup>-1</sup>), and with a 10% inoculum it would take 3 days to consume 5 mM DCP in a fermentor (30°C).

The 16S rRNA gene sequence of the strain MC4 had 100% identity to the rRNA gene of an uncultured gamma proteobacterium (accession no. AF529342.1), and 99% identity to 16S rRNA genes of *Pseudomonas putida* strains (accession no. AB008001, AY512610, AY391278.1). This classifies the organism as a strain of *P. putida*.

**Identification and purification of the 2,3-dichloropropanol dehalogenase.** For the conversion of *vic*-haloalcohols, several halohydrin dehalogenases of the SDR-superfamily of proteins have been described in the literature (55, 62). Therefore, we analyzed genomic DNA of strain MC4 for the presence of open reading frames similar to the respective genes for these enzymes. A series of PCR analysis with the primers derived from the sequences of the *hheA*, *hheB* and *hheC* genes (55, 62) indicated that no similar gene was present in strain MC4. Assays with addition of DCP to cell-free extracts also failed to give dehalogenase activity, whereas halohydrin dehalogenase activity is readily detected this way in control organisms.

Next, dehalogenase activity in cell-free extracts of strain MC4 grown on DCP was tested in a 2,6-dichlorophenolindophenol (DCPIP) reduction assay. The observed reduction of DCPIP was dependent on DCP, and was accompanied by release of chloride. The specific activity of the dehalogenase enzyme in cell free extract was 94 mU/mg protein in the presence of the artificial electron acceptors DCPIP and phenazine methosulfate (PMS). The electron acceptors DCPIP and PMS could not be replaced by NAD<sup>+</sup> or NADP<sup>+</sup>. This suggests that the initial step in DCP conversion is catalyzed by a dehydrogenase that simultaneously dechlorinates the substrate and transfers electrons to an acceptor that is not a nicotinamide coenzyme. Activity could also be monitored with ferricyanide as an artificial electron acceptor. We called the enzyme DppA.



**Fig. 1.** SDS-PAGE of the protein fractions obtained during purification of DppA. (A) Coomassie stain of the heme-stained gel. (B) Heme stained gels. Lanes: M, molecular weight marker proteins; 1, cell-free extract; 2,  $(\text{NH}_4)_2\text{SO}_4$  purified fraction; 3, DEAE Sepharose purified fraction; 4, Ceramic HAP purified fraction.

The DppA protein was purified in three steps: ammonium sulfate precipitation, ion-exchange chromatography on DEAE-Sepharose and separation on a ceramic HAP column (Fig. 1, Table 1). DCP-dependent ferricyanide reduction was measured at each purification step to detect the protein responsible for the oxidation of the substrate. The molecular weight of the DppA was estimated as 72 kDa by SDS-PAGE analysis and 73.5 kDa by gel filtration. This indicated that DppA exists as a monomer in its native state.

**Table 1.** Purification of DppA<sup>a</sup>

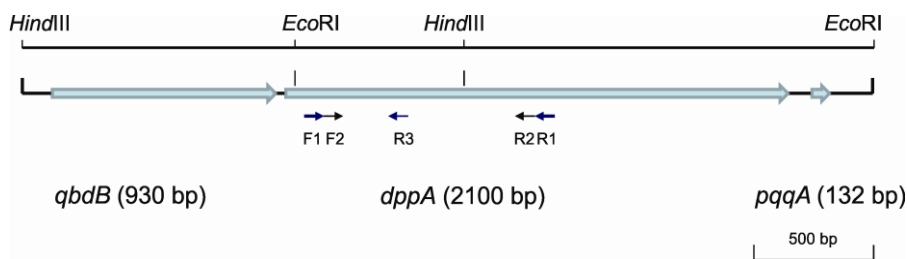
Fraction	Protein (mg/ml)	Activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification factor
CFE	15.3	6.6	265	0.4	100	1
$(\text{NH}_4)_2\text{SO}_4$ fractionation	13.8	18.4	148	1.3	56	3
DEAE Sepharose	0.5	5.8	69	10.9	26	25
Ceramic HAP	0.9	13.2	53	14.1	20	32

<sup>a</sup> Enzyme activity was measured at 25°C in 50 mM Tris- $\text{SO}_4$  (pH 8.0), containing 1 mM  $\text{K}_3[\text{Fe}(\text{CN})_6]$  and 5 mM 2,3-dichloropropanol.

**Cloning and analysis of dehalogenase gene.** To identify the gene responsible for DCP dehalogenation, the purified protein was subjected to N-terminal sequencing, which yielded  $\text{NH}_2\text{-QVDQAIIA}$ , and the NCBI non-redundant protein database was scanned for homologs. This led to several hits annotated as quinohemoprotein dehydrogenases. Multiple sequence alignments showed that these enzymes possess a highly conserved region at about 1 kb downstream of the obtained N-terminal coding sequence. Two degenerate primers, prF1 based on the N-terminus of the protein, and prR1 based on the conserved region, were used to amplify a segment

of the dehalogenase gene. The sequencing of this fragment confirmed that *dppA* encoded a quinohemoprotein.

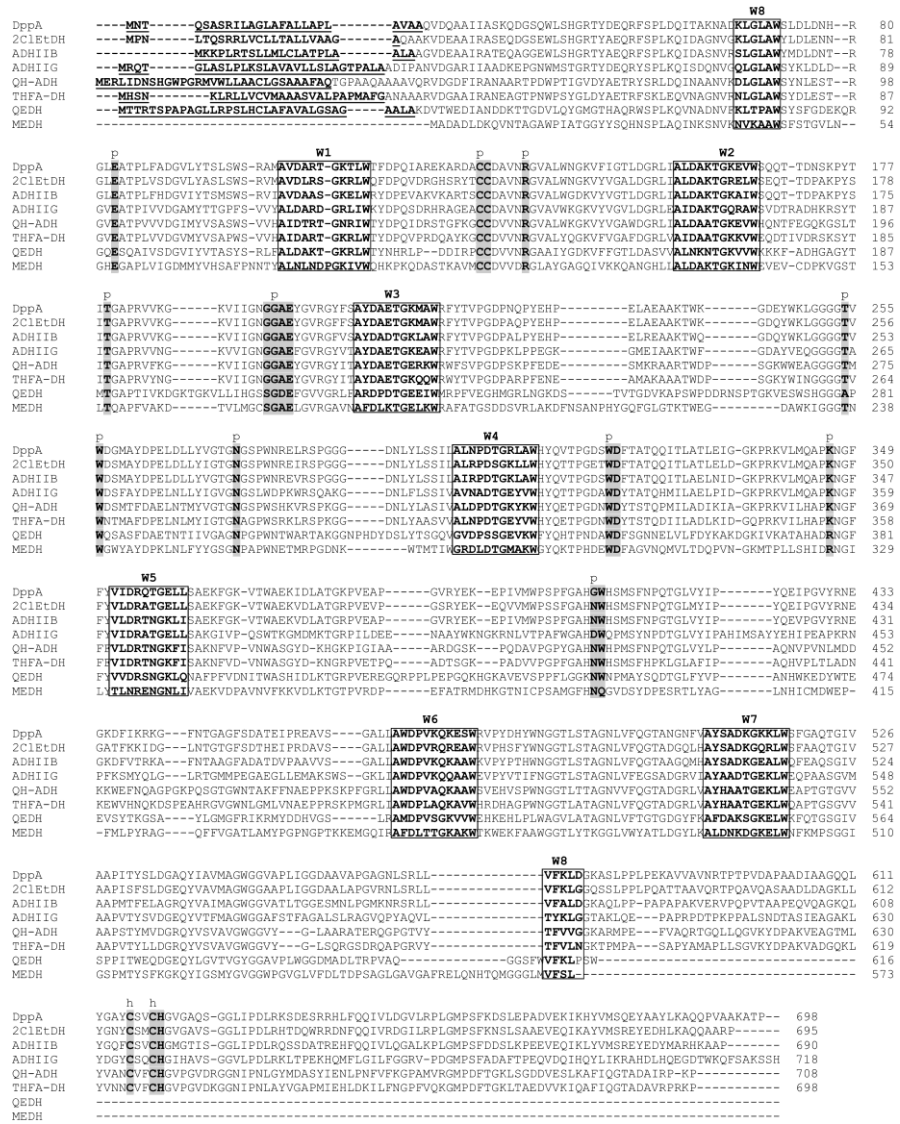
To isolate the complete MC4 dehydrogenase/dehalogenase gene, a gene library of chromosomal DNA of MC4 was constructed in pLAFR3. Two primers, prF2 and prR2, were designed based on the PCR-amplified sequence and used to screen the gene library by PCR. Out of 2,016 clones, six positive clones were identified. One of the positive clones was used to generate two separate sub-libraries and sequencing of two sub-library clones yielded DNA sequences that were assembled to obtain a 3555 bp contig containing the complete coding sequence for the DCP dehalogenase gene (*dppA*) along with its putative ribosome binding site and promoter sequence (Fig. 2).



**Fig. 2.** Schematic overview of the structural DCP dehalogenase gene. The direction of transcription is indicated by arrows. Small arrows indicate primer positions for amplification. The *dppA* gene (2.1 kb) encodes the DCP dehalogenase (698 aa). The *qbdB* gene encodes a putative protein which belongs to a superfamily of proteins involved in the meta pathway of phenol degradation. The *pqqa* gene encodes a short protein required for pyrroloquinoline quinone (PQQ) biosynthesis.

The complete *dppA*-encoded protein sequence including the signal peptide (698 aa) was highly homologous to type II quinohemoprotein alcohol dehydrogenases, particularly with 2-chloroethanol dehydrogenase from *Ps. stutzeri* (78% identity), the homologous alcohol dehydrogenases IIB (76%) and IIG (53%) from *Ps. putida* HK5 (51,52), a type I quinohemoprotein ethanol dehydrogenase from *Comamonas testosteroni* (51%) (41, 47) and a tetrahydrofurfuryl alcohol dehydrogenase from *Ralstonia eutropha* Bo (51% identity) (63). Homology with less than 50% identity was found with a quinohemoprotein ethanol dehydrogenase from *Ps. aeruginosa* (38% identity) (33, 16) and a methanol dehydrogenase from *Methylophilus methylotrophus* W3A1 (35% identity) (61). The first 25 amino acid residues of the encoded DppA protein constitute a typical signal sequence, which is responsible for the translocation of the protein to the periplasmic space. The presence of a signal peptide is a common characteristic of quinohemoproteins (53), which are located in the periplasm of gram-negative bacteria. The predicted size of the mature protein without signal peptide is 72.978 kDa, in agreement with the SDS-PAGE analysis.





**Fig. 3.** Sequence alignment of DppA with known quinohemoproteins and quinoproteins. Type II ADHs include 2-chloroethanol dehydrogenase from *Ps. stutzeri* (2C1EtDH, accession no. AAG09249.1), and the alcohol dehydrogenases ADH IIB (BAC15559.1, PDB 1KV9) and ADH IIG (BAD99293.1, PDB 1YIQ) from *Ps. putida* HK5. Type I ADHs include quinohemoprotein ethanol dehydrogenase from *C. testosteroni* (QH-ADH, Q46444.1, PDB 1KB0) and tetrahydrofurfuryl alcohol dehydrogenase from *R. eutropha* Bo (THFA-DH, AAF86335.1). Homologous quinoprotein alcohol dehydrogenases are ethanol dehydrogenase from *Ps. aeruginosa* (QEDH, CAA08896.1, PDB 1FLG) and methanol dehydrogenase from *Methylophilus* W3A1 (MEDH, AAA83765.1, PDB 4AAH). Signal sequences are underlined at the beginning of the sequence. Amino acids involved in PQQ and calcium binding are represented by the letter P on top of the alignment while those forming heme domain are indicated by h. The tryptophan docking motifs W1-W8 are indicated in boxes.

Sequence alignments with proteins of known structure indicated that the amino acids involved in PQQ and calcium binding in quinohemoproteins and quinoproteins are mostly conserved in DppA (Fig. 3). In the PQQ- and calcium-binding domain, which corresponds to the N-terminal part of the sequence, contains several residues which are commonly conserved amongst quinoproteins and quinohemoproteins (e.g. Glu83, Cys129, Cys130, Arg135, Thr179, Gly195, Glu197, Trp256, Asn274, Trp318 and Asp319), whereas other residues (Gly194, Ala196, Thr254, Lys346 and Trp407) are only conserved only in quinohemoproteins. An exception is Gly406 in the PQQ binding domain of DppA, which aligns with a conserved Asn present in most of quinohemo/quinoproteins with exception of ADH IIG, having Asp at this position. The conserved acidic residues in the PQQ and calcium domain that are involved in catalysis in quinohemo/quinoproteins of known structure are conserved as Asp319 and Glu197 in DppA (11). The heme-binding residues Cys616, Cys619 and His620, which are conserved in the C-terminal heme domain of all quinohemoproteins, are also present in DppA, in agreement with the biochemically observed heme binding. Finally, the partially conserved tryptophan docking motifs (W1 to W8), a typical feature of quinohemo- and quinoproteins, are present in DppA as well. In 7 out of the total 8 docking motifs found in these proteins, Ala, Gly and Trp are highly conserved at positions 1, 7 and 11 in each motif. In docking motif W5, these residues are replaced by different amino acids: Ala at position 1 is replaced by either Val or Thr and Trp at position 11 is replaced by either Leu or Ile while Gly at position 7 is conserved. These docking motifs are assumed to play a role in the stability of the  $\beta$ -propeller base structure of quinohemo/quinoproteins.

A small ORF encoding a peptide of 23 amino acids was present downstream of the *dppA* gene. Blast analysis indicated that this peptide contains glutamate and tyrosine residues for PQQ biosynthesis (43). Upstream of the *dppA* gene, there is an ORF encoding 310 amino acids. A Blast search indicated that the encoded hypothetical protein is similar to a putative protein from *Azoarcus* sp. BH72 (accession no. YP\_934348) and QbdB from *Pseudomonas* (accession no. BAC15558). QbdB is a hypothetical protein believed to be involved in the meta-pathway of phenol degradation.

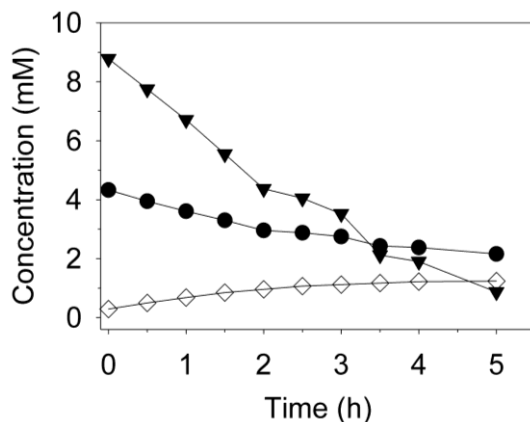
**Substrate range and kinetic parameters.** Using purified enzyme, the substrate profile of DppA was explored. Oxidation of halogenated and non-halogenated alcohols was measured by adding ferricyanide as an artificial electron acceptor. The data obtained were used to calculate  $K_m$  and  $k_{cat}$  values. Table 2 shows that the enzyme has a broad substrate range. The *n*-alcohols tested were well converted. The diols 1,2-propanediol and 1,3-propanediol showed considerably higher  $K_m$  values than the other substrates, as also reported for ADH IIG and ADH IIB (50). The  $k_{cat}$  value for DCP is  $17.8 \text{ s}^{-1}$  and the  $k_{cat}/K_m$  is  $2.3 \text{ s}^{-1}\text{mM}^{-1}$ , which indicates that DCP was well converted by DppA.

**Table 2.** Steady state kinetic parameters and substrate specificities of DppA.

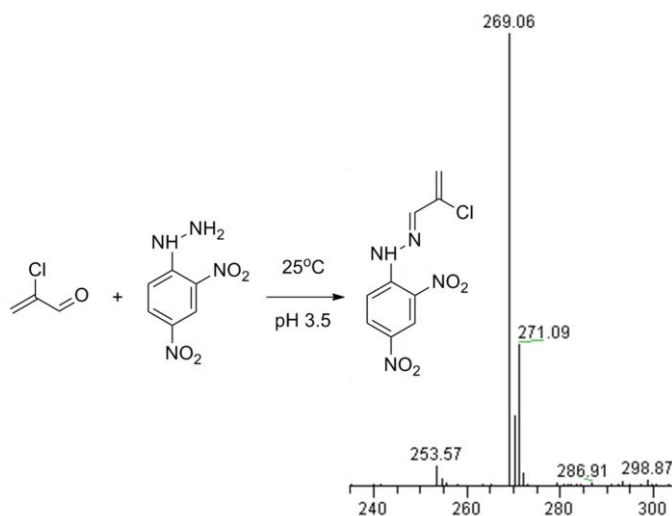
Substrate	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat}/K_m$ (s <sup>-1</sup> $\mu$ M <sup>-1</sup> )
Ethanol	14.9	788	0.02
Propanol	24.6	10	2.5
Butanol	20.2	1.5	13.2
Pentanol	25.3	3.3	7.6
Hexanol	23.9	4.4	5.5
Heptanol	16.1	1.5	10.5
Octanol	14.3	1.1	13.6
Allyl alcohol	17.1	15	1.1
1,2-Propanediol	7.1	5613	0.001
1,3-Propanediol	8.5	1033	0.008
2,3-Dichloropropanol (DCP)	17.8	7.6	2.3

The purification factor suggests that about 3% of the total protein in cell lysate is DCP dehalogenase, which in combination with the kinetic parameters of the dehalogenase and the assumption that about one-fourth of the total cell mass can be recovered as protein in cell-free extract suggests a possible DCP degradation rate of  $[S] \cdot 0.0075 \cdot 14.5 / (2.3 + [S]) \mu\text{mol/mg cells min}^{-1}$ . This would allow a growth rate of  $0.43 \text{ h}^{-1}$  at 1 mM substrate or  $0.06 \text{ h}^{-1}$  at 0.1 mM, assuming that the yield on DCP is the same as on glycerol (ca.  $0.06 \text{ mg cell dry mass}/\mu\text{mole}$ ) (64) and no energy generation from the dehalogenation reactions. Thus, the observed growth rate of less than  $0.02 \text{ h}^{-1}$  appears not to be rate-limited by the catalytic activity of the initial dehalogenase.

**Product identification.** To investigate the mechanism of dehalogenation, we examined the enzymatic conversion of DCP by purified DppA and examined intermediate and final products. Incubation of purified enzyme with substrate and potassium ferricyanide showed that DCP was converted with release of chloride, indicating that dechlorination and dehydrogenation are catalyzed by the same enzyme. Conversion of DCP (2.2 mM) yielded 1.2 mM of chloride, while 8 mM of ferricyanide was reduced (Fig. 4). This indicated that only one chlorine (or less) was released from the substrate during the dehydrogenase reaction. As homologous quinoxinoproteins are alcohol dehydrogenases that are known to act on the terminal hydroxyl group of alcohols and diols to form the corresponding aldehydes (4), a plausible mechanism of DCP conversion would be the oxidation to 2,3-dichloropropanal. This aldehyde could undergo elimination of HCl to form 2- or 3-chloroacrolein.



**Fig. 4.** Conversion of 2,3-dichloropropanol by DppA in the presence of ferricyanide as the electron acceptor. Symbols left-hand axis: ●, 2,3-DCP; ◇, Cl<sup>-</sup>; ▼, ferricyanide.



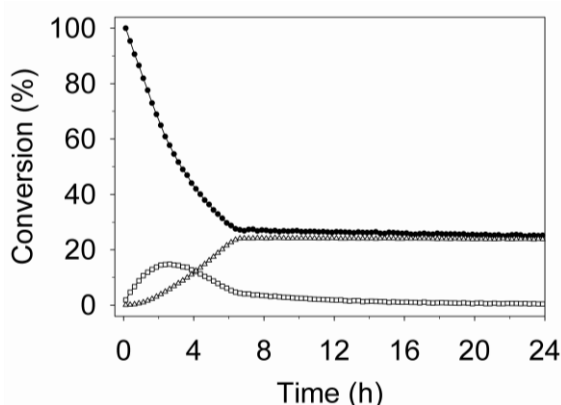
**Fig. 5.** Derivatization reaction of chloroacrolein with dinitrophenyl hydrazine. The presence of the chloroacrolein-dinitrophenylhydrazone adduct ( $m/z = 269.06$ ,  $M-H^+$ ) indicates that chloroacrolein is produced.

We did not observe an aldehyde product by gas chromatography, which could be due to its reactivity or instability, and therefore we derivatized the reaction samples with dinitrophenylhydrazine (DNPH) and analyzed possible adducts on LC-MS. In negative ionization mode, different adducts of DNPH with aldehydes should give different  $m/z$  values *viz*:  $m/z = 305$  (DNPH-derivative of 2,3-dichloropropanal),  $m/z = 271$  (DNPH-derivatives of 2- and 3-chloropropanal), or  $m/z = 269$  (DNPH-derivative of 2-chloroacrolein and 3-chloroacrolein). The negative mode ESI mass spectra indicated the appearance of a peak of  $m/z = 269$  (Fig. 5) which disappeared later during the conversion. This indicated that either 2-chloroacrolein or 3-chloroacrolein was formed. A DNPH adduct of propanal was also seen in minute amounts during the initial phase of the enzyme reaction, which may be formed by an unidentified side reaction or could be due to a substrate impurity or fragmentation in the LC-MS. No DNPH adducts were observed to

indicate the formation of 2-chloropropanal, 3-chloropropanal, or 2,3-dichloropropanal. This indicates that chlorine is rapidly removed from the substrate during the oxidative reaction.

To establish the position of the chlorine released from DCP, proton NMR was performed and chemical shifts were recorded on reaction mixtures containing enzyme, DCP and ferricyanide. All signals were studied in time which allowed us to link peaks to structures. Three signals in the NMR spectra of an intermediate product were assigned to 2-chloroacrolein (45). The time-course of its aldehyde proton (9.31 ppm) displayed the same trend as the two alkene protons (6.72 and 6.57 ppm). This again suggested that the oxidation of the hydroxyl group was accompanied by swift elimination of HCl, either in the active site of the enzyme or very rapidly after product release from DppA. Two other  $^1\text{H}$ -NMR signals (6.07 and 5.73 ppm) were assigned to the alkene protons of 2-chloroacrylic acid, which indicated further oxidation of 2-chloroacrolein to the acid by the same purified DppA enzyme. The acid apparently was not converted further under these conditions.

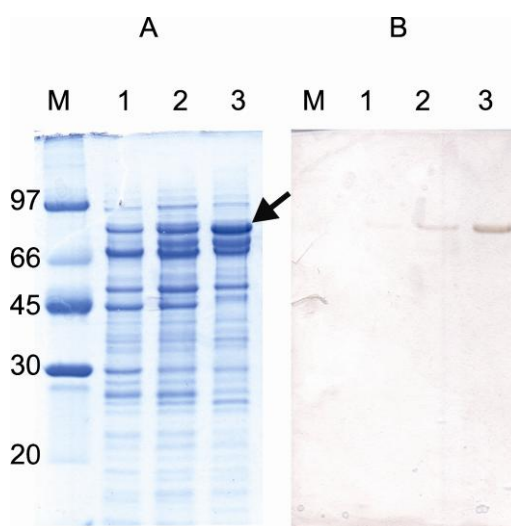
The best-separated  $^1\text{H}$ -NMR signals of the starting compound, as well as those of the intermediate product and the final product were integrated in all samples and used to visualize substrate conversion in time (Fig. 6). The results show that after 6 h, the reaction halted at approximately 72% conversion. This was probably due to complete consumption of the electron acceptor. Mass balances were not exactly stoichiometric since only 25% of the final product was detected based on integration of proton signals. This may be due to the high substrate concentration and the fact that the intermediate 2-chloroacrolein is a very reactive compound that could form dimers or polymers in aqueous solution (45), especially when produced by a pure enzyme with little possibilities for follow-up conversion of the final product. Some minor signals in the 1D  $^1\text{H}$ -NMR spectra were indeed observed, indicating formation of side products during the time course of these reactions, but these signals could not be related to a specific product. The results indicate that DppA can oxidize DCP to 2-chloroacrolein, and convert the latter to 2-chloroacrylic acid.



**Fig. 6.** Conversion of DCP by DppA followed by  $^1\text{H}$  NMR. Symbols: ●, 2,3-DCP; □, 2-chloroacrolein; △, 2-chloroacrylic acid.

**Heterologous expression.** Since the level of production of the native DppA in *Pseudomonas* strain MC4 is low, further work aimed at elucidating structure-

function relationships in this novel dehalogenase would benefit from better enzyme production. To facilitate heterologous periplasmic expression in *E. coli* (21), we fused the *dppA* gene to the 21 amino acid signal peptide sequence of *E. coli* alkaline phosphatase, yielding construct pNDL1. As quinohemoproteins require heme c maturation (49), the fusion protein was expressed in *E. coli* JCB712 and *E. coli* TOP10 in the presence of cytochrome c maturation factors, encoded on plasmid pEC86. We found that the recombinantly produced DppA has a better level of expression and heme incorporation in *E. coli* TOP10(pNDL1)(pEC86) than in *E. coli* JCB712(pNDL1)(pEC86) (Fig. 7), even though strain JB712 is known to incorporate heme effectively in the periplasmic space (26). The specific activities of DppA in cell-free extracts of these recombinant *E. coli* strains were 1.3 U/mg and 0.1 U/mg, respectively, as measured with ferricyanide reduction assays. Heme staining confirmed that the DppA protein contained covalently bound heme and the enzyme showed catalytic activity with DCP (Fig. 7).



**Fig. 7.** Coomassie stain (A) and heme stain (B) of an SDS-PAGE gel containing recombinant DppA expressed in *E. coli* JCB712 (lanes 1 and 2) and TOP10 (lane 3). Different concentrations of arabinose were used for induction: lane 1, 0.002%; lanes 2 and 3, 0.02%.

## Discussion

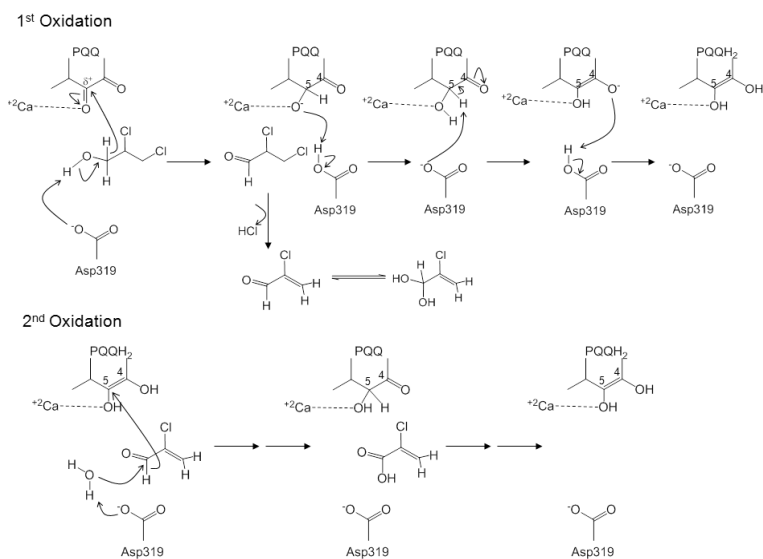
We report the isolation from a polluted site of *P. putida* strain MC4, which is capable of growth on DCP as sole carbon source. The strain grew aerobically on many other compounds as well, including sugars, several halogenated aliphatics, and non-halogenated alcohols. Bacterial cultures that utilize DCP and 1,3-dichloropropanol as growth substrates have been described earlier, but often substrate degradation is incomplete due to enantioselectivity of the catabolic enzymes, which restricts the possibilities to use such organisms for bioremediation applications (8, 38), whereas they may be attractive for production of optically active compounds (29, 30, 31, 32).

It is obvious that dehalogenation is a key step for microbial utilization of halogenated organic compounds (13, 27, 59). The dehalogenation of haloalcohols is

often catalyzed by haloalcohol dehalogenases (5, 38, 55), and we initially expected that strain MC4 would also contain such an enzyme, but no such activity could be detected and no homologous dehalogenase gene was present in strain MC4. Instead, activity measurements indicated that chloride release was electron acceptor dependent and led to the identification of a novel type of oxidative dehalogenase, which we called DppA. The enzyme had a broad substrate range encompassing a number of aliphatic alcohols and aldehydes. We also found that DppA accepts both the (*R*) and (*S*) enantiomers of DCP since conversion goes to completion with no sign of biphasic kinetics.

A blast search of the sequence of MC4 dehalogenase gene *dppA* in the NCBI database indicated that DppA was homologous to type II quinoxinoprotein alcohol dehydrogenases (ADH), which are mostly involved in the conversion of non-halogenated alcohols and contain both PQQ and heme as cofactors (4). The native DppA sequence contains a 25 aa signal peptide at the N-terminus that is cleaved off during maturation as apparent from the N-terminal sequence of the mature isolated protein. Furthermore, dehalogenase activity was detected in the periplasmic fraction prepared from strain MC4 by an osmotic shock method (data not shown). Other quinoxinoproteins also reside in the periplasm (53). The presence of an enzyme in the periplasmic space may have functional implications, such as improved protein stability and reduced proteolytic degradation (20). Besides, the presence of a dehydrogenase that forms a reactive and toxic metabolite in the periplasm could suppress potential toxic effects that may occur when formation of a reactive product occurs in the cytoplasm. The conversion of 2-chloroethanol by a periplasmic quinoxinoprotein was described earlier (56) and may have the same function: prevention of formation of highly reactive chlorinated aldehyde in the cytoplasm.

Since known quinoxinoproteins convert alcohols into corresponding aldehydes (53), we also expected the formation of an aldehyde during DCP conversion. The results indeed indicate that first step in the DCP catabolic pathway involves the conversion of DCP into 2-chloroacrolein, which is further converted into 2-chloroacrylic acid. Whether the same DppA is solely responsible for both steps was not certain, but NMR measurements indicated that purified DppA is active with 2-chloroacrolein as well. By analogy to the well-studied mechanism of quinoxinoproteins (53), we propose a catalytic mechanism of DppA (Fig. 8), which involves dehalogenation in the enzyme active site or immediately after product release. Thus, we propose that conversion of DCP by DppA proceeds in two steps: 1) an aldehyde is formed in the first step and halide ion is released immediately; 2) aldehyde is converted into corresponding acid by a second round of dehydrogenation. The <sup>1</sup>H-NMR experiments indeed suggest that release of chloride and a proton occur immediately upon formation of 2,3-dichloropropionaldehyde by hydride transfer to PQQ. This forms 2-chloroacrolein with a structure that is more stable due to resonance delocalization of the  $\pi$ -electrons. Whether the DppA enzyme mechanistically participates in halide release, e.g. through specific stabilizing interactions that facilitate cleavage of the carbon-halogen bond, such as occurring in haloalkane and haloalcohol dehalogenases (12, 14, 15), is uncertain at this moment.



**Fig.8.** Proposed mechanism for the two-step oxidation for DCP by DppA. The first oxidation results in 2-chloroacrolein and release of chloride. The second oxidation step converts the 2-chloroacrolein to 2-chloroacrylic acid.

A somewhat similar oxidative dehalogenation mechanism has been reported for a flavoenzyme from *Alcaligenes* sp. DS-S-7G, termed HDDase (30, 48). The enzyme oxidatively dechlorinates (*R*)-3-chloro-1,2-propanediol and produces acetic acid and formic acid. It was suggested that this conversion starts with formation of 3-chloro-2-oxopropanol, which could be cleaved by the reductive action of the FADH<sub>2</sub>-containing enzyme (29,48). The DppA-catalyzed dehalogenation is mechanistically completely different from the halohydrin dehalogenase catalyzed dehalogenation of chloroalcohols, where the vicinal halogen is released and an epoxide is formed by an intramolecular nucleophilic substitution (14).

Further degradation of 2-chloroacrylic acid was not studied in MC4, but possible pathways are hydrolytic dechlorination of 2-chloroacrylic acid, which yields pyruvate (34), or reduction of 2-chloroacrylic acid to 2-chloropropionic acid, which can be dehalogenated to lactate (37).

In summary, *P. putida* strain MC4 is able to completely degrade the environmental chemical 2,3-dichloropropanol. A novel dehalogenase gene with high similarity to quinohemoprotein alcohol dehydrogenases encodes a dehalogenase that mechanistically acts by alcohol group oxidation, causing dechlorination to 2-chloroacrolein.

## Acknowledgment

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## **Chapter 3**

### **A genetically engineered bacterium for 1,2,3-trichloropropane bioremediation**

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## Abstract

1,2,3-Trichloropropane (TCP) is a toxic compound that is recalcitrant to biodegradation in the environment and attempts to isolate TCP-degrading organisms by enrichment cultures have failed. A potential biodegradation pathway starts with hydrolytic dehalogenation to 2,3-dichloro-1-propanol (DCP), followed by oxidative metabolism. To obtain a practically applicable TCP-degrading organism, we introduced an engineered haloalkane dehalogenase with improved TCP degradation activity into a new DCP-degrading host bacterium. The dehalogenase gene (*dhaA31*) was cloned behind the constitutive *dhlA* promoter and introduced into the genome of *Pseudomonas putida* MC4 using a transposon delivery system. The transposon-located antibiotic resistance marker was subsequently removed using a resolvase step. Growth of the engineered recombinant bacterium *P. putida* MC4-5222 on TCP was indeed observed, and all organic chlorine was released as chloride. This genetically engineered strain is stable, free of gene transfer or transposition functions, and lacks a plasmid-encoded antibiotic resistance marker. It has the ability to completely mineralize TCP with quantitative stoichiometric release of inorganic chloride. The results show the applicability of an improved dehalogenase variant (developed by directed evolution) and genetic engineering for obtaining an effective whole-cell biocatalyst for the bioremediation of a recalcitrant chlorinated hydrocarbon.

## Introduction

1,2,3-Trichloropropane (TCP) is a toxic and carcinogenic non-natural compound. It is used in the paint industry, as a varnish remover or cleaning agent, and as an intermediate in the production of other chemicals, including polysulfone liquid polymers and hexafluoropropylene. However, a major source of TCP is the industrial synthesis of epichlorohydrin, where it is formed during chlorination of propylene, which generates a waste stream with TCP as the predominant component (29).

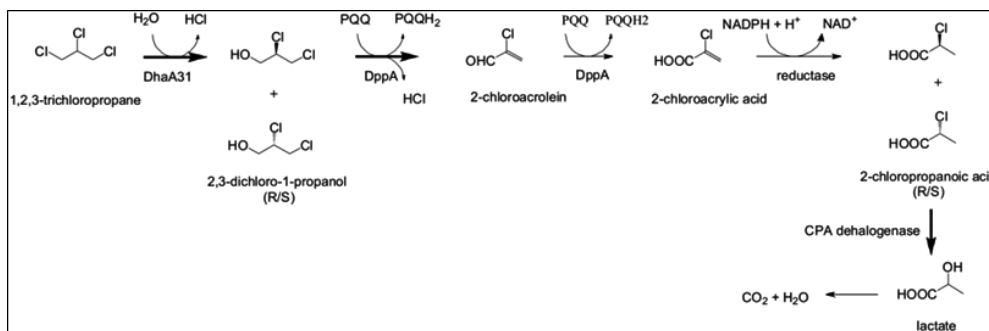
TCP is frequently detected as a groundwater pollutant because of improper waste disposal and due to its recalcitrance to biodegradation. It has a higher density than water, so it easily moves into deeper groundwater layers, leading to widespread contamination as a result of its high water solubility. This causes a serious risk to ecosystem quality as well as human health when it infiltrates drinking water supplies (25, 29). Cleanup of TCP-contaminated sites is problematic because of TCP's physico-chemical properties, including low sorption capacity of activated carbon. TCP can be degraded by reaction with metallic zinc, iron, or with zinc oxides (35, 36). Removal from contaminated water by biodegradation would be an attractive approach, provided that microbial cultures can be obtained that degrade TCP and use it as a growth substrate. By employing 1,2-dichloroethane-degrading bacteria, a full-scale groundwater treatment process has been developed for treatment of 1,2-dichloroethane contaminated groundwater, and since these compounds have similar physicochemical properties, the development of a treatment process should be feasible if suitable microorganisms are available.

To date, no naturally occurring organism has been described that can degrade TCP as sole carbon source under aerobic conditions. Reductive dechlorination of TCP may occur under anaerobic conditions a reaction that also may occur in the environment (45). We have previously described oxidative cometabolism of TCP by methanotrophs (1), and demonstrated that genetic engineering may be a strategy to obtain a strain for the aerobic degradation of TCP (2). Theoretical calculations have indicated that a number of transformations including reductive dechlorination, reductive  $\beta$ -elimination,

dehydrochlorination, and nucleophilic substitution by  $\text{OH}^-$  are thermodynamically favourable (5).

A possible pathway for TCP mineralization starts with the conversion of TCP to 2,3-dichloro-1-propanol (DCP) by a haloalkane dehalogenase that hydrolyzes a carbon-chlorine bond. Various haloalkane dehalogenases have been described (15, 18) but the only wild-type dehalogenase with significant activity on TCP is DhaA, which occurs in different strains of *Rhodococcus* (3). However, DhaA has a poor turnover number and a high  $K_m$  value. Bosma et al. (3) and Gray et al. (15) improved the kinetic properties of wild-type DhaA for TCP conversion by directed evolution. An engineered dehalogenase mutant (DhaAM2, containing the mutations Cys176Tyr + Tyr273Phe) was 3.5-fold more active with TCP than wild-type enzyme. In order to obtain a complete degradation pathway, this improved dehalogenase was cloned on a broad-host range plasmid under control of a constitutive promoter and transferred to a strain of *Agrobacterium radiobacter* that slowly degrades DCP. The recombinant strain indeed was able to degrade TCP (2), but there were several problems: (i) degradation was incomplete due to enantioselectivity of the haloalcohol dehalogenase HheC provided by the host whereas the introduced haloalkane dehalogenase produces a racemic mixture of (*R*)- and (*S*)-2,3-dichloropropanols; (ii) as evident from activity measurements (41) and structural analysis (8). HheC prefers vicinal haloalcohols with the halogen on the terminal carbon, i.e. 1,3-dichloro-2-propanol instead of 2,3-dichloropropanol; (iii), the plasmid-based system that was used for haloalkane dehalogenase expression is a broad host range cosmid derived from pLAFR3. It carries a tetracycline antibiotic resistance marker and mobilization functions, which implies that it is not desirable to use the strain in open applications, like an immobilized cell bioreactor from which cells may escape; (iv) the plasmid that was used has large segments of DNA that are not necessary for its function and that may contribute to plasmid loss, causing instability of the strain and loss of the *dhaA* gene. In agreement with these problems, attempts to degrade TCP in a continuous packed-bed bioreactor failed that was inoculated with the recombinant strain and fed TCP-contaminated water.

The current study aims to remedy the above-mentioned problems by construction of a recombinant strain that can completely degrade TCP through an improved catabolic pathway, that is free of any antibiotic resistance marker and that does not contain a transmissible plasmid. To construct such an organism, we used an improved DhaA variant as well as a novel degrading bacterial host for degrading 2,3-dichloro-1-propanol (DCP). The further improved haloalkane dehalogenase variant, called DhaA31, was recently obtained by rational design and directed evolution (27). It showed a 36-fold higher activity ( $k_{cat} = 1.3 \text{ s}^{-1}$ ) and 26-fold higher catalytic efficiency ( $k_{cat}/K_m = 1050 \text{ s}^{-1} \cdot \text{M}^{-1}$ ) than the wild-type enzyme. The expression of the gene for DhaA31 was achieved by using a strong constitutive promoter, as a high-level of cellular DhaA31 was expected to protect cells against toxic effects of TCP by increasing its conversion rate to DCP. As a DCP-mineralizing host, we used *Pseudomonas* strain MC4, a strain that was obtained from polluted soil of an industrial site by selective enrichment on DCP. The *dhaA31* gene was integrated into the chromosome of strain MC4 using a transposition system derived from transposon Tn5 (11). The results demonstrate that the recombinant strain MC4-5222 has the ability to completely degrade TCP according to the pathway given in Fig. 1.



**Fig. 1.** Proposed degradation pathway of TCP by *P. putida* strain MC4. Enzyme abbreviations: DhaA31, evolved haloalkane dehalogenase; DppA, dichloropropanol dehalogenase/dehydrogenase; CPA, 2-chloropropanoic acid dehalogenase. Thick arrows indicate the enzyme activities measured in this study.

## Materials and methods

**Strain and growth conditions.** The host organism used in this study was *Pseudomonas putida* sp. strain MC4, a 2,3-dichloropropanol degrading bacterium (I. Arif, G. Samin, D.B. Janssen, manuscript in preparation). The organism was grown in a synthetic mineral medium (MMY) that contained the following per liter: 5.4 g of Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 1.4 g of KH<sub>2</sub>PO<sub>4</sub>, 0.5 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.2 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 ml of trace elements solution and 5 mg of yeast extract (20). Cells were grown at pH 7.0 and 30 °C with shaking (200–250 rpm), and 1,2,3-trichloropropane (TCP) or 2,3-dichloro-1-propanol (DCP) was provided as a sole carbon source at 0.5–1.0 mM concentrations. Bottles were made gas-tight with screw caps having Viton septa, and samples were taken with a syringe to avoid loss of substrate. Inocula consisted of cells from a 5 ml overnight culture in LB medium. The cells were collected by centrifugation and resuspended in sterile MMY. Growth was measured turbidimetrically at 600 nm. For plates, 1.5% (w/v) agar was added.

For production of cells for testing enzyme levels, LB medium was used. A preculture was made by inoculating 5 ml of LB containing tetracycline (Tc, 25 µg/ml) with cells from a freshly streaked plate and after overnight growth; it was used to inoculate a main culture in LB medium with 100-fold dilution. The cultures were incubated overnight at 30 °C for strain MC4 and at 17 °C for 48 h for the strain *E. coli* TOP10. In case of a culture containing pIT31, 0.8 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added at the start for induction of haloalkane dehalogenase synthesis.

**Construction of broad-host-range expression plasmids.** The gene encoding improved haloalkane dehalogenase (*dhaA31*) was placed under control of the inducible *trc* promoter (4) or the constitutive *dhlA* promoter (20). Clones with the *dhaA31* gene behind the *trc* promoter were constructed in the broad-host-range vector pIT2, which is derived from pBBR1MCS2 (22) and carries a tetracycline resistance marker and the *trc* promoter. Primers PF1 and PR1 (see supporting information) were used for the amplification of *dhaA*. PCR was carried out as follows: 1 min at 92 °C, 20 cycles of 15 sec at 94 °C, 30 sec at 65 °C, 1 min at 68 °C and a final extension of 5 min at 69 °C. The PCR product was purified by using the Qiaquick (Qiagen) purification kit. Insert was ligated at 1:3 vector: insert ratio, and the mixture were subsequently transformed to *E. coli* TOP10 by chemical transformation to *E. coli* or to strain MC4 by electroporation. The plasmid was named pIT31. Transformants were plated onto LB agar plates containing tetracycline (25 µg/ml) and grown at 37 °C for *E. coli* TOP10 and 30 °C for strain MC4.



For placing the *dhaA31* gene under control of the strong constitutive *dhlA* promoter, we used a sequence of *X. autotrophicus* GJ10, including the ribosome binding site (RBS), but with a variation between the RBS and start codon. In one case, the three bases (TCT) preceding the start codon was made one base longer and the resulting segment was designated as translation initiation region A (one base insertion, TCAT). The second version, designated B, contained two mutations between the RBS and the start codon (TCT → CAT). Plasmid pIT31 was digested with *Bam*HI and *Hind*III to remove the *trc* promoter and ligated with a 517 bp PCR fragment harboring the *dhlA* promoter, ribosome binding site and translation initiation region A or B. For the isolation of a 517-bp fragment containing the *dhlA* promoter sequence, PCR was run with total DNA of *X. autotrophicus* GJ10 using primers PF2 and PR2a or PR2b (see supporting information), giving pIS31A and pIS31B, respectively. The PCR conditions used were 2 min at 94°C, 25 cycles of 30 sec at 94°C, 15 sec at 66°C, 20 sec at 68°C and a final extension of 5 min at 68°C. In the same way, another improved variant *dhaA27* (27) was cloned under the *trc* and *dhlA* promoters to yield pIT27 and pIS27, respectively.

**Chromosomal integration of *dhaA31B*.** The mini Tn5 system consists of transposon vector (pUT) and a delivery system (10). The transposon vector contains two insertion sequences (IS50L and IS50R), which govern transposition in the presence of a transposase, encoded by the *tnp* gene. Two segments are present between the insertion sequences. One segment carries a unique *Not*I cloning site, where the gene of interest (*dhaA31*) can be inserted. The other segment is a marker segment containing a gene for kanamycin resistance and the *xylE* gene for easy detection of insertion by a chromogenic reaction occurring when the encoded catechol 2,3-dioxygenase converts catechol. The marker segment is flanked by two resolution sites (*res*) and is used to screen exconjugants after transposition events. After transposition, the marker segment can be removed by expression of the RP4-derived resolvase gene *parA* as the *res* sequences are a substrate of the resolvase. The second part of the mini Tn5 system carries delivery functions and consists of a suicide plasmid that is introduced into target organism by means of mating. Due to the R6K origin of replication it can only be maintained in strains producing the replication protein  $\lambda$  (such as the host *E. coli*  $\lambda$  lysogens) and therefore is lost in *P. putida*. The delivery plasmid can mobilize the transposon vector into the target strain using the helper strain *E. coli* HB101(RK600) having an RP4 transfer function. The most salient feature of pUT vector is the presence of *tnp* gene required for transposition outside of transposon vector. After transposition event, the delivery vector is unable to replicate into the target strain and *tnp* gene is not present to express transposase and do further transfer of mobile segment (26).

To clone the *dhaA31* gene in the pUT vector, a 2-kb *dhaA31B* fragment containing promoter and terminator was amplified from pIS31B by PCR, using the primers PF3 and PR3 and PR3 (see supporting information). The PCR program was run for 1 min at 92°C, 20 cycles of 15 sec at 94°C, 30 sec at 65°C, 1 min at 68°C and final extension for 5 min at 72°C. The PCR product was purified by using Qiaquick purification kit for ligation and digested with *Not*I. Cloning of the *Not*I insert into the unique *Not*I site of pJMS11 gave rise to pUT31B. The presence of the 2-kb fragment harboring *dhaA31B* was confirmed by digestion with *Not*I restriction endonuclease and analysis on gel. The resulting plasmid pUT31B was used as a delivery plasmid.

The delivery plasmid was mobilized from *E. coli* CC118( $\lambda$ *pir*) into target cells by triparental mating. Overnight cultures of the required strains were prepared in 2 ml LB medium containing appropriate antibiotics. The recipient strain *P. putida* MC4 was grown at 30°C, *E. coli* HB101(RK600) was grown at 37°C with chloramphenicol (Cm) at 50  $\mu$ g/ml and *E. coli* CC118( $\lambda$  *pir*)(pUT31B) was grown at 37°C with kanamycin (Km) at 50  $\mu$ g/ml.

Mating was done by mixing 0.02 ml each of culture. After washing and resuspension in 0.02 ml MgSO<sub>4</sub> (10 mM), the mixture was spotted on an LB agar plate and incubated for 10-13 h at 30°C. Then the cells in the spot were resuspended into MgSO<sub>4</sub> (10 mM) and spread on M9 plates with citrate (10 mM) and Km (50 µg/ml) for selection. Colonies were tested for sensitivity to piperacillin (50 µg/ml) on an LB agar plate to ensure that the whole delivery plasmid was not integrated. Exconjugants obtained after the transposition event were screened in various ways, i.e. resistance to Km (75 µg/ml), sensitivity to piperacillin (50 µg/ml), and catechol 1,2-dioxygenase and dehalogenase activities. Catechol 2,3-dioxygenase was tested by spraying plates with catechol. The ring-cleavage reaction converts catechol to 2-hydroxymuconic semialdehyde, which gives a yellow color. Dehalogenase activities were screened in microtiter plates by suspending cells in water (50 µl) containing TCP or DBE as substrate. Plates were kept at 30°C for 4 h with DBE or for 24 h with TCP, and then halide detection reagents were added (20). Active cells produced a red color.

To remove inverted repeats and antibiotic markers, plasmid pJMSB8 which harbors the RP4 resolvase gene *parA* was introduced into dehalogenase-positive exconjugants by triparental mating (10, 26) and desired colonies were selected by testing for loss of resistance to kanamycin and the retained presence of haloalkane dehalogenase activity.

**Southern blotting.** To establish the copy number of dehalogenase gene after transposition event, Southern blotting was performed. The experiment was carried out with strains MC4, MC4-52 and MC4-5222. Genomic DNA was isolated with a genomic DNA extraction kit (Sigma) and digested with *SalI* restriction endonuclease. DNA fragments were separated on an 0.8% agarose gel and transferred to the nylon membrane by diffusion blotting. A 2-kb *NotI* insert containing *dhaA31* was used as a template to generate a probe by labeling with digoxigenin, using the non-radioactive DIG high prime kit (Roche). Hybridization was carried out at 60°C and detection was performed according to protocol provided by the manufacturer (Roche).

**Partial genome sequencing.** A partial genome sequence was obtained by Base Clear Leiden, using paired-end Illumina sequencing. De novo assembly was done with CLC-Bio software and the established contigs were analyzed using standalone Blast software to locate the insert carrying the haloalkane dehalogenase gene.

**Enzyme and protein assays.** For preparation of cell-free extracts, strain MC4 and derivatives thereof were cultivated in a 4 mM of 2,3-dichloropropanol (DCP) as a sole carbon source. The preculture was prepared by growing strain MC4 overnight in LB at 30°C. After growth, the cells were harvested by centrifugation, washed once with TMEG buffer, pH 7.5, and resuspended in TMEG (1/50 of the original culture volume; 10 mM Tris.SO<sub>4</sub>, 1 mM 2-mercaptoethanol, 1 mM EDTA, 0.01% NaN<sub>3</sub>). After ultrasonic disruption of the cells, the lysate was centrifuged (40,000 rpm for 45 min). The clear supernatant contains dehalogenases and was used for assays.

Haloalkane dehalogenase assays were performed by incubating an appropriate amount of cell-free extract at 30°C in 50 mM Tris-SO<sub>4</sub> buffer, pH 8.2, containing substrate. Dehalogenase activity was measured by determining halide release from substrates such as TCP, 2-chloropropanoic acid (CPA), or 1,2-dibromoethane (DBE). Samples were taken at different times (5 to 40 min), and halide concentrations (0.1 to 2 mM) were measured colorimetrically at 460 nm after the addition of mercuric thiocyanate and ferric ammonium sulfate (37). One unit of enzyme activity is defined as the amount of the enzyme that catalyzes the formation of 1 µmole of a halide per min.

Activity of dichloropropanol dehydrogenase (DppA) was measured at 30°C. Ferricyanide reduction by DppA activity was measured by following the decrease in  $A_{420}$  in a 1 ml reaction mixture consisting of 50 mM Tris-SO<sub>4</sub>, pH 8.0, 5 mM 2,3-dichloropropanol and 1 mM ferricyanide. The extinction coefficient of ferricyanide at 420 nm is 1.02 mM<sup>-1</sup>cm<sup>-1</sup>. One unit of enzyme activity is defined as the amount of enzyme catalyzing the reduction of 1 μmole of ferricyanide per min.

Protein concentrations of cell free extracts were measured with the Bradford reagent at 595 nm using the bovine serum albumin as standard.

**Analytical methods.** Concentrations of halogenated compounds were determined by gas chromatography. Samples (4.5 ml) were extracted with diethyl ether (1.5 ml) containing 0.05 mM mesitylene as an internal standard. The ether layer was analyzed by split injection of 2 μl samples on a gas chromatograph equipped with a flame ionization detector (Agilent) and a HP5 column (25 m × 0.25 mm × 0.2 μm) (Hewlett Packard). The carrier gas was nitrogen (50 kPa), and the temperature program was 5 min isothermal at 60°C followed by an increase to 110°C at a rate of 2°C/min and then to 130°C at a rate of 15°C/min.

Gas chromatography-mass spectrometry was performed on a HP5890 gas chromatograph equipped with an HP5 capillary column connected to a type 5971 mass-selective detector (Agilent). Helium was used as a carrier gas (0.9 ml min<sup>-1</sup>) and the temperature program was the same as above.

## Results and discussion

**Heterologous expression of a highly evolved haloalkane dehalogenase using different promoters.** Effective conversion of 1,2,3-trichloropropane (TCP) requires high-level expression of a dehalogenase that is active with TCP in a host that utilizes the product 2,3-dichloro-1-propanol (DCP). To achieve this, we constructed the broad-host range plasmids such as pIT31, pIS31A and pIS31B in which the gene encoding an improved haloalkane dehalogenase (27) is expressed under control of the *trc* and *dhlA* promoters (4, 20). For the *dhlA* promoter region, the triplet preceding the start codon was mutated in two different ways (17). In one variant, named as A, one extra base (A) was inserted at the -2 position, leading to the sequence GGAGGCTCATATG (ribosome binding site and start codon underlined). In variant B, TC at the -3 to -2 positions relative to the start codon was replaced by CA, leading to GGAGGCCATATG (ribosome binding site and start codon underlined). The same pBBR1MCS2-derived replicon (22) was used for all broad-host-range expression plasmids, so copy number did not affect the efficiency of expression.

As a host, we used a newly isolated strain of *P. putida* that utilizes DCP as a growth substrate. This organism degrades both stereoisomers of DCP, in agreement with the lack of enantioselectivity of the initial oxidative dehalogenase/dehydrogenase (DppA) that converts DCP to 2-chloroacrolein and 2-chloroacrylic acid (I. Arif, G. Samin, D. B. Janssen, submitted for publication). The organism was identified as a strain of *P. putida* by 16S rRNA gene sequencing.

Two genes encoding haloalkane dehalogenase variants that were improved by directed evolution were tested in strain MC4. Plasmid pIT27, containing haloalkane dehalogenase mutant 27 (27) gave a lower haloalkane dehalogenase level than variant 31 (Table 1). It was also observed that in the case of the *trc* promoter, the activity of the dehalogenase was low as compared to the use of the strong constitutive *dhlA* promoter (Table 1), in contrast with the results of Bosma et al., (3) who found little difference between the efficiencies of *trc* and *dhlA* promoters in *A. radiobacter* AD1 as the host. When the two variants of the *dhlA* promoter region were compared, the dehalogenase activity was

highest in case of pIS31B, which agrees with the notion that a C at position -3 relative to the first base of the start codon leads to elevated expression (17). The promoter and translation initiation region of pIS31B was therefore selected for further work.

**Table 1.** Expression of haloalkane dehalogenase in MC4 containing various plasmids carrying the *dhaA31* gene.

Plasmid	Haloalkane dehalogenase activity (U/mg)
pIT27	0.01
pIT31	0.12
pIS27A	0.01
pIS27B	0.03
pIS31A	0.10
pIS31B	0.19

Specific activities were measured with TCP using cell-free extracts from cells grown in LB medium.

**Chromosomal integration of mini-Tn5 containing *dhaA31* into *Pseudomonas*.** For chromosomal integration of the *dhaA31* gene with the constructed constitutive promoter region into *P. putida* strain MC4, we employed a Tn5-derived transposon vector (pJMS11) developed by de Lorenzo and coworkers according to their published protocols (10, 11). Strain MC4 is a 2,3-dichloropropanol utilizing bacterium that was isolated from contaminated soil. Plasmid DNA could be introduced either by transformation through electroporation or by conjugation through triparental mating. For introduction of the *dhaA31* gene in strain MC4, it was first cloned on the transposon delivery vector pUT2 in *E. coli* as described under Materials and Methods. After introduction of the transposition system into strain MC4 by triparental mating, 50 exconjugants were selected on basis of growth on M9-Km plates with 10 mM citrate as a carbon source. This selection permitted the *Pseudomonas* recipient strain to grow when it had acquired the Km resistance gene region, but did not allow growth of the *E. coli* donor strain. A total of 35 Km-resistant colonies were selected that were able to grow on citrate and DCP as sole carbon source, whereas 15 Km-resistant citrate-utilizing colonies lost the capacity to grow on DCP. Sensitivity to pipericillin indicated that the whole plasmid only rarely integrated into the chromosome of strain MC4. Of the 50 Km-resistant colonies, 45 were sensitive to pipericillin. The Km-resistant colonies also produced catechol 2,3-dioxygenase, as indicated by a yellow color that was observed after spraying colonies with a solution of catechol.

The next step was to remove the antibiotic-resistance selection markers by using the resolvase protocol (11). For this, 30 haloalkane dehalogenase-positive MC4-derivatives were separately subjected to triparental mating in which pJMSB8 was introduced into the recipient to express the resolvase gene *parA*. Colonies of strain MC4 were selected on M9 medium containing 5 mM citrate and tested for Km sensitivity and spraying with catechol was used to verify the loss of *xylE*. We observed that 90% of the colonies that appeared were Km sensitive and showed absence of the *xylE* marker. The presence of a 2-kb fragment having the *dhaA31* gene was confirmed by PCR analysis with the same primers as those used for cloning.

**Utilization of TCP as sole carbon source.** Different derivatives of strain MC4 carrying the *dhaA31B* gene on the chromosome were tested for growth on TCP. For this purpose, we performed batch incubations using mineral medium with TCP (0.5 mM) as sole carbon source. Several MC4-derived strains carrying *dhaA31* (e.g., MC4-5221, MC4-5222 and

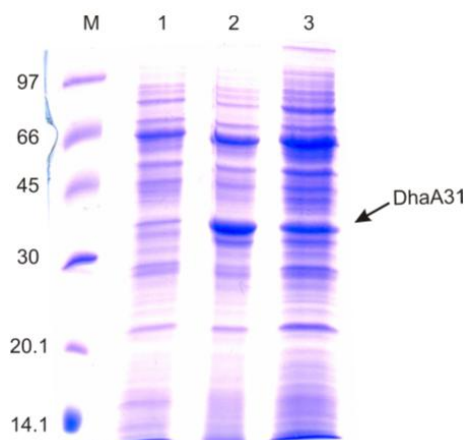
MC4-1331) showed growth on TCP. Disappearance of TCP was accompanied by the stoichiometric release of inorganic chloride ions as well as microbial growth. In negative controls, containing wild-type strain MC4, no microbial growth or release of chloride was detected. A derivative designated MC4-5222 showed the most rapid growth on TCP. To compare the expression level of the dehalogenase in different strains that carried insertions with the dehalogenase gene, cell-free extracts were made, and the haloalkane dehalogenase activities towards TCP were measured (Table 2). Strain MC4-5222 showed the highest haloalkane dehalogenase activity, indicating that the DhaA31 expression level and the growth rate were correlated.

**Table 2.** Specific activity of DhaA31 in MC4-31B strains towards TCP.

Strain	Properties	Haloalkane dehalogenase (DhaA31) (U/mg)
MC4	wild type 2,3-dichloropropanol degrader	-
MC4-52	<i>dhaA31</i> -inserted derivative of MC4	0.04
MC4-13	<i>dhaA31</i> -inserted derivative of MC4	0.03
MC4-5221	<i>dhaA31</i> -inserted derivative of MC4	0.04
MC4-5222	<i>dhaA31</i> -inserted derivative of MC4	0.10
MC4-1331	<i>dhaA31</i> -inserted derivative of MC4	0.03

To check if the plasmid-carrying strains producing haloalkane dehalogenase (strain MC4(pIS31B)) and a strain carrying chromosomally integrated *dhaA31* (MC4-5222) produced all three dehalogenases required for TCP degradation we measured the expression levels of DhaA31, haloalcohol dehydrogenase/dehalogenase (DppA) and 2-chloropropionic acid (CPA) dehalogenase in the wild-type strain MC4 and in the engineered derivatives (Fig. 2). DppA is a quinoxinoprotein alcohol dehydrogenase that converts 2,3-dichloropropanol to 2-chloroacrolein and 2-chloroacrylic acid in the presence of an artificial electron acceptor like ferricyanide (A. Irfan, S. Samin, D.B. Janssen, submitted). Assays with cell-free extracts prepared from cells grown in DCP (4 mM) showed that both strain MC4 and strain MC4-5222 produced dehalogenases for DCP mineralization and there were no significant differences between the DppA and CPA dehalogenase levels. The dehalogenases level were lower in the plasmid-based system MC4(pIS31B) (Table 3). Furthermore, we observed that the expression of DhaA31 was higher in the plasmid-based system MC4(pIS31B) than in the chromosomal integration MC4-5222. Possibly, the strong overexpression of DhaA31 in this plasmid-based system reduces expression of DppA and CPA dehalogenase.

The ability of strain MC4-5222 to mineralize TCP was examined in batch cultures with repeated addition of TCP. The optical density ( $OD_{600}$ ), chloride, TCP, and DCP levels were measured at different times. The culture was inoculated with overnight-grown cells in LB medium with a starting  $OD_{600}$  of 0.05. After the first addition of TCP (0.5 mM), it was noticed that the  $OD_{600}$  decreased, which might be due to toxic effects of TCP added to the cultures of low cell density. After 4 days, visible growth was observed as also shown by an increase of  $OD_{600}$ . This growth was continued when TCP was added with different time intervals. Furthermore, after 20 days, the degradation of TCP was rapid and the accumulated cells of *P. putida* strain MC4-5222 were able to degrade 1.1 mM TCP quickly. Overall, the added TCP (3.1 mM) was dechlorinated completely with stoichiometric release of chloride (9.3 mM) as shown in Fig. 3. Thus, the recombinant strain was capable of complete mineralization of TCP. From the optical densities, it was calculated that the growth rate  $\mu$  varied from 0.0012 - 0.008 h<sup>-1</sup>.

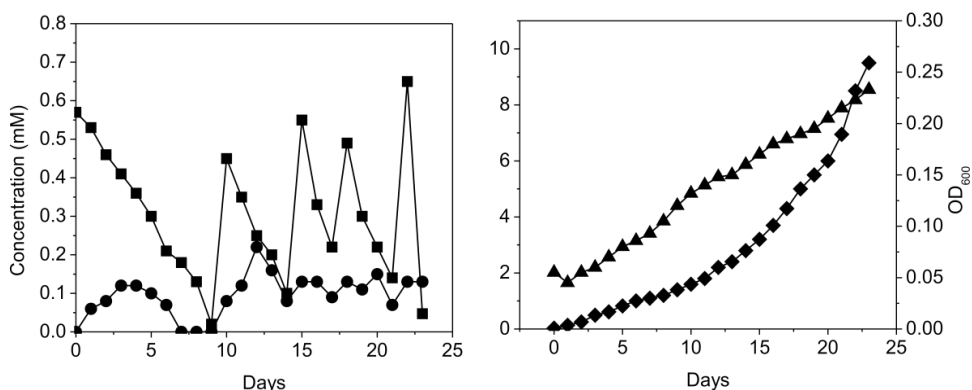


**Fig. 2.** Haloalkane dehalogenase expression in derivatives of *P. putida* MC4. Slots: M, marker proteins; 1, cell-free extract of strain MC4; 2, strain MC4(pIS31B); and 3, strain MC4-5222 carrying the chromosomally integrated *dhaA31* gene. All cultures were grown on DCP as carbon source.

**Table 3.** Specific activities of DhaA-, DppA- and CPA dehalogenase in cell-free extracts of MC4 and derivatives thereof.

Strain	Haloalkane dehalogenase (DhaA31) (U/mg)	Dichloropropanol dehalogenase (DppA) (U/mg)	2-Chloropropanoic acid (CPA) dehalogenase (U/mg)
MC4	0	0.13	0.28
MC4(pIS31B)	0.73	0.03	0.12
MC4-5222	0.37	0.16	0.57

Higher concentrations of TCP (>1 mM) appeared very toxic to strain MC4-5222, indicating that further mutations or genetic modifications may be required for improved conversion. However, extensive ethyl methanesulfonate (EMS) mutagenesis experiments with subsequent selection of cultures with increased resistance or repeated transfer of cultures exposed to TCP at the upper limit of tolerance did not yield improved strains. As outlined by Haro and de Lorenzo (16), the rate of metabolic fluxes, the formation of side-metabolites and the physiological control of degradative pathway may be a bottleneck that is as important as the presence of all the enzymes for a metabolic pathway.

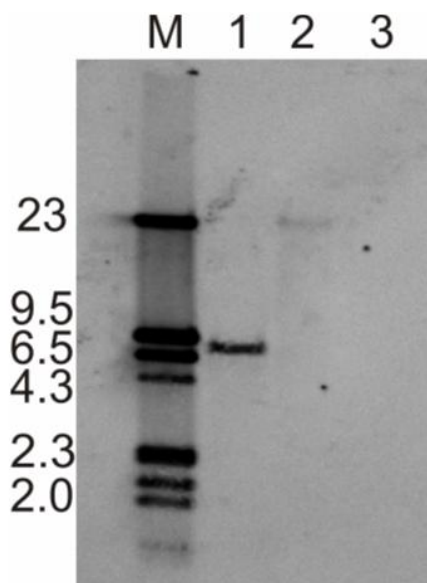


**Fig. 3.** Growth of *P. putida* MC4-5222 on TCP. Panels A and B represent data from the same culture. TCP was repeatedly added to a total concentration of 3.1 mM. Symbols: ▲, optical density at 600 nm; ■, TCP; ●, DCP; ◆, chloride.

**Stability of the TCP degrading strain MC4-5222.** To check the stability of the integrated gene on the chromosome, strain MC4-5222 was repeatedly streaked on LB agar plates without TCP. The presence of the dehalogenase gene was tested by measuring dehalogenase activity of isolated colonies as described above. No colonies that had lost the haloalkane dehalogenase gene were found after ten serial transfers, which indicated that strain MC4-5222 is genetically stable, in agreement with previous reports which described that the chromosomally inserted DNA is stably inherited (10).

**Localization of the chromosomal integration.** We checked the copy number of the integrated *dhaA31* gene by means of Southern blotting. Furthermore, partial genomic sequences of MC4 and MC4-5222 were obtained to identify the location of integrated genes. The results of Southern blotting indicated that strains MC4-52 and MC4-5222 each contain a single copy of the *dhaA31* dehalogenase gene at a different position (Fig. 4). The site of integration apparently influences the level of expression of the *dhaA31* gene.

The partial genomic sequence of strain MC4-5222, and comparison with the sequence of the wild-type strain MC4, revealed that a 2165 bp fragment carrying the *dhaA31* gene (accession number <to be included after acceptance>) was present as an insertion after position 1305 of a 26,912 bp contig of strain MC4. Upstream was a 288 codon ORF with no similarities to genes of known function. Downstream was a 911 codon ORF encoding a putative protein of the P-loop NTPase superfamily showing 76% identity to a putative transcriptional regulator of *Pseudomonas fluorescens* Pf0-1. The 27 kb contig in which the *dhaA31* gene was integrated further shows similarities to genomic segments of *P. auruginosa* SBW25, *P. mendocina* YMP and *P. aeruginosa* PA7, but query coverage was only 13-53%, which together with further sequence analysis showed that strain MC4 is not highly similar to other bacteria of which the genome sequence is published.



**Fig. 4.** Detection of chromosomally integrated *dhaA* DNA by Southern hybridization. Hybridization was performed with a 2-kb fragment (*dhlA* promoter, *dhaA31* gene and terminator) as a probe with non-radioactive DIG-labeling and *SalI* digested chromosomal total DNA of strain MC4-5222 (lane 1); MC4-52 (lane 2); and MC4 (lane 3).

**Prospects for TCP bioremediation with genetically engineered TCP-degrading bacteria.** The development of a biological process for on-site removal of TCP from groundwater is critically dependent on the availability microorganisms that degrade TCP in a manner that supports cellular maintenance and growth. The relevant physico-chemical properties of TCP are similar to those of 1,2-dichloroethane ( $K_{ow}$  values 1.98 and 1.48, Henry coefficients  $3.4 \cdot 10^{-4}$  and  $1.18 \cdot 10^{-4}$  atm $\cdot$ m $^3$  $\cdot$ mol $^{-1}$  for TCP and 1,2-dichloroethane, respectively). Due to these values, absorption by activated carbon and air stripping are problematic for TCP and 1,2-dichloroethane removal, making bioremediation especially attractive. A full-scale bioreactor-based treatment process has been installed for the removal of 1,2-dichloroethane from contaminated groundwater at a large contaminated site in Lübeck, Germany (40). This process employs the 1,2-dichloroethane degrader *Xanthobacter autotrophicus* GJ10, in which critical catabolic genes are located on a plasmid, closely linked to insertion sequences (19). The similarity in physical-chemical properties of TCP and 1,2-dichloroethane suggest that the development of a bioreactor treatment process is feasible once an effective organism is obtained.

Bacteria that degrade 1-chloro-*n*-alkanes gene are easily isolated from soil and they often produce the *dhaA*-type haloalkane dehalogenase that slowly degrades TCP (31). This includes *Rhodococcus* strain TB2, isolated by us from a major TCP-contaminated site in the USA. In view of the widespread occurrence of the *dhaA* gene it seems remarkable that TCP is so recalcitrant to aerobic biodegradation, since only 3-5 mutations are required for much better conversion (2, 27). Various attempts by ourselves and others to obtain a TCP-degrading culture by classical enrichment have failed, whereas compounds of similar structure and toxicity, such as 1,2-dibromoethane and 1,3-dichloropropene, were degraded after prolonged enrichment (30, 31). We propose that the limiting factor in the evolution of TCP-degrading bacteria is the extremely rare occurrence of the required mutations in a



*dhaA*-type haloalkane dehalogenase gene that resides in a host organism to which such mutations provide an advantage for growth. Such a host should be able to metabolize the dichloropropanol that is produced. Bacteria that degrade dihalopropanols have been found at various places, (13, 21, 31) but they appear quite rare, probably because these compounds also do not occur naturally at biologically significant concentrations. The same may be true for the further metabolites chloroacrolein and 2-chloroacrylic acid (Fig. 1). Thus, in a natural environment, mutations in the haloalkane dehalogenase gene that would improve its TCP hydrolysis activity may never occur in a host that grows on the degradation product. The toxicity of the TCP transformation products (epichlorohydrin) if incompletely metabolized may even pose a selective disadvantage on organisms possessing a TCP-active hydrolytic dehalogenase (44).

To circumvent problems of slow adaptation in natural environments, genetic engineering techniques have been proposed for obtaining strains with enhanced biodegradation potential. Several improved strains have indeed been constructed (6, 7, 14, 23, 28, 32, 34, 39, 42). These works provided excellent scientific insight in causes of recalcitrance and demonstrated that bottlenecks in catabolic pathways could be removed. Furthermore, tools for the construction of recombinant bioremediation bacteria were developed and several constructed strains exhibited remarkable new properties in the laboratory. On the other hand, success in terms of full-scale application is limited (6, 24, 43). The only well-documented field trial concerns *Pseudomonas fluorescens* HK44, a strain that degrades polycyclic aromatic hydrocarbons harboring a natural catabolic plasmid that is equipped with a *lux* bioluminescent reporter. The strain produces light in response to the presence of naphthalene or the aromatic degradation products salicylate and 3-methylsalicylate (33). We consider the main cause of the modest progress in this area the technical difficulty of constructing organisms for compounds that are really recalcitrant, chemicals such as 1,2,3-trichloropropane, 1,1,1-trichloroethane or trichloroethylene (38). Most of the earlier work was aimed at combining into a single strain the capacity to degrade mixtures of compounds, or to expand the degradation capacity of a single organism (32, 34, 43). The urgency for application may be unclear, since the performance in bioremediation of such organisms may not exceed that of mixed cultures or of other natural isolates.

The difficulty in obtaining more effective organisms by genetic construction may have different biochemical causes. Although transposons are very good tools for constructing genetically stable strains (12, 26), the obtained organisms do not always degrade the target substrate as expected even if the pathway looks fine on paper (9). For example, a *Pseudomonas* strain able to grow on 2-chlorobenzoate was still unable to grow on 2-chlorotoluene as sole carbon source, although it possesses all the genes in a functional state required for its degradation (16). Similarly, we have observed that the introduction of the 1,2-dichloroethane dehalogenase gene from *X. autotrophicus* in a 2-chloroethanol-degrading *Pseudomonas* does not allow the resulting recombinant strain to use 1,2-dichloroethane for growth. Even though the TCP-degrading *Pseudomonas* strain constructed here is may be the best example of a constructed organism that grows on a compound that is really recalcitrant, for unknown reasons the growth rate is not as high as the naturally evolved 1,2-dichloroethane-mineralizing *X. autotrophicus* GJ10 mentioned above.

An engineered bioremediation organism can likely survive only during an *in situ* or bioreactor treatment process when the target compound is the predominant pollutant in groundwater or in the waste stream, and if its metabolism supports growth, e.g., by use as electron donor or acceptor. If the contaminant is a minor component, the engineered strain may have difficulty to survive *in situ* (9, 24). If a waste stream contains a whole range of recalcitrant contaminants, adding a constructed organism that degrades only one or two

components will contribute too little to the overall process. Furthermore, there is only a chance for successful application of bacteria with a constructed pathway when the lack of a pathway really is the factor that restricts biodegradation, instead of substrate bioavailability or oxygen supply. Due to their application as solvents and intermediates and their formation in specific synthetic chemical processes, compounds such as 1,2-dichloroethane, TCP and trichloroethene occur as sole or major contaminant at many sites, making them good targets for this type of studies.

## Conclusions

This study set out to construct a TCP degrader, which can be used to treat TCP contaminated water. For application in groundwater treatment systems, open processes such as the one developed for 1,2-dichloroethane will be needed. Therefore, we aimed at constructing an organism that is free of antibiotic resistance marker and plasmid encoded genes that can be transferred into other organisms by transposition or conjugation. The transposon delivery system developed by de Lorenzo and coworkers appears excellently suited for that purpose. Moreover, the constitutive expression of essential genes can be another advantage since it avoids the necessity of high concentrations of inducing substrates. Previous work indicated the potential, but success was very modest in view of the poor activity of the best available dehalogenase and the poor growth of the host on DCP, which is the first hydrolytic product. An improved organism for TCP bioremediation was obtained here by using an engineered haloalkane dehalogenase variant with a higher catalytic activity (DhaA31) followed by constitutive expression into a suitable host. Integration into the genomic DNA of the newly isolated host *P. putida* strain MC4 yielded a strain that slowly grew on TCP as sole carbon source. The conversion of TCP into DCP was catalyzed by the improved dehalogenase variant and further conversions were carried out by the enzymes of the MC4 host strain. The resulting engineered bioremediation bacterium strain MC4-5222 is able to utilize TCP as a sole carbon source with complete dechlorination. The strain has no introduced antibiotic resistance marker and is free of plasmid-encoded mobile genes. Taken together, these results suggest that the recombinant strain MC4-5222 should be applicable for the removal of TCP from contaminated groundwater.

## Acknowledgements

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## **Chapter 4**

### **Periplasmic localization of haloalkane dehalogenase and its use in protein targeting for whole-cell biocatalysis**

Ghufrana Samin, Edwin van Bloois, Marco W. Fraaije and Dick B. Janssen

## Abstract

The expression of heterologous proteins in the *Escherichia coli* periplasm can have important advantages over cytoplasmic expression because it may improve protein folding, reduce proteolytic degradation, and facilitate protein isolation. In case of whole-cell processes, periplasmic localization of enzymes can improve substrate access and reduce product toxicity. To enable export to the periplasm, a signal sequence can be fused to the target protein or the target protein is fused to an authentic periplasmic protein. Here, we report the identification of a modified *Rhodococcus* haloalkane dehalogenase (DhaA31) as a novel export fusion partner for periplasmic expression in *E. coli*. We observed that this haloalkane dehalogenase, of which another variant is used in the HaloTag reporter gene system, localizes to the periplasm of *Pseudomonas putida* and *E. coli*, independent from a signal sequence. Additionally, DhaA31 is able to trigger periplasmic localization when genetically fused to thioredoxin and alditol oxidase, two unrelated cytoplasmic enzymes. The haloalkane dehalogenase – alditol oxidase hybrid enzyme catalyzed the whole-cell conversion of xylitol. Our data show that DhaA31 can be exploited as a platform for the expression of enzymes in the periplasm, thereby opening new avenues for its use in biotechnological applications.

## Introduction

The production of recombinant proteins in sufficient quantities is often the first requirement for many biotechnological applications as well as for biochemical and structural studies. The high-level production of recombinant proteins requires, in addition to an efficient genetic expression system, a suitable expression host. To this end, different prokaryotic or eukaryotic microorganisms are commonly used (39, 60). Despite the plethora of known expression hosts, the Gram-negative bacterium *E. coli* is often the preferred host because of the ease of handling and genetic manipulation, as well as its rapid growth rate and capability to produce recombinant proteins in high yields (3, 49). *E. coli* cells have distinct cellular compartments i.e. the cytoplasm and the cell envelope. The latter comprises the cytoplasmic and outer membranes, separated by the periplasmic space. This compartmentalization offers the advantage that proteins can be expressed in each of these cellular compartments, depending on the characteristics of the target protein. Periplasmic expression of target proteins may, for example, be favored over cytoplasmic expression in some cases as it improves protein folding, reduces proteolytic degradation and, moreover, the periplasm represents a cellular compartment with less contaminants relative to the cytoplasm, thereby allowing a simpler and more straightforward purification of expressed proteins (12, 30).

Under various physiological conditions, a further implication of periplasmic expression can be reduced toxicity of enzymatic transformation products. This is of importance when natural organisms are exposed to synthetic chemicals, such as halogenated hydrocarbons. Especially in the case of xenobiotic compounds and oxidative reactions, cytoplasmic conversion may yield reactive metabolites such as aldehydes and epoxides, which may decrease viability or completely block metabolism (57). During bacterial conversion of 1,2-dichloroethane and 1,2-dibromoethane, sequential conversion by haloalkane dehalogenases and alcohol dehydrogenases can produce highly toxic bromoacetaldehyde (37, 55). Periplasmic

expression may lead to temporary accumulation of reactive intermediates in the medium rather than in the cells and thereby reduce toxicity. Thus, the conversion of 2-chloroethanol by periplasmic quinoprotein alcohol dehydrogenase can be explained this way (19, 55).

Furthermore, the HCl-eliminating hexachlorohexane dehalogenase (LinA) and the 1,2,4,5-tetrachlorohexacyclohexadiene-hydrolyzing haloalkane dehalogenase (LinB) from bacteria degrading  $\gamma$ -hexachlorocyclohexane (lindane) have been shown to occur in the periplasm (31). The latter enzymes can act on a mixture of lindane degradation isomers and form various toxic side products that the cell may prefer to keep outside (32, 42, 43).

The translocation of proteins to the periplasm is usually accomplished by employing endogenous protein translocation pathways. The vast majority of the bacterial proteins that function outside of the cytoplasm (secretory proteins) are synthesized with an N-terminal signal sequence that directs the protein to a specialized membrane-embedded translocation machinery. This translocation system facilitates the vectorial transport across the cytoplasmic membrane (7). A large number of studies show that *E. coli* possesses two distinct translocons, Sec and Tat, in addition to several highly specialized mechanisms of pathogenic *E. coli* strains for the secretion of virulence factors (7, 8, 26). Most secretory proteins are exported by the Sec translocon in an unfolded state (7). In contrast, the Tat translocon is able to translocate fully folded and often cofactor-containing secretory proteins (26).

Different strategies have been presented for the export of recombinant proteins in *E. coli* and other bacteria, which usually revolve around fusing a signal sequence genetically to the target protein (54). However, in many cases this does not result in periplasmic export of the target protein and this can frequently be solved by attachment of a larger proteinaceous moiety that functions as an export signal, or secretion partner. Known examples of a secretion partner include a truncated variant of *Staphylococcus aureus* protein A and OsmY from *E. coli* (15, 41).

Here, we report on the subcellular localization of haloalkane dehalogenase (DhaA31) and its use as a novel export signal for generic periplasmic expression in *E. coli*. DhaA31 is a directed-evolution improved variant of the bacterial haloalkane dehalogenase DhaA (34). The latter has been identified in different strains of the Gram-positives *Rhodococcus* and *Mycobacterium*, as well as in the Gram-negative *Pseudomonas cichorii* where it is involved in the hydrolytic dehalogenation of xenobiotic aliphatic organohalogenes (37, 38). The enzyme belongs to the  $\alpha/\beta$ -hydrolase superfamily of proteins and catalyzes carbon-halogen bond cleavage via covalent catalysis, as shown by numerous biochemical studies and the crystallographic structure (33). When the histidine of the catalytic triad of such a haloalkane dehalogenase is replaced by mutagenesis, an inactive variant is obtained, in which the covalent alkyl-enzyme intermediate can be trapped (36) because the first half-reaction - dehalogenation and enzyme alkylation - still can proceed but the second half reaction - hydrolysis of the covalent intermediate is blocked (40). This property allowed the use of a further modified haloalkane dehalogenase in the HaloTag localization reporter system where the dehalogenase is genetically fused to a target protein yielding a hybrid that subsequently can be detected with a fluorescent alkyl halide pseudo-substrate (28).

Biochemical fractionation experiments that we report here show that DhaA31, which is considered to be a cytoplasmic enzyme involved in alkyl halide

detoxification, is present in the periplasmic space when expressed in *P. putida* and *E. coli*. Remarkably, DhaA31 does not contain a typical signal sequence and was not processed upon periplasmic export. Furthermore, we found that DhaA31 is able to facilitate the periplasmic export of two cytoplasmic model proteins, including an oxidase that was catalytically active in whole cells. Our data show that DhaA31 can be used as a platform for the periplasmic expression of different enzymes in a functional form, thereby opening avenues for use of this system in various biotechnological applications.

## Materials and methods

**Reagents, enzymes and sera.** Restriction enzymes were obtained from New England Biolabs (Beverly, USA). T4 DNA ligase and DNA polymerase were from Invitrogen. ECL Western-blotting detection reagent was from Amersham biosciences. Horseradish peroxidase was from Fluka. All other chemicals were supplied by Sigma and of analytical grade. Antisera against DnaK and DsbA were kindly provided by A. Mogk and H.D. Bernstein, respectively. Antiserum against AldO was described before (53) and antiserum against the hexahistidine tag was from Abcam.

**Strains, plasmids and growth conditions.** The *E. coli* strain TOP10 (Invitrogen) was used as a routine host for all plasmid constructs. *E. coli* strains MC1061 (6) and TOP10 were used for subcellular localization experiments and whole-cell conversions of xylitol. *P. putida* strains MC4 and MC4-5222 were used for subcellular localization studies. The latter strain is a genetically modified derivative of *P. putida* strain MC4 in which the dehalogenase gene (*dhaA31*) is integrated in the genome and expressed under the constitutive *dhlA* promoter of *Xanthobacter autotrophicus* (21).

For the expression of DhaA31, plasmids pIT31 and pIS31 were used, which are based on the medium copy vector pIT2 (46), and were described previously (Samin et al. in preparation). For fusing AldO and TrxA with DhaA31, we used a three-step PCR approach in which both model proteins were appended to the C-terminus of DhaA31 separated by a flexible linker peptide (Pro-Gly-Gly). In the first round, the TrxA coding sequence (accession number P0AA25) was obtained using genomic DNA from *E. coli* TOP10 as template, whereas the *aldO* gene was amplified from pBAD-mbpAldO as template (16). The final PCR products were digested with *NdeI/HindIII* and ligated into the corresponding sites of the medium copy plasmid pIT2 (46), yielding pIT2-DhaA31-TrxA and pIT2-DhaA31-AldO, respectively. Primer sequences are available on request and the nucleotide sequence of all constructs was confirmed by DNA sequencing (GATC Biotech, Germany).

For the expression of DhaA31 or the DhaA31-TrxA fusion, cells were grown to saturation overnight at 30°C (TOP10 and MC1061), or for 48 h at 17°C (*P. putida* MC4). Cells were grown as previously described for the expression of AldO, or the DhaA31-AldO hybrid (16). No inducer was included because sufficient expression of all constructs was obtained by un-induced background expression. Strain MC4-5222 was grown in minimal media containing 4 mM 2,3-dichloro-1-propanol as sole carbon source. All strains were routinely grown in Luria Bertani medium (LB, containing per l 10 g tryptone, 5 g yeast extract, 5 g NaCl) under aerobic conditions unless indicated otherwise. Where appropriate,



ampicillin (100 µg/ml) or tetracycline (12.5 µg/ml) was added to the culture medium.

**Cell fractionation.** *E. coli* or *P. putida* cells expressing DhaA31 or its derivatives were grown as described, 15-20 OD<sub>600</sub> units of cells were harvested and periplasmic extracts of these cells were obtained either by osmotic shock or cold shock procedure as described (17, 45). Protein concentrations of the different subcellular fractions were measured with Bradford reagent at 595 nm using bovine serum albumin as standard.

**Enzyme assays.** The dehalogenase activity of DhaA31 or CPA dehalogenase was analyzed by incubating an enzyme-containing sample with 5 mM 1,2,3-trichloropropane in 50 mM Tris-SO<sub>4</sub> buffer, pH 8.2, at 30°C. Dehalogenase activities were measured by determining halide release spectrophotometrically (Perkin-Elmer Lambda Bio40) at 460 nm after the addition of mercuric thiocyanate and ferric ammonium sulfate at different times (47). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 µmole of halide ion per min. The oxidase activity of cellular fractions and whole cells expressing AldO or DhaA31-AldO was determined by coupling the production of H<sub>2</sub>O<sub>2</sub> by AldO or its derivatives to a horseradish peroxidase-mediated oxidation of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid. The resulting pink adduct can be detected spectrophotometrically at 515nm ( $\epsilon_{515} = 26 \text{ mM}^{-1} \text{ cm}^{-1}$ ) (10). For the detection of oxidase activity, phosphate-buffered saline (pH 7.4) was used as assay buffer containing 0.1 mM 4-aminoantipyrine, 1 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 3 units of horseradish peroxidase, 5 mM xylitol, 0.01 and OD<sub>600</sub> units of cells. All experiments were performed at least in duplicate.

**SDS-PAGE and immunoblotting.** Samples of cellular fractions containing equal amounts of protein were analyzed on standard 12% SDS-PAGE gels followed by protein staining, or immunoblotting. Proteins were transferred to nitrocellulose membrane (Amersham Biosciences) using a semidry apparatus from Biorad. Immunodetection was performed essentially as described before (53).

**Ni<sup>2+</sup>-NTA agarose purification of DhaA31 and MALDI-TOF analysis.** His-tagged DhaA31 was isolated from an *E. coli* TOP10 periplasmic fraction by a one-step nickel-NTA procedure as recently described (52). To assess whether DhaA31 was processed during periplasmic export, the mass of the purified enzyme was determined by mass spectrometry on a MALDI-TOF/TOF 4800 Proteomics Analyzer (Applied Biosystems) in the range of m/z 600-4000, in positive ion mode, essentially as described (2).

**Sequence analysis.** The presence of a potential signal sequence was analyzed by using the Signal P and PSORT online prediction tools (9, 59). For the prediction of non-classically secreted proteins SecretomeP 2.0 was used (4).

## Results

**Periplasmic expression of haloalkane dehalogenase.** Dehalogenases catalyze the hydrolysis of the carbon-halogen bond of organohalides. Owing to this, these enzymes represent important potential tools for different applications such as, for

example, the removal of organohalides from soil and groundwater (20). Consequently, dehalogenases have been the focus of much research to engineer variants with improved properties as nicely illustrated by DhaA31. DhaA31 represents an optimized variant of the *Rhodococcus* haloalkane dehalogenase DhaA (19, 25) with drastically improved catalytic properties (19, 34).

Currently, we use DhaA31 in combination with *P. putida* MC4 as a basis to engineer a bacterial strain for bioremediation purposes. During these efforts we noticed that DhaA31 appeared in periplasmic fractions obtained by an osmotic shock procedure, which is rather unexpected because it is considered to be a cytoplasmic enzyme. We decided, therefore, to carefully assess the subcellular localization of DhaA31 in *P. putida* MC4. To this end, two different DhaA31 expression plasmids were constructed equipped with either a *trc* promoter (pIT31), which is an inducible promoter, or the *X. autotrophicus dhla* promoter (pIS31), which is a strong constitutive promoter for the expression of the *dhaA31* gene in MC4 (21). Importantly, pIT31 and pIS31 are medium copy plasmids (pBBR1 origin of replication (23)), resulting in a moderate overexpression of DhaA31 and were therefore chosen for all subsequent fractionation experiments to minimize the risk of artifacts related to overexpression levels.

Following growth at 30°C as described under Materials and Methods, MC4 cells expressing DhaA31 were harvested and initially subjected to the osmotic shock procedure to obtain a periplasmic and spheroplast fraction (which contains the cytoplasmic and total membrane fraction). The dehalogenase activity in these fractions was analyzed by measuring the halide release spectrophotometrically. As shown in Table 1 this revealed that in all strains tested the major portion of active DhaA31 was found in the periplasmic fraction. As a control to monitor the efficiency of the fractionation protocol, we analyzed the activity of the cytoplasmic enzyme 2-chloropropionic acid (CPA) dehalogenase (Samin et al. in preparation) in all fractions (Table 1). This showed that the activity of this enzyme was restricted to the spheroplast fraction, thereby indicating that the fractionation procedure was effective. Care must be taken with interpreting these results as the fractionation procedure used can have a profound influence on the experimentally determined subcellular localization of certain proteins, as shown by a recent proteomic study (18). Therefore, we studied the subcellular localization of DhaA31 again using another fractionation protocol based on the osmotic shock method, which yielded almost exactly the same results as with the cold shock procedure (data not shown).

Despite the moderate expression of DhaA31 from a medium copy vector, it could be possible that (only) overexpression of DhaA31 results in its periplasmic localization, e.g. due to leakage or aberrant behavior of highly overexpressed protein. Therefore, cells of strain MC4-5222 were included as an additional control. In this strain, the *dhaA31* gene has been inserted in the chromosome by transposon-mediated integration, which results in low expression of this enzyme. The specific haloalkane dehalogenase activity was 0.37 U/mg in MC4-5222 cell-free lysates obtained by sonication, as compared to 0.73 U/mg for extract of MC4(pIT31). As observed before, the major portion of active DhaA31 was found in the periplasmic fraction of MC4-5222 cells (Table 1) whereas the activity of CPA dehalogenase was almost exclusively found in the spheroplast fraction. Thus, the periplasmic localization of DhaA31 is not caused by its overexpression and the results strongly indicate that DhaA31 is indeed transported to the periplasm in different expression constructs.

Encouraged by these results we next wished to confirm the periplasmic localization of DhaA31 by using further controls, including *E. coli* cells because most fractionation procedures have been optimized for this organism (14, 50). To this end, cellular fractions were prepared according to the osmotic shock method from *P. putida* MC4 or *E. coli* TOP10 expressing DhaA31 from pIT31 or pIS31, respectively. The cellular fractions derived from TOP10 and MC4 cells were subjected to SDS-PAGE analysis followed by protein staining of the gel (Fig. 1A). This showed that DhaA31 (indicated by an asterisk) was expressed in all strains and, moreover, a substantial enrichment of DhaA31 was observed in the periplasmic fractions, consistent with the results of Table 1.

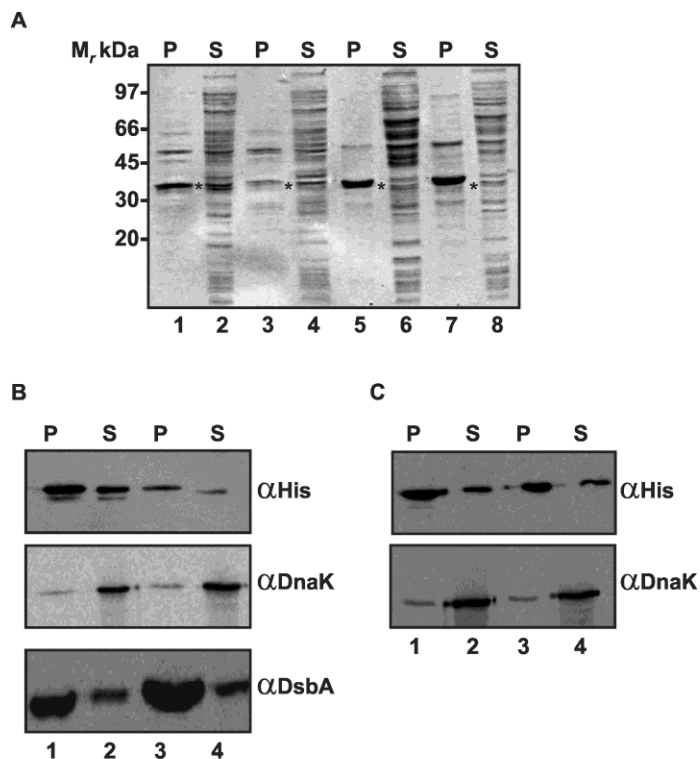
**Table 1.** Activity of DhaA31 and CPA dehalogenase in cellular fractions of *P. putida* MC4.

Strains <sup>b</sup>	Activity <sup>a</sup>			
	DhA31		CPA dehalogenase activity	
	Periplasmic fraction	Spheroplast fraction	Periplasmic fraction	Spheroplast fraction
MC4 (pIT31)	2.20 (77)	0.66 (23)	0.17 (2)	8.64 (98)
MC4 (pIS31)	1.65 (72)	0.64 (28)	0.27 (6)	4.40 (94)
MC4-5222	0.10 (83)	0.02 (17)	0.05 (3)	2.25 (97)

<sup>a</sup> Activity is expressed as specific activity and (%).

<sup>b</sup>pIT31: DhaA31 expression plasmid equipped with the *trc* promoter; pIS31: DhaA31 expression plasmid equipped with the *dhla* promoter.

To assess the presence of DhaA31 in the periplasmic fractions, we next analyzed all subcellular fractions by immunoblotting. To enable immunodetection in these experiments, DhaA31 was equipped with a C-terminal His affinity purification tag, allowing its identification by means of an antiserum against the His tag. When these samples were probed with a His antibody, a small amount of DhaA31 was detected in the spheroplast fractions whereas a significant fraction of DhaA31 was indeed observed in the periplasmic fraction of *E. coli* TOP10 and *P. putida* MC4 (Fig. 1B and C), thereby confirming our previous observations. To monitor the efficiency of the fractionation procedure, additional control blots were performed with antisera against DnaK or DsbA, which serve as a cytoplasmic or periplasmic marker, respectively. This showed that DnaK is mainly observed in the spheroplast fraction as expected and DsbA is predominantly detected in the periplasmic fractions of *E. coli* TOP10. Unfortunately, we were unable to detect DsbA in periplasmic fractions of *P. putida* MC4 using our DsbA antiserum against *E. coli* DsbA. Importantly, DnaK and DsbA are commonly used as fractionation markers by us and others in similar localization studies (35, 51-53). Combined, the results demonstrate that the fractionation procedure was efficient and, therefore, clearly show that DhaA31 is exported out of the cytoplasm to the periplasm.

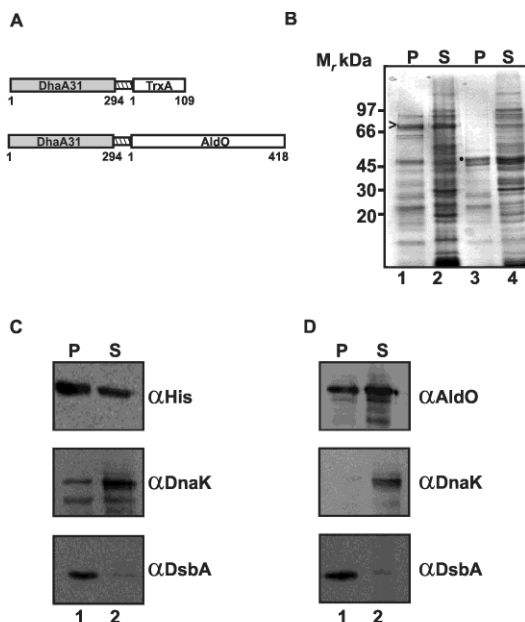


**Fig. 1.** Periplasmic localization of haloalkane dehalogenase. DhaA31 was expressed from two distinct expression plasmids in *E. coli* TOP10 or *P. putida* MC4. Plasmid pIT31 is equipped with a *trc* promoter, and pIS31 contains the *dhlA* promoter. Cells were grown as described under Materials and Methods and subsequently fractionated into a periplasmic (P) and spheroplast (S) fractions, according to the osmotic shock method. Samples were analyzed by SDS-PAGE and Coomassie staining (A). The position of DhaA31 is indicated by an asterisk. TOP10 transformed with pIT31; lane 1 and 2, or with pIS31; lane 3 and 4. MC4 transformed with pIT31; lane 5 and 6, or pIS31; lane 7 and 8. Immunoblot analysis of the periplasmic and spheroplast fractions shown in panel A from TOP10 (B) or MC4 (C), using the indicated antisera. DhaA31 produced from pIT31; lane 1 and 2, or from pIS31; lane 3 and 4.

**DhaA31 is exported independently of a cleavable signal sequence.** The vast majority of proteins that function outside of the cytoplasm are synthesized with an N-terminal signal sequence that is cleaved off upon translocation (7). Most established signal sequences are easily recognized in the primary sequence of a protein by online prediction tools such as SignalP and PSORT (9, 59). Therefore, we used these tools to investigate whether DhaA31 is equipped with such an established signal sequence. Remarkably, no signal sequence was predicted by these programs. Some proteins are exported out of the cytoplasm independent of a standard N-terminal signal sequence. Potential candidates of this group of such so-called non-classical secreted proteins can be identified by the online prediction tool SecretomeP (4). However, DhaA31 was not recognized by this algorithm as a secreted protein that does not require an N-terminal signal-sequence. To assess the absence or presence of a cleavable signal sequence experimentally, we determined the mass of DhaA31 purified from a periplasmic preparation of *E. coli* TOP10 by

MALDI-TOF mass spectrometry. If DhaA31 is produced as a precursor protein that possesses a signal sequence that is removed by proteolysis upon translocation (7), one would expect that the periplasmic form of DhaA31 has a significantly lower mass than the protein predicted by the gene sequence. However, the results of the MALDI-TOF analysis revealed that the mass difference of periplasmic DhaA31 and its theoretical mass was less than one amino acid, which does not correspond to removal of a standard signal sequence with an average length of 20 residues. This indicates that DhaA31 does not contain a cleavable signal sequence, thereby strongly suggesting that DhaA31 is exported signal sequence independently.

**DhaA31 as a platform for periplasmic expression.** Our finding that DhaA31 is exported to the periplasm of *E. coli* and *P. putida* independent of a cleaved signal sequence has interesting implications with respect to the potential use of this enzyme as a platform for the periplasmic expression of other proteins. Therefore, we investigated the ability of DhaA31 to facilitate the periplasmic export of two different cytoplasmic model proteins, namely thioredoxin (TrxA) and alditol oxidase (AldO). TrxA is a small (ca. 15 kDa) cytoplasmic endogenous *E. coli* protein (13). AldO is a carbohydrate oxidase of 45 kDa from the bacterium *Streptomyces coelicolor* and contains covalently bound FAD as cofactor (11, 16). Both model proteins were genetically fused to the C-terminus of DhaA31 with a His tag and flexible linker peptide (Pro-Gly-Gly) in between, resulting in genes encoding hybrid DhaA31-TrxA or hybrid DhaA31-AldO, respectively (Fig. 2A). Both constructs were introduced into the medium copy vector pIT2 (see Materials and Methods) and transformed into *E. coli*. Subsequently, a spheroplast and periplasmic fraction was prepared from cells producing DhaA31-TrxA or DhaA31-AldO according to the osmotic shock procedure. Next, the different cellular fractions were subjected to SDS-PAGE analysis followed by protein staining of the gel to establish the expression of the constructed hybrids (Fig. 2B). This showed that DhaA31-TrxA and DhaA31-AldO were expressed efficiently and migrated in the gel at a position corresponding to their expected size, i.e. 49 kDa for DhaA31-TrxA (Fig. 2B, lanes 3 and 4, indicated by a closed circle), and 80 kDa for DhaA31-AldO (lanes 1 and 2, indicated by an arrowhead). Interestingly, a considerable fraction of both chimeras was observed in the periplasmic fraction (lanes 1 and 3).



**Fig. 2.** DhaA31 as a platform for generic periplasmic expression. (A) Schematic representation of the TrxA (DhaA31-TrxA) and AldO (DhaA31-AldO) fusion constructs used in this study. The constructs comprise the first 294 amino acids of DhaA31, a short sequence of 9 residues (hatched) containing a his tag and a flexible linker and amino acids 1-109 of TrxA, or residues 1-418 of AldO. (B) Analysis of subcellular localization. The DhaA31-TrxA and the DhaA31-AldO hybrid were expressed in wild-type *E. coli* cells as described under Materials and Methods and subsequently fractionated into a periplasmic (P) and spheroplast (S) fraction, according to the osmotic shock procedure. Samples were analyzed by SDS-PAGE and Coomassie staining. The position of DhaA31-AldO (lane 1 and 2) is indicated by an arrowhead and the position of DhaA31-TrxA (lane 3 and 4) is indicated by a closed circle. Immunoblot analysis of the DhaA31-TrxA (C) or DhaA31-AldO (D) content in the fractions shown in panel A, using the indicated antisera.

To confirm the presence of DhaA31-TrxA and DhaA31-AldO in the periplasmic fractions, we performed an immunoblotting experiment using an antibody against the His tag for detection of DhaA31-TrxA (Fig. 2C), or an antiserum against AldO for the identification of DhaA31-AldO (Fig. 2D). This revealed that a substantial amount of both hybrids was present in the periplasmic fractions, thereby verifying the results from the SDS-PAGE analysis. Moreover, a considerable fraction of DhaA31-TrxA and DhaA31-AldO was also observed in the spheroplast fraction. There was no apparent degradation of the TrxA chimera but the AldO hybrid showed some degradation, suggesting that DhaA31-AldO may be less stable than DhaA31-TrxA and may be prone to proteolytic degradation. As an additional control to monitor the efficiency of the fractionation procedure, we analyzed the levels of the cytoplasmic marker DnaK and the periplasmic protein DsbA in the same samples by immunoblotting. This showed that DnaK was mainly observed in the spheroplast fraction whereas a substantial amount of DsbA was detected in periplasmic fraction (Fig. 2C, D), showing that the fractionation procedure was effective. Therefore, these data show that two vastly different proteins, TrxA and AldO, can be successfully transported to the periplasm by fusing them to DhaA31.

**Application of the DhaA31-AldO hybrid in whole-cell biocatalysis.** Although the results described above emphasize the potential of DhaA31 as a platform for periplasmic expression, they do not reveal whether TrxA or AldO are translocated in an active form. With regards to this issue it is interesting to note that a strong fluorescent band was observed in the spheroplast and periplasmic fraction of cells expressing DhaA31-AldO when the SDS-PAGE gel shown in Fig. 2 was briefly incubated in acetic acid and placed under UV light prior to protein staining (data not shown). This indicates that DhaA31-AldO contains covalently bound FAD and, therefore, suggests that it is folded into its active conformation. Inspired by this finding we investigated the activity of DhaA31-AldO in more detail to establish if the system can be used in biotechnological applications. Therefore, we analyzed whether wild-type *E. coli* cells expressing the DhaA31-AldO hybrid could be employed in the conversion of xylitol. Xylitol represents the preferred substrate of AldO and is converted into D-xylose with the concomitant production of H<sub>2</sub>O<sub>2</sub> (16, 56). The formation of the latter can be monitored spectrophotometrically by using a peroxidase-mediated reaction in which the H<sub>2</sub>O<sub>2</sub>, inherently formed upon oxidase activity, reacts to form a colored product.

**Table 2.** Conversion of xylitol by *E. coli* cells expressing AldO or DhaA31-AldO fusion protein.

Protein	Toluene permeabilized	Whole cell activity ( $\mu\text{M}\cdot\text{min}^{-1}\cdot\text{OD600}^{-1}$ )
AldO	-	0
	+	15.2 $\pm$ 1.4
DhaA31-AldO	-	5.3 $\pm$ 0.2
	+	10.5 $\pm$ 1.1

Table 2 shows that control cells, expressing cytoplasmic AldO, did not show significant conversion of xylitol, suggesting that no substantial lysis of the cells has occurred under these conditions. However, xylitol was readily converted by these cells upon permeabilization with toluene. This shows that cytoplasmic AldO is unable to react with xylitol and suggests that xylitol does not readily pass the cytoplasmic membrane, as noted before (53). Cells expressing the DhaA31-AldO hybrid were able to convert xylitol efficiently without permeabilization, indicating that xylitol was available to the DhaA31-AldO hybrid, in agreement with the presence of the protein in the periplasm. After toluene treatment, these cells displayed moderately improved oxidase activity.

These data show that DhaA31 is able to facilitate the periplasmic export of AldO in an active form as judged by the activity of cells expressing the DhaA31-AldO hybrid towards xylitol. Thus, DhaA31 can be applied as a vehicle for the periplasmic expression of AldO in a functional form, indicating its potential use as a platform for generic periplasmic expression of active enzymes.

## Discussion

Expression of target proteins in the periplasm of Gram-negative bacteria can have major advantages over cytoplasmic expression as, for example, it improves protein

stability and reduces proteolytic degradation. In case of whole-cell biocatalysis, periplasmic expression of enzymes that form toxic and/or reactive side products may reduce cellular toxicity. Moreover, the purification of proteins expressed in the periplasm can be done with a simple extraction procedure (12, 30). To obtain periplasmic expression of a target protein, it has to be exported out of the cytosol across the cytoplasmic membrane, which is usually achieved by fusing a signal sequence to the protein of interest in order to funnel it into a distinct protein translocation pathway (54). In many cases, however, this approach does not result in the periplasmic export of the target protein, which can frequently be solved by fusing it to a so-called secretion partner, which functions as an export signal. A secretion partner is larger than a signal sequence and often comprises a full-length protein or a truncated variant thereof, such as *S. aureus* protein A and OsmY from *E. coli* (15, 41). In the work reported here we show that DhaA31 can be utilized as a platform for periplasmic expression. Specifically, data are presented suggesting that: (i) DhaA31 is localized to the periplasm; (ii) it does not contain a typical signal sequence and is not processed upon translocation; and (iii) DhaA31 is able to facilitate the periplasmic export of TrxA and AldO, two unrelated cytoplasmic enzymes, which were genetically fused to the C-terminus of DhaA31. Moreover, the DhaA31-AldO hybrid was successfully applied in the whole-cell conversion of xylitol.

DhaA31 is an optimized variant of DhaA containing 6 altered residues that are part of its main access tunnel towards the active site (34). Furthermore, DhaA31 is equipped with a His affinity purification tag. It is therefore conceivable that the translocation of DhaA31 may be caused by these mutations or the His tag. To exclude these possibilities, we performed an additional control experiment, showing that recombinant wild-type DhaA containing a His tag is also localized to the periplasm, similar to a DhaA31 variant lacking a His tag (data not shown). Combined, this shows that the translocation of DhaA31 is not an indirect effect caused by mutations or a His tag; rather, this appears physiologically relevant. If the function of DhaA translocation to the periplasm indeed is related to a preference for keeping the metabolism of reactive substrates and products outside the cells is at present unclear. The DhaA-type haloalkane dehalogenase has been detected in various different host strains (see Introduction), and can function in the metabolism of diverse compounds such as 1-halo-*n*-alkanes, 1,2-dibromoethane, 1,3-dichloropropylene, and maybe other compounds (37, 38).

Our data indicate that DhaA31 is transported to the periplasm in a signal sequence-independent fashion. This is not unprecedented as several examples of secretory proteins that are translocated signal sequence independently have been described previously, including the unrelated dehalogenase LinA and the  $\alpha/\beta$ -hydrolase fold haloalkane dehalogenase LinB mentioned above (1, 22, 24, 31, 44). Furthermore, an increasing number of these proteins are discovered by recent proteomic studies analyzing the secretome of different bacteria (18, 22, 27, 58), giving rise to the term non-classical protein secretion as opposite to classical, signal sequence-dependent export (5). Non-classically secreted proteins can be identified by the online prediction tool Secretome P (4). Remarkably, DhaA31 and both LinA and LinB are not recognized by this algorithm despite their experimentally verified periplasmic localization (31). This may suggest that the export of DhaA31 does not depend on specific information present in its primary sequence. However, when we fused the signal sequence of DppA, a periplasmic protein of *P. putida* MC4, to the N-terminus of DhaA31 its export was blocked in MC4 as well as in *E. coli* (details



not shown). This shows that periplasmic export of DhaA31 can be impaired by N-terminal modification, indicating that its export is sequence specific, and thereby ruling out non-specific export mechanisms such as leakage.

It is well established that in the case of classical protein export, secretory proteins are directed to either the Sec or Tat translocon by virtue of their N-terminal signal sequence (7). Moreover, the wealth of genetic, biochemical and structural data for these systems has resulted in a detailed mechanistic understanding of signal sequence-dependent protein translocation (7). In contrast, this information is not available for non-classically secreted proteins, raising the question what mechanism is employed for their translocation? With regards to this question it is interesting to note that accumulating evidence indicates that both the Sec and Tat translocon are involved in signal sequence-independent translocation as well. Firstly, the *E. coli* Tat translocon is able to translocate multi-subunit complexes of which one subunit contains a signal sequence, which is sufficient for translocation of the entire complex by a so-called hitchhiker mechanism (45). Secondly, a recent study showed that *Rhizobium leguminosarum* bv. *viciae* SodA, a periplasmic protein devoid of a classical signal sequence, was exported by a SecA-dependent mechanism (24). These recent findings may point towards a prominent role of established translocation machineries in non-classical protein secretion. This notion is supported by our results obtained with two unrelated cytoplasmic enzymes, TrxA and AldO, as model proteins to explore the ability of DhaA31 to facilitate the periplasmic export of passenger proteins. TrxA is a small (~ 15 kDa) cytoplasmic endogenous *E. coli* protein and is involved in maintaining the redox balance of the cytoplasm (13). AldO is a carbohydrate oxidase of 45 kDa from the bacterium *S. coelicolor* and contains covalently bound FAD as cofactor (11, 16). Recent translocation studies showed that TrxA can be exported functionally to the periplasm via the cotranslational SRP-dependent pathway as well as Tat-dependently probably because the rapid folding of TrxA prevents its post-translational export (29, 48). In contrast, we have shown that AldO can exclusively be transported in a functional form Tat-dependently to the periplasm because of its covalently bound FAD cofactor, which is autocatalytically incorporated during protein synthesis (53). Therefore, the ability of DhaA31 to translocate TrxA and, more specifically, AldO in a functional form to the periplasm points towards a Tat-dependent mechanism. This may suggest that DhaA31, and possibly other non-classically exported proteins, utilize the Tat pathway. Attempts to elucidate the export mechanism of DhaA31 by examining its translocation in *E. coli* cells with an impaired Tat or Sec pathway were inconclusive (data not shown) and, therefore, a novel translocation mechanism for DhaA31 cannot be excluded at present.

The ability of DhaA31 to facilitate the translocation of TrxA and AldO, which represent two structurally different enzymes, emphasizes the remarkable tolerance and flexibility of DhaA31 as a platform for the generic periplasmic export of heterologously expressed proteins. In addition, periplasmic expression is a frequently used strategy in the design of whole-cell biocatalytic systems as it dramatically improves substrate accessibility of the relevant enzymes (54). Our data illustrate this by showing that wild-type *E. coli* cells expressing the DhaA31-AldO hybrid are able to convert xylitol, whereas cells expressing wild-type AldO cannot.

The commercially available HaloTag reporter system uses another derivative of the DhaA dehalogenase as a marker protein in localization studies

(28). Due to the removal of the histidine that forms the base in catalytic triad, the covalent intermediate formed during the first half-reaction of the catalytic cycle is trapped. This way, the enzyme can capture fluorescent groups that are bound to a substrate moiety via a linker, and a genetic fusion to a target protein can report on the localization of the fusion when examined by fluorescence spectroscopy. Our unexpected finding that DhaA31 is a periplasmic protein may have ramifications for this system. It would be interesting to investigate whether the DhaA variant employed in the HaloTag system behaves similarly as DhaA31 and to explore if the peculiar localization behavior of the dehalogenase appears in a broader range of organisms.

In summary, our data show that DhaA31 is exported to the periplasm signal sequence independently and can be exploited as a generic platform for the heterologous expression of biotechnologically relevant enzymes in the periplasm. This opens avenues for the use of this system in various biotechnological applications such as whole-cell biocatalysis as shown in the present study.

## Acknowledgements

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## **Chapter 5**

### **Remediation of 1,2,3-trichloropropane-contaminated water by genetically engineered bacteria in a packed bed bioreactor**

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## Abstract

Trichloropropane is an emerging groundwater contaminant that is highly toxic and recalcitrant to biodegradation under aerobic conditions. The applicability of *Pseudomonas putida* MC4 strains that are genetically engineered to degrade TCP was investigated with a continuous flow packed-bed bioreactor. In these strains, an improved haloalkane dehalogenase is inserted in the chromosome, enabling hydrolytic conversion of TCP to 2,3-dichloro-3-propanol, which serves as a growth substrate for strain MC4. The reactors contained ceramic rings or sintered porous glass as packing material, and were inoculated with a suspension of precultivated cells to develop a biofilm. The extent of degradation was analyzed by measuring the effluent concentrations of TCP and 2,3-dichloropropanol (DCP) and the release of chloride ions. At low dilution rate, efficient removal was observed but when dilution rate was enhanced, removal efficiency decreased. A bioreactor inoculated with a mixed culture of different variants of engineered TCP-degrading bacteria and an additional dichloropropanol degrading strain showed improved performance with continuous 95% TCP removal at 23 h residence time over a period of 4 weeks.

## Introduction

During recent decades, research on the biodegradation of short chain haloaliphatics was mainly focused on chloromethanes, chloroethanes and chloroethenes, which are industrially produced in large amounts and often encountered as environmental contaminants. However, so far too little attention has been paid to the biodegradation of 1,2,3-trichloropropane (TCP), which is another non-natural, toxic and recalcitrant chemical (30, 33). It is used as an intermediate in chemical production, as a solvent, paint remover and degreasing agent. It is also formed as a byproduct during the industrial synthesis of epichlorohydrin, a chemical that was widely used to prepare adhesives, coatings, epoxy resins, etc. TCP is introduced into the environment mainly by accidental release or improper waste disposal and it is found as a groundwater pollutant in different parts of the United States (2). Long-term exposure to TCP may cause kidney failure, body weight reduction, and can induce formation of tumors in experimental animals. The Guidelines for Carcinogen Risk Assessment reported that TCP likely is likely carcinogenic to humans (30).

Remediation of TCP-contaminated sites is difficult due to its chemical stability, recalcitrance to biodegradation, and its unfavorable physiochemical properties (28). TCP can be converted by chemical reactions that involve oxidation, reduction, substitution, or hydrolysis (21) but complete removal is troublesome. A biological treatment process could be an efficient and cheap strategy for the purification of TCP contaminated water, but there are very few reports describing biodegradation (5, 15, 35). No naturally occurring bacteria are known to degrade TCP under aerobic conditions. Bosma et al. (2002) constructed TCP-degrading recombinant bacteria, which were able to grow slowly when TCP was added as the sole carbon source. The degradation capacity is based on the introduction of a TCP-hydrolyzing haloalkane dehalogenase into a strain of *Agrobacterium radiobacter* (now called *Agrobacterium tumefaciens*) that slowly grows on 2,3-dichloropropanol (5).

This first TCP-degrading strain was not optimal for environmental application for several reasons: 1) the growth rate and degradation kinetics are



slow, probably due to the modest activity of the improved haloalkane dehalogenase; 2) there is no good match between the enantioselectivity of the haloalkane dehalogenase (which produces mainly (*S*)-2,3-dichloro-1-propanol and the host organism, which prefers the (*R*)-enantiomer; 3) the vector system used for introduction of the improved dehalogenase gene into the host strain is plasmid-based, mobilizable (although not self-transmissible), and carries a tetracycline antibiotic resistance marker. The use of organisms with a transmissible antibiotic resistance gene in an open environmental application is undesirable.

Recently, we succeeded in engineering new recombinant organisms for TCP biodegradation, in which most of these issues are partially solved. First, a better haloalkane dehalogenase was used (DhaA31,17). Second, the improved haloalkane dehalogenase gene is inserted into the chromosome without an antibiotic resistance marker using a transposon delivery system (7). Third, the new host, a strain of *Pseudomonas putida* called MC4, has an enhanced capacity to degrade 2,3-dichloropropanol and there is no selectivity for one of the enantiomers (Samin et al. manuscript in preparation). In view of these improvements, it is tempting to test the new recombinant strains for removal of TCP in laboratory-scale continuous-flow bioreactors in order to evaluate the possibilities and limitations of application for groundwater cleanup, and to identify which further improvements in strain development are required.

The organisms tested in this study are *P. putida* MC4-5222, MC4-5221 and MC4-1331 (Samin et al. submitted for publication). They are derived from the same parent strain, *P. putida* MC4, which was enriched on 2,3-dichloro-1-propanol as sole carbon source. The derivatives carry a chromosomal insertion of the improved DhaA31 variant of the *Rhodococcus erthropolis* haloalkane dehalogenase (14, 17). This insertion was introduced with the help of a Tn5-derived transposon system (8). Using a resolvase (16), selection markers including the kanamycin-resistance gene, were removed. The only coding sequence introduced in this engineered organism is gene for the improved haloalkane dehalogenase. The wild-type version of this gene has been detected in various gram-positive and gram-negative bacteria, and can be easily retrieved from soil by enrichment with 1-chlorobutane or 1-chlorohexane (18, 19).

For the removal of chlorinated hydrocarbons from water and air, the use of immobilized cells in a packed-bed reactor is a commonly applied technology (9, 10, 27). Various biofilm reactor types were used successfully to treat wastewater or groundwater contaminated with chlorinated xenobiotic compounds (25, 29). In comparison with the use of suspended cells, immobilization of microorganisms on an inert support has a number of advantages, such as a higher biomass concentration, a higher tolerable hydraulic and substrate loading, a better protection of cells against toxic substances, and prevention of suspended biomass in the effluents. Furthermore, higher degradation efficiency and improved operational stability were reported for systems using immobilized cells (13).

Especially the work of Stucki et al. (25, 26) is of interest since their experiments were done with a reactor inoculated with a defined culture of 1,2-dichloroethane-degrading bacteria. Furthermore, this system was fully scaled up and used for the treatment of a 1,2-dichloroethane contaminated field site near Lübeck, Germany. The relevant physico-chemical properties of 1,2-dichloroethane and 1,2,3-trichloropropane are very similar, and it is justified to argue that once a good organism for aerobic biodegradation of TCP becomes available, the road to a full-scale treatment technology is straightforward. Since many prominent TCP-

polluted sites received the contaminant from epichlorohydrin-manufacture wastes, TCP occurs as the predominant pollutant in many cases, which would facilitate the competitive maintenance of a TCP-degrader in a bioreactor receiving groundwater from such a practical site.

In this study, mineralization of TCP by the engineered strain *P. putida* MC4-5222 is investigated. Continuous biological removal of TCP from an aqueous stream under aerobic conditions is demonstrated for the first time. However, residence times had to be kept low for efficient removal. When further engineered strains were inoculated into the reactor (MC4-5221, MC4-1331 and *Agrobacterium radiobacter* AD1 (32), performance improved. Up to 80-95% TCP was biologically removed at varying residence times with negligible air stripping over a period of three months.

## Materials and Methods

**Chemicals.** All chemicals used were of analytical grade and were supplied by Sigma, Aldrich or Merck. The Raschig rings were purchased from AceChemPack Tower Packing Co. and glass beads were ordered from ROBU-Glasfilter-Geraete GmbH.

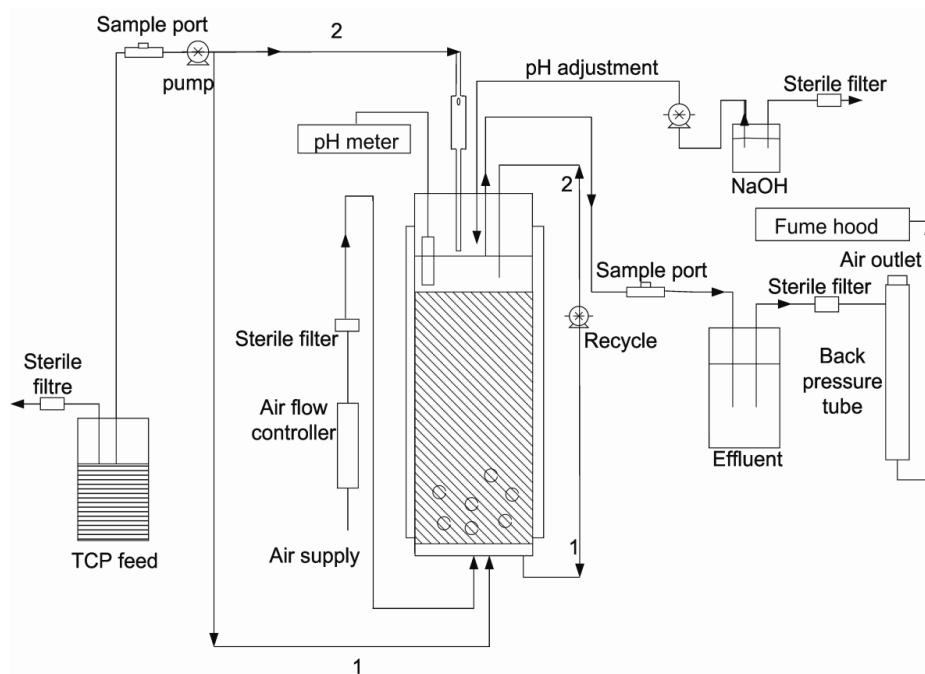
**Growth conditions and media.** The construction of *Pseudomonas putida* MC4-derivatives MC4-5222, MC4-5221 and MC4-1331 is described elsewhere (Samin et al. 2011, submitted for publication). *Agrobacterium radiobacter* AD1 is able to grow on 1,3-dichloro-2-propanol and slowly degrades 2,3-dichloro-2-propanol (32). The MC4-derivatives were grown on TCP (0.1-1 mM) at 30°C in a medium (MMY) that contained (per liter): 5.4 g of Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 1.4 g of KH<sub>2</sub>PO<sub>4</sub>, 0.5 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.2 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 ml of trace element metal solution and 5 mg of yeast extract (11). To obtain growth, 0.5 mM TCP was initially added and batch cultures of MC4-derivatives were incubated at 30°C with shaking. With intervals of a few days, 0.5-1 mM TCP was added until the OD<sub>600</sub> was 0.4-0.5. The cells from a 4 l culture were collected by centrifugation at 5,000 rpm for 20 min, resuspended in 700 ml 50 mM phosphate buffer, pH 7.2, and then introduced into the reactor. Strain *A. radiobacter* AD1 was grown on LB medium at 30°C (32).

**Bioreactor setup.** The reactor was a double-walled cylindrical glass vessel with a volumetric capacity of 1.3 l and working volume of 0.7 l. Sampling ports were present at the top, the bottom, and at the middle of the vessel. Two reactor setups were used, which were same in their design except for the TCP inlet, recirculation method, and type of packing material. In reactor setup 1, the reactor was filled with 300 g ceramic Raschig rings as packing material and the TCP inlet was positioned at the bottom of the reactor, thus achieving an upward flow through the reactor. Water was removed from the top of the reactor for recirculation, analysis, and effluent discharge. In setup 2, the packing material consisted of sintered glass beads (1.5 mm), and the TCP inlet was at the top. Recirculation was from the opposite side of the TCP inlet. The sampling port for analysis and effluent discharge were at the top of the reactor. A process flow diagram of both reactor setups is shown in Fig. 1. In both cases, part of the water stream from the outlet was recirculated using a peristaltic pump operated at 1 to 25 ml/min, as shown in the figure. For both reactors, the pH was monitored and controlled at pH 7 with 0.1 M NaOH. The temperature was maintained at 30°C with the help of an outer heating jacket. Viton

fluoroelastomer tubing was used in the whole system to minimize TCP loss by evaporation or diffusion. The reactors were operated under non-sterile conditions.

The tendency of the support material to adsorb substrate such as TCP and DCP and to release chloride ions was measured in duplicate shake-flask experiments. Each flask contained MMY medium (50 ml), substrate and support material, and was incubated at 30°C for four weeks. After this, the concentration of substrates by analyzed by gas chromatography and release of chloride ions by colorimetric analysis as described below.

**Biofilm formation.** The Raschig rings and glass beads were washed twice with 50 mM phosphate buffer (pH 7.2) and sterilized by autoclaving. A 700 ml suspension of TCP-grown cells was introduced in the reactor, which was left for 4 days with no supply of TCP. During this period, air was introduced at a rate of 1 ml/ min in the form of bubbles from the bottom of the reactor and the temperature was kept at 30°C. Four days after inoculation, addition of TCP (1-1.5 mM) was started at 0.1 ml/min. To evaluate the performance of the reactor, TCP inlet concentrations were varied from 0.1 to 0.3 mM, inlet flow rates were varied from 0.1 to 2 ml/min, and recirculation was varied from 1 to 25 ml/min.



**Fig. 1.** Process flow diagram of packed-bed bubble column bioreactor. In reactor setup 1, Raschig rings were used as biofilm support material with TCP feed in upward direction (1) and in reactor setup 2 sintered glass beads were employed as carrier material with TCP inlet downwards (2).

**Sampling and analysis.** Syringes were used to take 5 ml samples from the outlet and inlet for TCP, DCP and chloride measurements (Fig. 1). Concentrations of TCP and DCP were determined by gas chromatography as follows: samples of 4.5 ml were extracted with diethyl ether containing 0.05 mM mesitylene as internal

standard. Samples of 2  $\mu\text{l}$  were analyzed on a HP5 column (25 m  $\times$  0.25 mm  $\times$  0.2  $\mu\text{m}$ , Agilent Technologies) installed in a gas chromatograph equipped with a flame ionization detector. The carrier gas was nitrogen (50 kPa), and the temperature program was 5 min isothermal at 60°C followed by an increase to 110°C at a rate of 2°C/min and then to 130°C at a rate of 15°C/min.

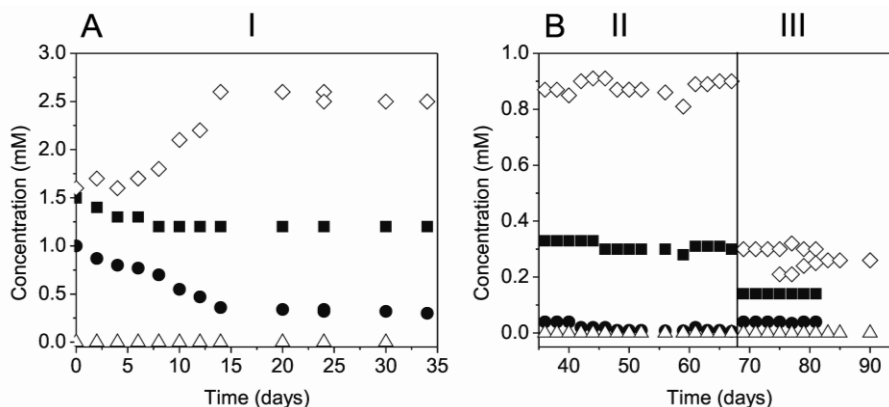
Chloride concentrations were measured colorimetrically at 460 nm after the addition of mercuric thiocyanate and ferric ammonium sulfate as described (4).

## Results and Discussion

**Packed-bed reactor.** To study the continuous removal of TCP by the engineered recombinant bacteria, we opted for bioreactor system with a packing material to which the cells can attach and form a biofilm. As packing materials, ceramic (Raschig) rings and sintered glass beads were tested, since both have been used previously for the removal of synthetic contaminants in fixed film reactors, including chlorinated hydrocarbons (23, 25, 27, 34). To measure the possible adsorption of TCP and DCP by ceramic rings and sintered porous glass beads, we followed the procedure described under Materials and Methods. We found that neither packing material adsorbed TCP or DCP or released chloride ions to a significant extent.

**Biofilm development and TCP degradation in reactor 1.** Attachment of cells was started by incubating the reactor with a cell suspension of *P. putida* strain MC4-5222. The cells were prepared in 50 mM phosphate buffer as described in Materials and Methods and the support material was also prewashed with 50 mM phosphate (10). Cells saturated with phosphate are more hydrophobic and have a higher tendency to flocculate and attach to surfaces (6). After inoculation, the system was left for 4 days without TCP supply since it was reported that attachment of cells can proceed faster when substrate is completely consumed (9). The temperature was kept constant at 30°C, which is optimal for the *Pseudomonas* strain used, since it has been reported that cultivation at the optimal growth temperature can increase biofilm formation, e.g. through production of extracellular polymeric substances (EPS) involved in surface adhesion (1).

After four days, TCP supply (1.2 - 1.5 mM) was started at low dilution rate (0.1 ml/min, residence time 116 h) to allow cell growth and an increase of biofilm size. The effluent concentrations of TCP, DCP and chloride ions were measured with different time intervals by GC-FID and colorimetric analysis, respectively. Effluent concentrations are the same as concentrations in the reactor since due to the high recirculation rate (5 ml/min) as compared to the feed rate (mostly 0.1 ml/min), the overall reactor contents can be considered as ideally mixed, although there may be local variations in TCP levels close to the inlet port.



**Fig. 2.** Degradation of TCP by genetically engineered *P. putida* MC4-5222, reactor setup I with cells immobilized on ceramic rings. A) Reactor startup and TCP removal at high initial concentrations with dilution rate 0.008; B) TCP removal at different steady states with varying TCP inlet concentrations and dilution rates. Symbols: ■, TCP influent; ●, TCP effluent; △, DCP; ◇, chloride.

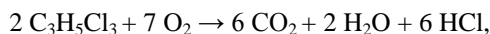
The analytical results indicated that the immobilized genetically engineered MC4-5222 cells could remove TCP over a prolonged period of time (90 days). In the beginning, the degree of removal was around 33%, and it gradually improved to 75% TCP removal (Table 1, Figure 2A). The data also showed that TCP was completely degraded since DCP was never detected as a product (detection limit 0.01 mM) and chloride concentrations in the effluent increased stoichiometrically. The increasing degree of removal during the first 14 days of operation indicated that the engineered cells were growing within the column and stayed in the reactor (Fig. 2). Both TCP removal and chloride release continued over a 20-day period indicating that a stable degradation process was achieved due to immobilized organisms. Slight amounts of biomass appeared in the effluent collection vessel. However, after the initial startup period of 15 days, the degree of removal by strain MC4-5222 did not increase a lot anymore and (less than 5% further increase) under these reactor operation conditions.

Experiments were continued with a lower TCP concentration in the influent (0.33 mM)(Fig. 2, phase II). To prevent stripping of TCP via the exit air, the airflow was kept low throughout all experiments (0.5-1.5 ml/min) and the TCP solution (0.33 mM) was supplied at low feed rates (ca. 0.1 ml/min). This raised the question if oxygen supply might be limiting. In view of the TCP Henry coefficient ( $H=3.2-3.4 \cdot 10^{-4}$  atm·m<sup>3</sup>/mol, which converts to a dimensionless Henry coefficient (or partitioning coefficient) of  $[TCP]_g/[TCP]_l = 0.012$ ) and the air flow rate, the amount of TCP that could leave the reactor via the gas phase is calculated from:

$$TCP_{out,g}/TCP_{out,l} = F_{gas} * [TCP]_g / F_l * [TCP]_l, \text{ and} \\ [TCP]_g/[TCP]_l = 0.012$$

This suggests that at the conditions with the strongest aeration (liquid flow rate of 0.1 ml/min, air flow rate of 1.5 ml/min) still only 18% of the TCP could leave the reactor via the gas phase as compared to the TCP that leaves via the liquid phase. At residence times of 116 h and 23 h, this value was 2.4% and 0.6% respectively.

Assuming that TCP degradation is a completely aerobic oxidative process and biomass formation is negligible, TCP degradation can be described by;



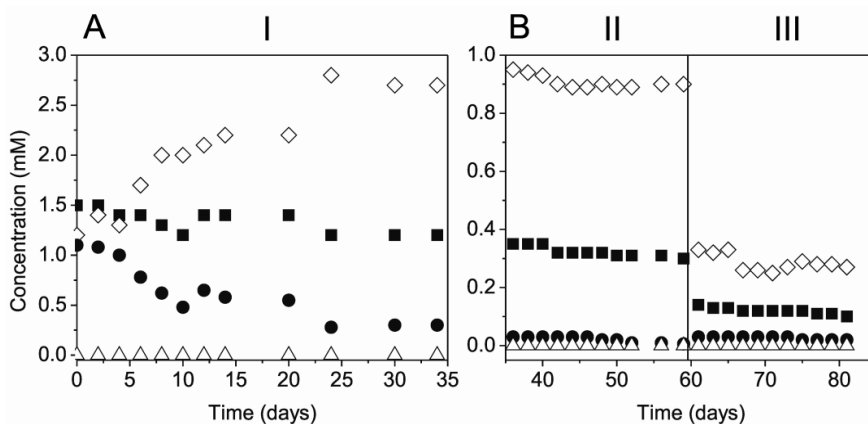
From this, it follows that the ratio between TCP feed ( $\mu\text{mole/ml}\cdot\text{ml}/\text{min}$ ) and air flow ( $\text{ml}/\text{min}$ ) should be lower than 1: 0.4. Since under the operation conditions used (TCP inlet 0.33 mM at 0.1 ml/min, lowest air flow 1.0 ml/min) this ratio is 2.5-fold higher, the system cannot be oxygen-limited. Thus, the modest performance in terms of % removal must have another cause.

To establish if TCP removal can be improved, we first tested variations in TCP loading and residence time in the reactor. These conditions could have an effect on biofilm growth and TCP degradation kinetics. Hydrodynamic conditions can influence biofilm density, which in turn will influence the diffusion of nutrients through the biofilm and thereby have an effect on reactor performance (3, 24).

In phase II, after decreasing the TCP feed concentration to 0.33 mM TCP at a residence time of 116 h, a lower level of TCP was found in the effluent and increased degree of TCP removal (87-93%). At day 48, mineralization of TCP had increased to 95-97% with only a few mg/l remaining in the effluent and with stoichiometric release of chloride ions. No DCP was detected in the effluent and the same results were seen in the reactor for about 67 days.

To test the possibility of TCP mineralization at a shorter water residence time, flow rates were increased from 0.1 ml/min to 0.5 ml/min and the TCP concentration was decreased from 0.33 mM to 0.14 mM (Fig. 2, Phase III). It was found that TCP removal efficiency was reduced to 70-80%. Apparently, the kinetics of the system did not allow a high hydraulic loading without losing performance in terms of removal efficiency. If degradation is in a first-order regime, which is not unlikely in view of the modest affinity of the dehalogenase for TCP, the removal efficiency is expected to decrease (22). After 90 days of operation, reactor 1 was stopped because of back growth of cells into the TCP feed solution, and it was decided to test a modified setup.

**Modified reactor setup: sintered glass packing and upward flow.** To test if improved performance could be obtained by changing the reactor setup, we decided to run the reactor with sintered glass beads as biofilm support and changed the substrate inlet to prevent back growth (reactor setup 2). Glass beads were found to be the best support material for mineralization of low-chlorinated biphenyls by *Sphingomonas* sp. (23) and trichloroethylene degradation using a pure culture expressing *ortho*-monooxygenases (27). Porosity of glass beads works well for the biofilm formation and inside the pores high fluid velocity has less effect on biofilm destruction (19). Furthermore, the time required for biofilm formation may depend on the type of carrier material used for immobilization (9).



**Fig. 3.** Reactor setup 2, employing a packed-bed bioreactor with sintered glass beads as biofilm support. Panel A shows TCP removal at higher concentration (1.1-1.5 mM) with constant dilution rate during startup. Panel B represents continuous degradation under different loading regimes. Symbols: ■, TCP influent; ●, TCP effluent; △, DCP; ◇, chloride. The detailed operation conditions of the system are given in Table 1.

Startup of reactor 2 proceeded in a similar way as in the previous experiment. In the beginning, TCP (1.2-1.5 mM) was supplied with flow rate of 0.1 ml/min. As shown in Fig. 3A, during the first 24 days, the outlet concentration gradually decreased from 1.1 to 0.28 mM, corresponding to a degree of removal of 75%. Various conditions, such as TCP feed rate and recirculation rate, were changed as mentioned in Materials and Methods. The reactor was run until 34 days with same TCP concentration (1.2-1.5 mM) but no further improvement was observed.

At day 34, the reactor was supplied with 0.35 mM TCP at the same flow rate of 0.1 ml/min and 116 h residence time. From that time on, 92-98 % TCP removal was observed. However, the water-loading rate was rather low so after 59 days, the flow rate was increased 5-fold (0.5 ml/min) to investigate if TCP removal continued at lower residence time. At the same time, TCP concentration decreased to 0.14-0.1 mM. At the new residence time of 23 h, TCP degradation reduced to 75-80% with stoichiometric release of chloride.

The results presented in Table 1 indicate that an increase of organic loading may have an inhibitory effect on the developed biofilm as reported earlier by Emanuelsson et al. (2006). With the decrease of organic loading when switching from phase I to phase II ( $1.0$  to  $0.26 \text{ mg h}^{-1} \text{ l}^{-1}$ ), the TCP removal efficiency of reactor 2 increased from 75 to 93% and a similar behavior was found in the reactor 1 filled with Raschig rings. However, when the organic loading was increased from 0.25 to 0.5 mg/l/h (phase II to phase III), in both types of reactor the decrease of removal efficiency was more than what we expected from the comparison of phase I and II. These results suggested that in addition to the effect of organic loading, a high flow rate of the TCP feed (phase III in both reactors) might have an inhibitory effect on biofilm performance and reduce the TCP removal capacity of the reactor. No back growth was observed in reactor 2 over the whole period.

**Table 1.** Summary of operation conditions used in continuous bioreactors 1 and 2.

Reactor	Phase	Days	Organic loading (mg TCP h <sup>-1</sup> l <sup>-1</sup> )	Residence time (h)	Removal efficiency (%)	Strains
1	I*	34	1.0-1.3	116	33-77	MC4-5222
	II	67	0.26-0.29	116	87-98	
	III	90	0.4-0.6	23	71-80	
2	I*	34	1.0-1.3	116	26-75	
	II	59	0.25-0.30	116	91-98	
	III	81	0.44-0.61	23	78-80	
	IV	121	0.40-0.44	23	75-95	MC4-5221, MC4-5221, MC4-1331, AD1
	V	137	1.74-1.76	06	50-51	

\*Phase I represents the time immediately after inoculation.

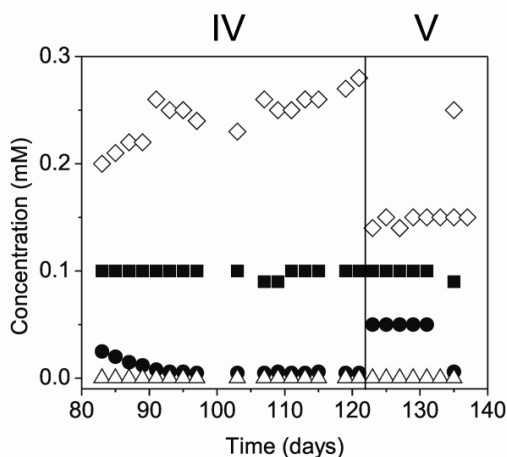
**Use of a mixed culture of TCP and DCP degraders.** During construction of the TCP degrading derivatives of *P. putida* strain MC4 by transposition, we observed that, for unknown reasons, a large fraction of the dehalogenase-positive derivatives lost their ability to grow on DCP. This raised the suspicion that the modest performance of the bioreactor at higher loading rates may be caused by some intrinsic weakness of the recombinant strain MC4-5222. Since independent transposition events should yield MC4 derivatives with the haloalkane dehalogenase gene integrated in different positions of the genome, we tested if inoculation with additional independently obtained recombinants could enhance performance. In addition, cells of *Agrobacterium radiobacter* AD1 were added to the reactor to afford biodegradation of any possible side products or excreted intermediates formed in the TCP and DCP degradation pathways. Van den Wijngaard et al. (1993) observed that the use of defined mixed culture, obtained by adding the 2-chloroethanol-degrader *Pseudomonas* GJ1, may strongly improve the performance of a 1,2-dichloroethane degrading culture of *Xanthobacter autotrophicus* GJ10 that was operated in a chemostat. In that case, cross-feeding with vitamins in return of secretion of the intermediate 2-chloroethanol stimulated growth of the mixed culture (31).

The performance of bioreactor 2 significantly improved after a mixed culture of MC4-5222, MC4-5221, MC4-1331 and *Agrobacterium radiobacter* AD1 was added to the reactor. At a residence time of 23 h, TCP removal efficiency was enhanced to 94% and only a low level of TCP was present in the effluent (Fig. 4, Phase IV). This indicates that the removal efficiency and overall stability of the TCP-mineralization process were not only influenced by the growth kinetics parameters of a particular strain but also by the presence of other species and possibly organic growth factors (31). With the mixed culture, continuous removal of TCP was achieved over a period of 20 days.

Next, while keeping the TCP concentration constant (0.1 mM), we decreased the residence time further to 6 h. Under these conditions, the TCP removal efficiency was only 50%. Thus, in addition to the effect of organic loading, the flow velocity of TCP affects the bioreactor performance. This may be



due to intrinsic kinetics of the TCP-degrading organisms or diffusion of substrates through the biofilm (24). Previously, it was observed that various environmental factors such as lack of availability of a suitable carbon source or some nutrient may hinder the success of inoculation (11, 36). It is well possible that unidentified factors (e.g. a vitamin) are required for strain MC4 derivatives to exhibit their full TCP degradation potential. In addition, a low concentration of TCP might limit cell growth and in the removal efficiency of the TCP-degrading biofilm. For example, if the decay rate of the organism would be high, the low substrate loading applied in our experiments would not suffice to obtain a continuous increase in active biomass in the reactor, which would influence the overall kinetics of the reactor.



**Fig. 4.** Degradation of TCP by a mixed culture of *P. putida* strains MC4-5221, MC4-5221, MC4-1331 and *A. radiobacter* AD1 in reactor 2 operated in a continuous mode. Symbols: ■, TCP feed; ●, TCP effluent; ◇, chloride release. Loading rates and residence times are mentioned in Table 1.

## Conclusions

In this study, the capacity of genetically modified organisms to mineralize TCP was employed to obtain, for the first time, continuous removal of TCP from an aqueous stream under aerobic conditions. Two different packed bed bioreactors in which TCP-degrading cells were immobilized were operated for more than 130 days under different conditions (Table 1), and both setups allowed removal of TCP up to 90%. TCP that was removed was essentially mineralized as no side products were detected and all organic chlorine that was removed was detected as inorganic chloride in the reactor effluent. Thus, the use of immobilized cells of a genetically modified strain such as MC4-5222 in a packed bed bubble column bioreactor may offer an effective way to cleanup TCP contaminated water.

Limitations are the high residence time that was required and remaining concentrations of TCP in the effluent (0.02-0.05 mM). At shorter residence time, removal efficiency decreased, for unknown reasons. We suspect that strain MC4 is not a very robust organism, as it was observed that during batch cultivation, the steady state growth phase was quickly followed by a phase in which the cell

density is reduced, suggesting that the organism may have a high intrinsic maintenance demand or decay rate. Thus, further improvement of the host may be needed for practical application. The low levels of TCP remaining in the bioreactor effluent may be removed in a practical situation with an activated carbon filter, as was also used during the full-scale treatment of 1,2-dichloroethane contaminated groundwater with a defined bacterial culture (26).

An intriguing observation was that with a mixed culture of TCP degrading genetically engineered derivatives of MC4 and an additional 2,3-dichloropropanol degrading organism (strain AD1), the performance of the reactor was remarkably improved. This suggests that composing an effective consortium should be included in the design of improved versions of the TCP removal process presented here.

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## **Chapter 6**

### **Summary and concluding remarks**

The use of microbes for the remediation of polluted sites is an attractive alternative to traditional methods, which are mostly based on extraction and separation. However, the feasibility of a biodegradation process depends on the availability of an organism able to degrade the environmental compounds that are present. The existence of a wide diversity of microorganisms that degrade synthetic chemicals, including xenobiotic organohalogenes, indicates that nature itself is able to partially solve the problem of increasing pollution. However, many synthetic halogenated compounds remain difficult to degrade by naturally occurring organisms in the environment (13, 33).

Among the group of chlorinated hydrocarbons, 1,2,3-trichloropropane (TCP) is recognized as an emerging contaminant. It is very recalcitrant towards degradation, suspected to be carcinogenic, and may cause several health hazards depending on the concentration and time of exposure (21). It is present as a contaminant in groundwater and drinking water at various places in the United States and Europe (1). The work described in this thesis is focused on the biodegradation of TCP under aerobic conditions with the aim to obtain an organism that can grow on TCP and mineralize it to innocent products.

Chapter 1 presents an overview of the properties of TCP and the possibilities for degradation and remediation. Clean-up of 1,2,3-trichloropropane contaminated water is difficult and challenging due to the physical properties of TCP and the limited possibilities of chemical and biological transformation (24, 25). Aerobic degradation is possible only with the methanotrophic bacteria and bacteria that possess a dehalogenase gene that catalyzes hydrolytic dehalogenation of TCP to dichloropropanol (4). The absence of a naturally occurring organism for aerobic TCP degradation indicates that perhaps the evolution process of microbes towards the degradation of this synthetic compound is quite slow. However, the availability of genetic tools allows us to boost the evolution process in the laboratory and to construct genetically engineered strains for biodegradation. The introduction also describes why improved organisms for TCP degradation will be useful. For application in environmental biotechnology, a TCP-degrading strain should have the following properties: 1) genetic stability, including lack of mobility of recombinant genes; 2) absence of antibiotic resistance markers; 3) fast and complete degradation and mineralization of TCP.

The first step towards the construction of an efficient TCP-degrading strain was the use of the improved TCP-hydrolyzing haloalkane dehalogenases that were obtained by Pavlova et al. (22) through directed evolution of an enzyme variant that was studied earlier by Bosma et al. (4). The best haloalkane dehalogenases, such as DhaA27 and DhaA31, exhibited a 36-fold higher activity than the natural enzyme towards TCP. We decided to use DhaA27 and DhaA31 for enhanced transformation of TCP into 2,3-dichloro-1-propanol (DCP). The next requirement was a host strain that can use DCP as sole carbon source.

**Isolation of 2,3-dichloro-1-propanol (DCP) degrading strain.** Chapter 2 describes a bacterial strain, called MC4, which was isolated from contaminated soil on basis of its capacity to utilize DCP. This Gram-negative, motile rod was able to grow aerobically on various other substrates as well, including sugars, alcohols and several halogenated compounds such as 2-bromoacrylic acid, 2,3-dichloropropanoic acid and 2,3-dichloro-1-propanol. The most important feature of strain MC4 was its growth on both the (*R*) and (*S*) enantiomers of DCP, which offers the possibility of complete degradation of DCP produced by DhaA31, which is a mixture of stereoisomers. The 16S rRNA gene sequence of strain MC4 has 99% identity to the 16S rRNA genes of different *Pseudomonas putida* strains, which classifies the organism as a strain of *P. putida*.

A novel dehalogenase, called DppA, was identified in cell extracts of strain MC4 grown on DCP. It converts DCP with liberation of two equivalents of chloride. The compound is first oxidized to 2-chloroacrolein, which is further transformed into 2-

chloroacrylic acid. The *dppA* gene was not homologous to halohydrin dehalogenase genes such as the *hheA*, *hheB* and *hheC* genes encoding enzymes of the short-chain dehydrogenase/reductase family (20, 32, 34). DppA showed dehydrogenase activity with DCP in a 2,6-dichlorophenolindophenol (DCPIP)-reduction assay or in an assay with ferricyanide as an artificial electron acceptor, thus indicating that the initial step in DCP conversion is both a dehydrogenase and a dehalogenase reaction. The enzyme simultaneously dechlorinates the substrate and transfers electrons to an acceptor during oxidation of the alcohol group.

The DppA sequence was used as a query in searches of the NCBI non-redundant protein database which led to several homologs annotated as quinoxinoprotein dehydrogenases (2). A sequence alignment of DppA with proteins of known structures indicated that the amino acids involved in the PQQ and calcium binding sites are mostly conserved. To our knowledge, DppA is the first PQQ-dependent protein that is attributed with a dehalogenase function.

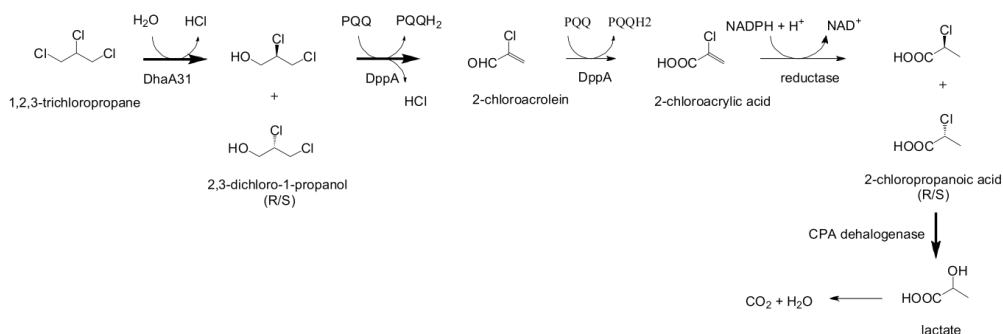
We propose in Chapter 2 that DCP dehalogenation by DppA takes place in two steps: 1) an aldehyde is formed through the dehydrogenase activity with immediate release of chloride through elimination, yielding 2-chloroacrolein; 2) the aldehyde is converted to the corresponding 2-chloroacrylic acid by a second dehydrogenase reaction. A somewhat similar reaction mechanism was proposed for the oxidative dechlorination of dichloropropanol with hydroxyacetone formation, as catalyzed by a flavoenzyme from *Alcaligenes* sp. DS-S-7G (15, 28).

Whether the DCP-dehalogenating dehydrogenase really is adapted to carry out dehalogenation reactions remains uncertain at this moment. Thus, we do not know if the enzyme possesses a halide-binding site that facilitates the elimination of HCl and double bond formation during or after the oxidation of the alcohol group of DCP. HCl elimination may also be non-catalyzed. Structural characterization of the enzyme and measurement of dehalogenase activities of DppA homologs that do not originate from dehalogenating organisms could provide further insight in this issue.

**Construction of genetically engineered strains for TCP degradation.** The low activity of the wild-type DhaA enzyme against 1,2,3-trichloropropane (TCP) is the main factor that limits the suitability of this enzyme for the constructing of a TCP bioremediation organism. In Chapter 3, we propose that if a set of enzymes for the complete mineralization of TCP is present in a single strain; such a strain should be able to grow on TCP as sole carbon source. To obtain such an organism, we first established the expression of the haloalkane dehalogenase variants DhaA27 and DhaA31 under two different promoters. The *trc* promoter (5) is an inducible (repressible) promoter in *E. coli* and the *dhlA* promoter (14) is a strong constitutive promoter in several gram-negative bacteria. Both were used in the broad host range vector pIT2 (16, 26) for driving dehalogenase production. It was found that the DhaA31 mutant showed the highest activity when expressed under the *dhlA* promoter (plasmid pIS31B), with a mutated *dhlA*-derived ribosome binding site, and the resulting recombinant strain *P. putida* MC4(pIS31B) was able to grow on TCP as sole carbon source.

In the degradation pathway of TCP, DhaA31 and DppA are the key enzymes (Fig. 2). The DhaA31 protein was expected to be a cytoplasmic protein because of the absence of any known signal peptide, whereas DppA clearly is a periplasmic protein with a signal peptide, as mentioned in Chapter 2. When we considered to improve the degradation properties of the recombinant strain, we assumed that the presence of both dehalogenating enzymes in the periplasm may enhance the TCP degradation by protecting the cytoplasm against 2-chloroacrolein, which is a functional toxic product, and by preventing useless translocation steps, i.e. uptake and export of TCP and DCP, respectively. To test if the co-localization of these

enzymes in the periplasm is beneficial, we made a fusion protein of DhaA with the signal peptide of the DppA protein. During this work, we noticed that even without the signal peptide added the DhaA31 protein was transported to the periplasm. The details of this work are described in Chapter 3 (see below).

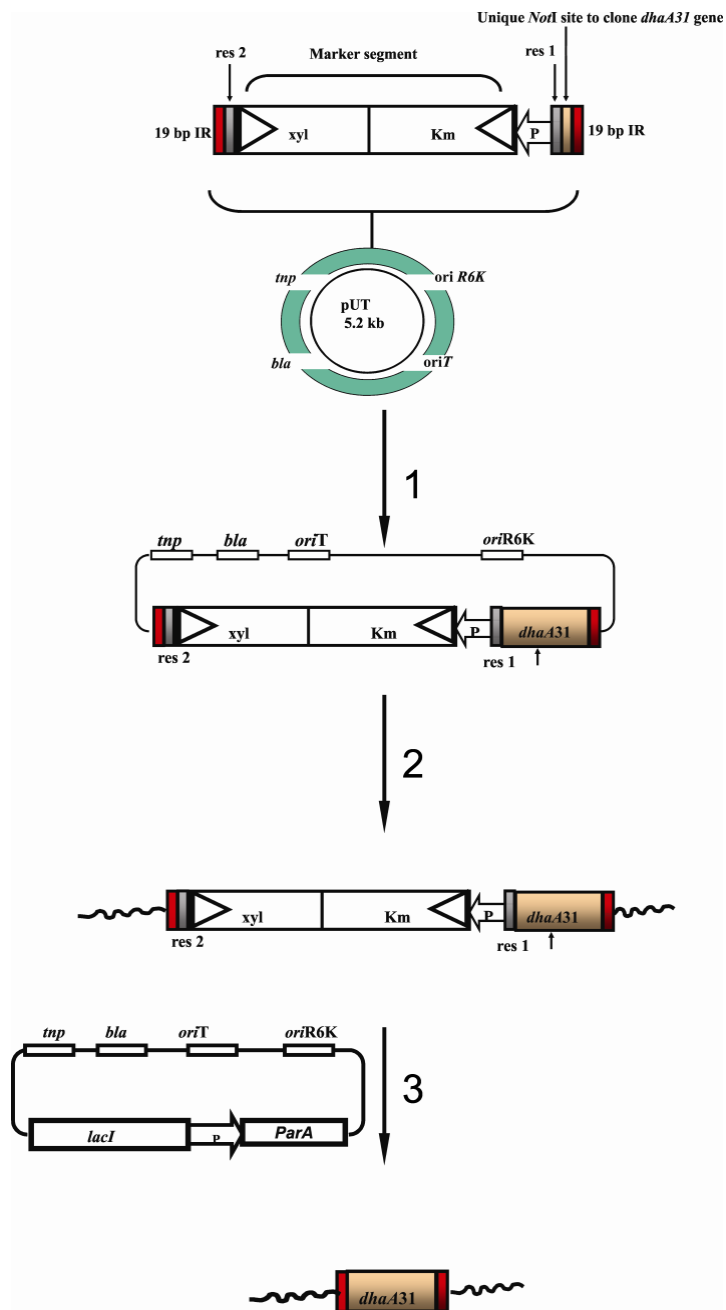


**Fig. 1.** Proposed degradation pathway for TCP by strain MC4-5222. Enzymes: DhaA31, evolved haloalkane dehalogenase; DppA, dichloropropanol dehalogenase/dehydrogenase; CPA, 2-chloropropionic acid dehalogenase. Thick arrows indicate the enzyme activities measured in this thesis.

The use of a plasmid vector as a tool during strain construction is convenient but for environmental applications such a plasmid-based expression is undesirable. Furthermore, the presence of antibiotic selection markers could cause spreading of antibiotic-resistance genes in the environment, which is also unwanted. These considerations triggered de Lorenzo and coworkers to develop transposon-based systems for gene integration in Gram-negative bacteria (8). The Tn5-derived transposon system used in Chapter 3 can mediate integration of a foreign gene into the chromosome. It provides several advantages such as stable integration and the possibility to remove the selection markers by a subsequent resolvase step. Such cloning vectors have been used to construct bacteria for environmental applications (23). Using this delivery system, we integrated the gene encoding the modified haloalkane dehalogenase variant DhaA31 (22) behind the constitutive *dhla* promoter into the chromosome of strain MC4 (Fig. 2).

After performing the transposition and resolvase step, we found that several MC4-derived strains were unable to grow on TCP although the integrated dehalogenase gene was present and functional. In addition, these strains were unable to grow on DCP as growth substrate. This observation suggested that several biochemical factors are involved in functioning of the enzymes involved in metabolic pathway for TCP, and that knockout of the TCP-positive phenotype can easily occur. However, three different MC4 derivatives that did grow on TCP were obtained (MC45221, MC45222, and MC41331). Strain MC4-5222 was found to be the best strain for growth on TCP. The presence of the haloalkane dehalogenase gene was confirmed by PCR, Southern hybridization and partial genome sequence analysis of *P. putida* MC4-5222. Moreover, activities of key enzymes involved in the TCP degradation pathway such as DhaA31, DppA and chloropropanoic acid dehalogenase (CPA-dehalogenase) were measured. The results indicated that activities of these enzymes were sufficient to support cellular growth on TCP as sole carbon source, according to the pathway shown in Fig. 1.





**Fig. 2.** Integration of the *dhaA31* gene into the genome of strain MC4. Steps: 1) Construction of pUT31B by cloning the *dhaA31* gene with the *dhlA* promoter into the unique *NotI* site, which is present between the insertion sequences of the pUT delivery vector; 2) Introduction of pUT31B into strain MC4 by triparental mating. The exconjugants obtained were tested for kanamycin resistance, yellow coloration with catechol, haloalkane dehalogenase activity and growth on DCP as sole carbon source; 3) The resolvase gene *parA* on vector pJMSB8 was introduced into MC4 derivatives by triparental mating (8, 23). The resulting colonies were checked for loss of the marker segment.

During a growth experiment of MC4-5222 on TCP with repeated addition of TCP, we found that the growth rate ( $\mu$ ) was quite low, suggesting that at increasing concentrations TCP may be toxic to cells. Further mutations or genetic modification may be needed to improve the organism towards TCP degradation. However, mutagenesis experiments with ethyl methanesulfonate followed by selection for fast-growing derivatives on TCP did not yield any mutant strains having improved resistance towards TCP or an enhanced growth rate. As reported earlier, the rate of metabolic fluxes, the formation of toxic side-metabolites and an unfortunate balance between (rapid) decay and (slow) growth could be causes of the observed modest growth of the engineered constructs on TCP (11).

**Localization of haloalkane dehalogenase and its use as fusion partner for periplasmic targeting.** During the construction of different recombinant TCP-degrading bacteria, we discovered that the improved dehalogenase variant DhaA31 showed unexpected localization behavior. The haloalkane dehalogenase was found to be localized in the periplasm, both in *E. coli* and in *P. putida* MC4 derivatives. This localization behavior was unexpected since the haloalkane dehalogenase gene sequence does not suggest the presence of a targeting signal, such as an N-terminal signal sequence. Previously, two different types of dehalogenases, LinA and LinB, were also reported to localize into the periplasm without any evident signal sequence being present and without processing (19). Both LinA and LinB originate from the  $\gamma$ -hexachlorocyclohexane biodegradation pathway, LinA being a dehalogenase that eliminates HCl with formation of a double bond, whereas LinB is a haloalkane dehalogenase that is phylogenetically related to the DhaA derivatives used in this thesis. Several other proteins that are transported out of the cytoplasm into the periplasm without the presence of any signal sequence have been identified, and these are jointly called non-classical secreted proteins. Many of these proteins can be recognized by the SecretomeP, software (3). However, DhaA31 as well as LinA and LinB are not recognized by this algorithm as non-classical secreted proteins, despite their experimentally verified periplasmic localization.

In the localization experiments, we carefully checked if the presence of the DhaA31 protein in the periplasm space was due to overexpression or cell lysis during fractionation. For this, two different DhaA31 expression plasmids were used which were equipped with either a *trc* promoter (pIT31), which is an inducible promoter, or the *X. autotrophicus dhla* promoter (pIS31), which is a strong constitutive promoter for the expression of the *dhaA31* gene in *P. putida* MC4. In addition MC4-5222 in which *dhaA31* gene is present as a single copy inserted into the chromosome of strain MC4 was also included in this study. To test the efficiency of the fractionation process, we analyzed the presence of various cytoplasmic and periplasmic marker proteins by means of SDS-polyacrylamide gel electrophoresis, Western immunoblotting and enzyme activity measurements on different subcellular fractions. Our results clearly indicate that the fractionation process was efficient and that the apparent periplasmic localization of DhaA31 was not due to protein overexpression or cell lysis.

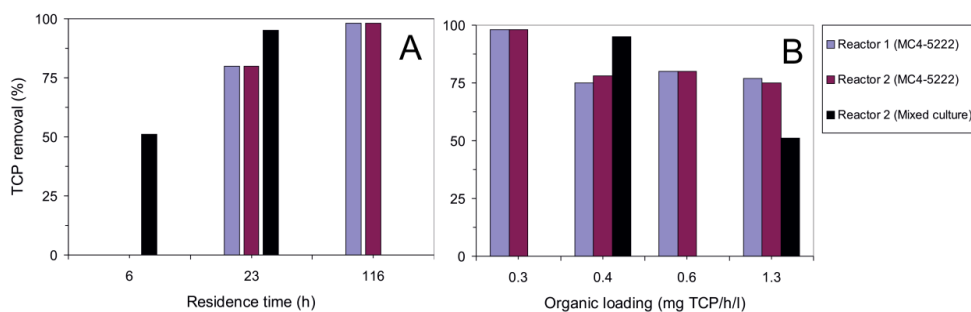
Upon translocation, a signal sequence-containing precursor protein is proteolytically converted into the signal sequence-less periplasmic form (9). Therefore, the periplasmic form of DhaA31 should have a lower mass if the enzyme is subjected to proteolytic removal of a signal sequence during translocation. However, the results of the MALDI-TOF analysis revealed that the mass of the periplasmic form corresponded nicely with its predicted mass, showing that DhaA31 is not proteolytically processed during periplasmic export. Moreover, removal of the codons for the first 20 N-terminal amino acids or the hexahistidine tag present on C-terminus of DhaA variants did not disturb translocation of DhaA31. Thus, the N-terminal end of the enzyme is not required for the observed translocation. The targeting mechanism is not yet clear.

The work presented in Chapter 3 not only describes that DhaA31 is transported to the periplasm without the presence of any known signal sequence and without processing, but also that DhaA can be used as a fusion partner to trigger the export of unrelated proteins. This was demonstrated with thioredoxin and alditol oxidase. The latter hybrid, DhaA31-AldO, was successfully used in a whole-cell conversion of xylitol. These results indicate the potential use of DhaA as a tag for the periplasmic export of heterologously expressed proteins, thereby opening avenues for the use of this system in various biotechnological applications such as whole-cell biocatalysis.

Periplasmic localization also may be preferred over cytoplasmic expression as it can improve protein folding and reduces proteolytic degradation. Moreover, the periplasm represents a cellular compartment with fewer proteins as compared to the cytoplasm, thereby allowing a simpler and more straightforward purification of expressed proteins (10, 18).

**Remediation of TCP in fixed-bed bioreactor by genetically engineered strains.** The construction of a genetically stable TCP-degrading strain that has no antibiotic resistance marker is a key step in the development of a biological groundwater treatment process for TCP. The successful application of 1,2-dichloroethane-degrading bacteria in a full-scale process to remove 1,2-dichloroethane from groundwater has been described earlier (27). Based on the similarities of the physio-chemical properties of TCP and 1,2-dichloroethane, there is a good reason to assume that a biological process for the removal of TCP from groundwater can be developed when an engineered bacterium for TCP degradation becomes available.

The strains *P. putida* MC4-5221, MC4-5222 and MC4-1331 were immobilized on two different kinds of packing material i.e. ceramic raschig rings and sintered porous glass beads in a fixed bed bioreactor system to purify TCP-contaminated water. In addition, *Agrobacterium radiobacter* AD1, which is a naturally occurring DCP degrading organism (31) was also inoculated in the reactor. The degradation of TCP was measured at various organic loadings. TCP degradation was demonstrated by measuring the in- and outlet concentration of TCP, and by measuring effluent levels of DCP and chloride.



**Fig. 3.** TCP removal in fixed-bed bioreactors at different residence times (panel A) and organic loading rates (panel B).

Initial experiments were done with the genetically modified TCP-degrading strain MC4-5222 only. With this culture, the reactor removed TCP (80-98%) at relatively high residence time (116 h and 23 h). However, when an increase of the dilution rate or a higher organic loading was applied, the TCP removal efficiency was decreased (Fig. 2A and 2B).

In the second part of the work, a mixed culture was used (*P. putida* MC4-5221, MC4-5222, MC4-1331, *A. radiobacter* AD1). This improved the performance of the reactor,

suggesting that an effective consortium could overcome some of the drawbacks of the use of a single culture of a TCP-degrading organism. However, when the residence time was decreased from 23 h to 6 h while keeping the TCP concentration constant (0.14 mM), the TCP removal efficiency was again reduced (50%), thus suggesting that the higher TCP influx still was not well accommodated by the reactor. One explanation could be that the higher rate of TCP and water supply caused an inhibitory effect on the biofilm due to TCP toxicity, which in turns decreased the performance of the reactor (Fig. 2B). Another possible reason could be that a low concentration of TCP limits the substrate uptake by the cells. Loss of chloroacrolein may be another possibility; if produced in the periplasm it could be lost from the cells and have a detrimental effect on the biofilm. The precise physiological reasons remain unclear, and further studies will be needed to identify and solve weaknesses in the current TCP degraders.

Despite these limitations, the utilization of these genetically modified strains to remove TCP in a continuous process is an attractive approach, which could be used to develop an ex-situ bioremediation process for TCP-contaminated water, which is entered with low dilution rates or is combined with a classical adsorption technique employing an activated carbon filter. Such a combination of biodegradation and activated carbon sorption was also employed in the full-scale process for 1,2-dichloroethane bioremediation implemented by Stucki and Thuer (27).

**Prospects and concluding remarks.** Despite the presence of highly diverse microbial communities in the environment and the occurrence of numerous natural organohalogenes in the biosphere, TCP appears to be beyond the degradation capabilities of naturally occurring microorganisms. Therefore the work described in this thesis was designed to construct a genetically engineered TCP-degrading bacterium, which could be used to treat TCP-contaminated water. For application in groundwater treatment systems, open processes will be needed and effluent sterilization is not an option. Therefore, using the transposon system developed by de Lorenzo and coworkers, we constructed TCP degrading organisms that are free of an antibiotic resistance marker and plasmid encoded genes that pose a risk of transferring genes to other organisms. Several strains have been constructed that exhibit remarkable properties in the laboratory but their success in terms of full-scale application is limited due to different biochemical and microbiological factors (6, 17, 29). For example, a recombinant *Pseudomonas* strain was unable to grow on 2-chlorotoluene as sole carbon source, although it possesses all the genes in a functional state required for its degradation (11). Likewise, earlier work in our laboratory showed that the introduction of the dehalogenase gene (for 1,2-dichloroethane conversion) from *X. autotrophicus* in a 2-chloroethanol-degrading *Pseudomonas* did not allow the resulting recombinant strain to grow on 1,2-dichloroethane as sole carbon source.

The work described in this thesis for the construction of TCP-degrading *Pseudomonas* strain probably is in our view the best example of a genetically modified organism that grows on a chlorinated hydrocarbon. Based on the similarity in physical-chemical properties of TCP and 1,2-dichloroethane and the successful application of a full-scale bioreactor-based treatment process for the removal of 1,2-dichloroethane from contaminated groundwater at a large contaminated site in Lübeck, Germany (27), we believe that the development of a bioreactor treatment process for TCP removal is feasible once an effective organism is obtained. However, there are still some limitations.

The first and major hurdle is the low growth rate of the strain MC4 derivatives. It is lower than that of the naturally evolved 1,2-dichloroethane-mineralizing bacterium *X. autotrophicus* GJ10. Although strain MC4 has the advantage of degrading both the (*R*) and (*S*) enantiomers of DCP, the moderate growth rate still is a likely bottleneck. Moreover, it

was observed that during batch cultivation, the steady-state growth phase was quickly followed by a cell decay phase. We presume that strain MC4 may have a high intrinsic maintenance demand or decay rate. The observed improvement of the performance of the reactor after addition of a second 2,3-dichloropropanol degrading organism (strain AD1) indicates that MC4 has weaknesses and that a further improvement of the host strain may be beneficial.

In summary, the work described in this thesis shows that genetically modified TCP degrading strains having a productive catabolic pathway can be constructed and that further genetic improvements may be useful. These genetic improvements are likely to be very rare in nature but may possibly be achieved in the laboratory by employing powerful tools of molecular biology and protein engineering.

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## **Nederlandse samenvatting**

Op veel plaatsen in de bodem zitten vervuilende stoffen die veelal afkomstig zijn van (oude) fabrieken, tankstations, vuilstortplaatsen of landbouwbedrijven. Op deze plaatsen is in het verleden met slecht afbreekbare stoffen gewerkt die soms ook een bedreiging voor de volksgezondheid vormen. Er zijn verschillende bodem-vervuilende stoffen bekend, maar de belangrijkste bodemvervuilers zijn oude bestrijdingsmiddelen, zware metalen, cyaniden, aromatische koolwaterstoffen en gechloreerde koolwaterstoffen. De verbinding 1,2,3-trichloropropaan (TCP) behoort tot de laatste groep en wordt gezien als een bodem- en watervervuiler die op steeds meer plaatsen in Europa en de Verenigde Staten wordt aangetroffen. TCP vormt een ernstige bedreiging voor de volksgezondheid mede omdat het waarschijnlijk kankerverwekkend is en bovendien is deze verbinding zeer lastig afbreekbaar. Het doel van dit proefschrift, zoals beschreven in hoofdstuk 2-5, is gericht op de microbiële afbraak van TCP tot onschadelijke producten.

**De eigenschappen van TCP.** De eigenschappen van TCP en de huidige manieren om deze verbinding op te ruimen of af te breken zijn beschreven in hoofdstuk 1. Hieruit blijkt dat het erg lastig is om een TCP verontreiniging op te ruimen omdat TCP zeer stabiel is waardoor er een beperkt aantal mogelijkheden is om deze verbinding om te zetten op een chemische of microbiële manier. Voorts geeft hoofdstuk 1 aan dat er enkele belangrijke voorwaarden zijn waar een micro- organisme aan moet voldoen om TCP om te kunnen zetten en om succesvol toegepast te kunnen worden. Deze zijn: genetische stabiliteit, de afwezigheid van antibioticumresistentie-genen en een snelle en volledige omzetting van TCP.

**De isolatie van een 2,3-dichloro-1-propanol afbrekende bacterie.** De experimenten beschreven in hoofdstuk 2 zijn gericht op de isolatie van een bacterie uit grond die in staat is om 2,3-dichloro-1-propanol (DCP) af te breken. Deze experimenten hebben een *Pseudomonas putida* stam opgeleverd, MC4, die in staat is onder aerobe omstandigheden op diverse verbindingen te groeien, zoals suikers, alcoholen en verschillende gechloreerde verbindingen waaronder DCP. De laatste eigenschap van deze bacterie is zeer belangrijk omdat dit de volledige afbraak van TCP met microbiële enzymen mogelijk maakt. Verdere bestudering van deze bacterie geeft aan dat het enzym, DppA, dat verantwoordelijk is voor de afbraak van DCP door stam MC4 een dehalogenase is. Verassend genoeg is DppA niet verwant is aan bekende dehalogenases en bovendien vertoont het zowel dehydrogenase als dehalogenase activiteit. Hieruit kan worden geconcludeerd dat DppA een nieuw soort dehalogenase is. Dit laatste wordt onderstreept door de nauwe verwantschap van DppA met een groep van andere enzymen, namelijk quinohemoproteïn dehydrogenases. Deze enzymen komen veel voor in aerob levende bacteriën en hebben voor hun werking verschillende cofactoren nodig, zoals pyrroloquinoline quinone (PQQ), calcium en heem. DppA is dus het eerste PQQ-afhankelijke enzym met een dehalogenase functie.

**Het ontwerp van een bacteriële stam voor TCP afbraak.** Indien een micro-organisme beschikt over alle enzymen die nodig zijn voor de afbraak van TCP, dan is het de verwachting dat dit organisme ook op TCP kan groeien. De genetische experimenten beschreven in hoofdstuk 3 hebben tot doel dit organisme te ontwerpen. Hiervoor is gebruik gemaakt *Pseudomonas putida* MC4 in combinatie met een geoptimaliseerde dehalogenase variant, DhaA31. Dit enzym katalyseert de omzetting van TCP naar DCP. De laatste verbinding kan verder omgezet kan worden door MC4 cellen, zoals aangetoond in hoofdstuk 2. De experimenten uit hoofdstuk 3 tonen aan dat wanneer DhaA31 wordt geproduceerd in MC4 cellen, deze cellen inderdaad in staat zijn om te groeien op TCP wanneer dit toegevoegd is aan het groeimedium. Tijdens vervolgent experimenten is de genetische stabiliteit



van dit systeem verhoogd door middel van een insertie van het gen dat codeert voor DhaA31 in het chromosomale DNA van MC4 cellen. Dit heeft enkele MC4 varianten opgeleverd waarvan MC4-5222 het beste groeit op TCP. Bovendien hebben genetische experimenten de aanwezigheid van het coderende gen in het chromosomale DNA van MC4 cellen bevestigd. Ondanks de goede groei van MC4-5222 op medium dat TCP bevat, tonen experimenten aan dat herhaaldelijke toevoeging van TCP waarschijnlijk giftig is voor deze cellen.

**De subcellulaire localisatie van DhaA31 en de toepassing van dit enzym als fusiepartner voor export naar het periplasma.** Alle enzymen worden in het cytoplasma geproduceerd, maar vele enzymen functioneren buiten het cytoplasma, zoals in het bacteriële periplasma. Dit betekent dat deze enzymen na of tijdens productie naar de plaats worden getransporteerd waar ze werkzaam zijn. Om dit transport mogelijk te maken zijn enzymen die buiten het cytoplasma functioneren vaak uitgerust met een export signaal. Hoewel DhaA31 geen bekend export signaal bevat, vormen de resultaten uit hoofdstuk 3 een basis om te veronderstellen dat dit enzym toch werkzaam is in het periplasma. In hoofdstuk 4 wordt de localisatie van DhaA31 in bacteriële cellen onder de loep genomen en wordt de rol van dit enzym als fusiepartner voor de export van andere enzymen naar het periplasma onderzocht. De resultaten geven aan dat DhaA31 aanwezig is in het periplasma van MC4 cellen en *Escherichia coli* cellen. Bovendien kan DhaA31 gebruikt worden voor het transport van andere enzymen naar het periplasma in een functionele vorm. Dit laatste resultaat is belangrijk met betrekking tot eventuele biotechnologische toepassingen van DhaA31 als fusie partner voor export naar het periplasma. Aanvullende experimenten beschreven in hoofdstuk 4 tonen aan dat dit systeem inderdaad gebruikt kan worden voor biotechnologische toepassingen, bijvoorbeeld de enzymatische omzetting van xylitol door complete cellen.

**Microbiële afbraak van TCP uit verontreinigd water.** De experimenten uit hoofdstuk 2 en 3 hebben geleid tot het ontwerp van een bacterie, MC4-5222, die over alle enzymen beschikt om TCP af te breken tot onschadelijke producten, die bovendien genetisch stabiel is én geen antibioticumresistentie genen bevat. Hiermee voldoet deze bacterie in principe aan de voorwaarden gesteld in hoofdstuk 1 om succesvol toegepast te kunnen worden voor de microbiële afbraak van TCP. De experimenten die zijn beschreven in hoofdstuk 5 onderzoeken de mogelijkheden van MC4-5222 voor de afbraak van TCP uit verontreinigd water. Uit deze experimenten blijkt dat deze bacterie in staat is om TCP voor 80-90% te verwijderen uit water verontreinigd met deze stof. Bovendien tonen de resultaten aan dat de efficiëntie van dit systeem verhoogd kan worden door gebruik te maken van meerdere bacterie- stammen tegelijkertijd. Jammer genoeg blijkt ook uit deze experimenten dat onder bepaalde condities de efficiëntie waarmee TCP verwijderd wordt drastisch omlaag gaat. Ondanks deze beperkingen, tonen de experimenten beschreven in hoofdstuk 4 aan dat het opruimen van een TCP verontreiniging met behulp van speciaal ontworpen bacteriecellen een veelbelovende strategie is, die wellicht op een grotere schaal toegepast kan worden.

**Conclusie en vooruitblik.** TCP wordt gezien als een belangrijke bodemvervuiler en vormt lokaal een ernstige bedreiging voor de water- en bodemkwaliteit. Jammer genoeg is het aantal mogelijkheden om deze verbinding om te zetten op een chemische of microbiële manier beperkt. Dit omdat er geen micro-organismen zijn die TCP kunnen afbreken ondanks de veelvoud aan micro-organismen in de natuur én de aanwezigheid van natuurlijke gechloreerde koolwaterstoffen in het milieu. Om toch microbiële afbraak van TCP mogelijk te maken is er een bacteriestam ontworpen, zoals beschreven in dit proefschrift, die alle vereiste enzymen bevat. De resultaten van de experimenten beschreven in hoofdstuk 2-5

geven aan dat deze bacterie inderdaad TCP kan afbreken en gebruikt kan worden om TCP op te ruimen uit water dat is verontreinigd met deze stof. Ondanks deze succesvolle resultaten zijn er nog wel enkele beperkingen, die met verdere optimalisatie van dit systeem verholpen kunnen worden. De resultaten geven aan dat met genetische technieken een bacterie kan worden verkregen die TCP kan omzetten in onschadelijke producten en dat mogelijk op grotere schaal TCP verontreinigingen biologisch opgeruimd kunnen worden.

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**“Gratitude is the memory of the heart”**

### List of publications

- **Samin G**, Janssen DB. A review: Degradation and transformation of 1,2,3-trichloropropane. (in press).
- Arif MI, **Samin G**, van Leeuwen JGE, Oppentocht J, Janssen DB. A novel dehalogenase mechanism in *Pseudomonas putida* strain MC4 for 2,3-dichloropropanol utilization (submitted).
- **Samin G**, Pavlova M, Arif MI, Damborsky J, Janssen DB. A genetically engineered bioremediation bacterium for 1,2,3-trichloropropane degradation (submitted).
- **Samin G**, van Bloois E, Marco WF, Janssen DB. Periplasmic localization of haloalkane dehalogenase and its use in protein targeting for whole-cell biocatalysis (in preparation).
- **Samin G**, Weitzes P, Janssen DB. Remediation of 1,2,3-trichloropropane-contaminated water by genetically engineered bacteria in a packed bed bioreactor (to be submitted).
- Duque AF, Hasan SA, Bessa VS, Carvalho MF, **Samin G**, Janssen DB, Castro PM (2011). Isolation and characterization of a *Rhodococcus* strain able to degrade 2-fluorophenol. Appl Microbiol Biotechnol 11: 3696-2.
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- Zia KM, Iqbal M, Nawaz H, **Samin G** (1999). Langelier calcium carbonate saturation determination by table values. Int J Agri Biol 01: 353-355.

### Work presentation at scientific meetings

- Poster presentation at Netherlands Biotechnology Congress (NBC), Ede, the Netherlands, 11 and 12 Mar, 2010.
- Poster presentation at 5<sup>th</sup> International Congress on Biocatalysis (Biocat 2010), Hamburg, Germany, 29 Aug-2 Sep, 2010.
- Poster presentation at Groningen Biomolecular Sciences and Biotechnology Institute (GBB) Conference, Groningen, the Netherlands, 9 Sep, 2010 (awarded 2<sup>nd</sup> poster prize).
- Poster presentation at 13<sup>th</sup> EuCheMS International Conference on Chemistry and the Environment ICCE 2011, Zurich, Switzerland, 11-15 Sep, 2011.
- Oral presentation at Groningen Biomolecular Sciences and Biotechnology Institute (GBB) Conference, Groningen, the Netherlands, 8 Sep, 2011 (awarded 3<sup>rd</sup> presenter prize).
- Oral presentation Symposium on Engineering Sciences at University of Punjab, Quaid-i-Azam campus, Lahore, Pakistan, 1 Mar, 2011 (awarded 2<sup>nd</sup> presenter prize).
- Lecture at National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan, 20 Feb, 2011.

## Stellingen

Behorende bij het proefschrift

van Ghufrana Samin  
te verdedigen op 11th May 2012

1. In the design of biodegradation processes for organohalogens, more emphasis should be given to the use of effective consortia (chapter 2).
2. Haloalkane dehalogenase can be exploited as a platform for the expression of enzymes in the periplasm, thereby opening new avenues for its use in biotechnological applications (chapter 4).
3. A degradation pathway that looks fine on paper may not work in a cell.
4. The design of bioremediation process employing genetically modified organisms is restricted more by biochemical hurdles than by ecological constrains.
5. Despite the wide spread occurrence of natural organohalogens in the biosphere, many chlorinated compounds are beyond the degradation capabilities of naturally occurring microorganisms offering great challenges for protein engineering and microbial physiology.
6. Sometimes it is easy to produce a result that is difficult to reproduce.
7. Research collaboration is a source of stimulation and creativity.
8. The blood groups of Dutch people should be checked to introduce a new blood group called 'Coffee positive'  
(non-scientific observation).
9. Language barriers are one of the main causes of social segregation.
10. Seek knowledge from the cradle to the grave (Hadiath).