A bacterial strain, designated *Pseudomonas* sp. strain DCA1, was isolated from a 1,2-dichloroethane (DCA)-degrading biofilm. Strain DCA1 utilizes DCA as the sole carbon and energy source and does not require additional organic nutrients, such as vitamins, for optimal growth. The affinity of strain DCA1 for DCA is very high, with a $K_m$ value below the detection limit of 0.5 $\mu$M. Instead of a hydrolytic dehalogenation, as in other DCA utilizers, the first step in DCA degradation in strain DCA1 is an oxidation reaction. Oxygen and NAD(P)H are required for this initial step. Propene was converted to 1,2-epoxypropane by DCA-grown cells and competitively inhibited DCA degradation. We concluded that a monooxygenase is responsible for the first step in DCA degradation in strain DCA1. Oxidation of DCA probably results in the formation of the unstable intermediate 1,2-dichloroethanol, which spontaneously releases chloride, yielding chloroacetalddehyde. The DCA degradation pathway in strain DCA1 proceeds from chloroacetalddehyde via chloroacetic acid and presumably glycolic acid, which is similar to degradation routes observed in other DCA-utilizing bacteria.

### Materials and Methods

**Isolation of bacteria.** Enrichments in batch cultures were set up in closed bottles containing mineral salts medium (10), with mixtures of soil and water from various sources as inocula. Incubations were carried out with 1 mM DCA at different temperatures (21 and 30°C).

In a second approach, a 2-liter fermenter containing 1 liter of mineral salts medium (10) was used for the enrichment. The mineral salts medium was initially supplemented with 0.02 g of yeast extract per liter. The dilution rate was 0.03 h$^{-1}$. DCA was supplied at 1.2 mmol/h via the gas phase. A biofilm sample from a groundwater purification plant, as described by Stucki and Thier (17), was used as the inoculum. The temperature of this contaminated groundwater varied between 8 and 12°C. For practical reasons, the fermenter used for the enrichment was kept at 21°C. From the mixed culture which developed in the fermenter, a pure culture was isolated by using serial dilutions in mineral salts medium with DCA and yeast extract.

**Analytical methods.** Concentrations of DCA were determined by analyzing 100-µl headspace samples on a Chrompack CP9000 gas chromatograph equipped with a CP-Sil 5CB column (Chrompack B.V., Middleburg, The Netherlands). The oven temperature was kept at 100°C. All experiments were performed at 25°C unless stated otherwise. The partition coefficient for DCA between the gas phase and the liquid phase at this temperature is 0.05 (1). Concentrations of CO$_2$ were measured by injecting 100-µl gas phase samples into a Hewlett-Packard 6890 gas chromatograph containing a Chrompack Poraplot Q column. Qualitative determination of the chiral composition of the formed 1,2-epoxypropane was performed by headspace analysis on a Carlo Erba Strumentazione (series 4200) gas chromatograph with a β-cyclodextrin 225 column (Supelco, Zwijndrecht, The Netherlands) at 50°C. Samples were heated to 60°C and 500-µl headspace was injected into the column. Retention times were 8.9 and 9.3 min for the $R$- and $S$-enantiomers, respectively.
Batch cultures. Growth experiments in batch cultures were performed in 250-ml serum bottles containing 50 ml of mineral salts medium (10). In the case of volatile compounds, such as DCA, Boston bottles with Teflon caps (Mininert; Wadsworth: Wadswinchen, Germany) were used in order to prevent substrate loss by evaporation. In the case of DCA as the carbon and energy source, the substrate concentration was 1 mM unless stated otherwise. Growth rates were assessed by measuring CO₂ production during the exponential phase.

To perform a comparative DCA degradation experiment between *Pseudomonas* sp. strain DCA1 and *X. autotrophicus* GJ10, the phosphate buffer concentration in the medium was 2.5 times higher than in the other experiments. The initial DCA concentration was 5 mM. Incubations with *X. autotrophicus* GJ10 were supplemented with 12 μg of vitamin B₆ per liter, which was filter sterilized by using 0.2-μm pore-size disposable filters (Schleicher & Schuell, Dassel, Germany). Incubations with strain GJ10 were performed at 30°C.

The growth of strain DCA1 was tested on chloroacetate, chloroacetic acid, 2-chloroethanol, and ethanol at different concentrations (1, 2, and 5 mM). Strains were grown at 21°C in stirred batch cultures by using 5-liter Erlenmeyer flasks containing 5 mM chloroacetic acid. At different times, 100-ml samples were taken and chloroacetate concentrations were determined metrically. Cell extract was incubated in 50 mM Tris-HCl (pH 7.5) with NAD in a final concentration of 1 mM. After the addition of chloroacetaldehyde (final concentration of 5 mM), the formation of NADH was monitored at 340 nm and at a constant temperature of 25°C.

Preparation of cell extracts. Cells were centrifuged for 10 min at 16,000 g and then washed in an equal volume of 50 mM Tris-HCl buffer (pH 7.5). The pellet was resuspended in a 100-times smaller volume, and the cells were disrupted by sonication for 1 min on ice followed by centrifugation (20 min at 30,000 g). The supernatant was immediately used for the determination of enzyme activities. The protein content was determined by using BCA Protein Assay Reagent (Pierce, Ill.).

Enzyme assays. DCA monooxygenase activity in cell extracts of strain DCA1 was determined in 25-ml glass vials (Supleco, Zwillinrecht, The Netherlands) closed with Teflon valves (Mininert). Cell extract was diluted in 50 mM Tris-HCl (pH 7.5) to a final volume of 2 ml. NADH or NADPH was added to a final concentration of 2 mM. DCA was added as an initial concentration of 100 μM unless stated otherwise, and DCA degradation was followed by headspace analysis as described above. For anaerobic incubations, 35-ml serum bottles with rubber septa were used. Bottles were flushed with nitrogen for 10 min before the DCA was added. A control incubation showed that no significant loss of DCA occurred during the time scale of the experiments. Monooxygenase activity in washed whole cells was determined with resuspended cells in a total volume of 2 ml of mineral salts medium. Monooxygenase activities in cell extracts as well as in whole cells were determined at 25°C.

Chloroacetate dehydrogenase activity was determined spectrophotometrically. Cell extract was incubated in 50 mM Tris-HCl (pH 7.5) with NAD in a final concentration of 1 mM. After the addition of chloroacetate (final concentration, 5 mM), the formation of NADH was monitored at 340 nm and at 30°C. Activities were corrected for NADH oxidase activity by measuring the disappearance of NADH (0.1 mM) when incubated with cell extract.

Chloroacetic acid dehalogenase activity was assayed at 30°C by measuring the release of chloride. Cell extract was incubated in 50 mM Tris-HNO₃ (pH 9.0) containing 5 mM chloroacetic acid. At different times, 100-μl samples were taken and chloride concentrations were determined by using a colorimetric method (4).

Effect of growth substrate on enzyme activities in strain DCA1. To determine the effect of different growth substrates on enzyme activities, cells of strain DCA1 were grown at 21°C in stirred batch cultures by using 5-liter Erlenmeyer flasks containing 1 liter of mineral salts medium (10). The concentration of phosphate buffer was 2.5-times higher than in the standard mineral salts medium, and growth substrates were added at a concentration of 5 mM. Flasks were inoculated with cells that were pregrown on the same substrate. Cells were harvested in the exponential phase. One part of the cell suspension was washed in mineral salts medium (10) and used immediately for the determination of monooxygenase activity in whole cells. The other part of the cell suspension was washed and concentrated in 50 mM Tris-HCl buffer (pH 7.5) and stored at −20°C until the preparation of cell extracts, and the determination of enzyme activities was performed as described above.

Competition experiments. Competitive inhibition of the monooxygenase was measured with DCA-grown cells of strain DCA1 harvested from the fermenter. Cells were diluted in mineral salts medium (10) to a final OD₆₀₀ of 0.13. Incubations were performed in 135-ml serum bottles with an initial DCA concentration of 250 μM. Different amounts of propene were added to the bottles. Based on the partition coefficient of 0.12 between liquid and air phases at 25°C (14), propene concentrations in the liquid phase were 0.8, 4, and 80 μM. Bottles were shaken vigorously at 25°C, and DCA degradation was measured as described above.

RESULTS

Isolation of DCA-degrading bacteria. In order to isolate bacteria with a high affinity for DCA, a fermenter was inoculated with a biofilm sample, kindly provided by G. Stucki, that originated from a rotating biological contactor treating groundwater polluted with DCA as the sole contaminant (17). Within 3 weeks, a DCA-degrading mixed culture was obtained. This mixed culture did not grow very well on mineral salts medium agar plates with DCA vapor present as carbon and energy source; therefore, serial dilutions in liquid cultures were used to obtain a pure culture. The obtained bacterial culture gave one colony type on rich (yeast-glucose) medium agar plates. The new isolate was identified by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) as a bacterium belonging to the genus *Pseudomonas*. The partial sequence of the 16S ribosomal DNA showed 99.4% similarity with *P. fluorescens* and 99.1% similarity with *P. viridiflava*. However, important characteristics such as fluorescence and hydrolysis of gelatin, which are typical for these subspecies, were all negative for the new isolate. The new isolate was designated *Pseudomonas* sp. strain DCA1.

Temperature. In preliminary experiments with strain DCA1 it was observed that, when precultured at 25°C, inocula were not always viable at higher temperatures. Therefore, the optimum temperature for growth of strain DCA1 was determined. As can be seen in Fig. 1, the temperature has a pronounced effect on the growth rate of strain DCA1. The highest maximum growth rate of 0.14 h⁻¹ was at 25°C. At 30°C, the maximum growth rate was twofold lower. No growth of strain DCA1 occurred at 35°C.

Growth kinetics. We could not determine the *Kₘ* value for DCA with washed whole cells of strain DCA1 since this value was below the detection limit of approximately 0.5 μM. Therefore, a comparative growth experiment between *Pseudomonas* sp. strain DCA1 and *X. autotrophicus* GJ10 was performed to study the kinetic differences between these two strains at low substrate concentrations. Substrate depletion curves in closed batch cultures were determined for both strains while they were growing on DCA at an initial concentration of 5 mM. In Fig. 2, the last part of the DCA depletion curves is shown. It is evident that the DCA degradation rate of strain GJ10 decreased dramatically at lower concentrations. Strain DCA1, however, did not show any decrease in the DCA depletion rate, even when the substrate concentration became very low, indicating a much higher affinity for DCA.

**FIG. 1.** Influence of temperature on maximal specific growth rates of *Pseudomonas* sp. strain DCA1 growing on 1,2-dichloroethane (1 mM).
First step in DCA degradation by *Pseudomonas* sp. strain DCA1. In the absence of a cofactor, no DCA degradation was observed in cell extracts of DCA-grown cells of strain DCA1 (Fig. 3). This suggests that a hydrolytic DCA dehalogenase, as is involved in the DCA degradation pathway of *X. autotrophus* GJ10, is apparently not present in strain DCA1. Alternatively, the first step in DCA degradation could be an oxidation reaction by a monooxygenase (24). In that case, oxygen and a source of reducing power (NADH or NADPH) would be required for DCA degradation in cell extracts. Degradation of DCA in cell extract did indeed occur when NADH was added to the reaction mixture (Fig. 3). An oxygen dependency of DCA degradation was first observed with whole cells of strain DCA1 (results not shown). In the absence of oxygen, no DCA degradation occurred. However, after the addition of air to the incubation mixture, DCA degradation was restored. Similarly, DCA was only degraded after the addition of air when nitrogen-flushed cell extract was used (Fig. 3). These results indicate that the first step in DCA degradation by *Pseudomonas* sp. strain DCA1 requires molecular oxygen and NAD(P)H, suggesting that DCA is oxidized by a monooxygenase.

Competitive inhibition of DCA degradation. Alkane monooxygenases generally have a broad substrate specificity and are often capable of performing both epoxidation and hydroxylation reactions (8). Oxidation of alkenes by cells expressing alkane monooxygenase activity often results in the accumulation of the corresponding epoxides. To determine whether the DCA-oxidizing enzyme would act similarly, DCA-grown cells of strain DCA1 were incubated with propene. This resulted in the formation of 1,2-epoxypropane, confirming the presence of monooxygenase activity in whole cells. Analysis of headspace samples of the reaction mixture on a chiral column revealed that both enantiomers of 1,2-epoxypropane were formed.

It is expected that, if one and the same monooxygenase oxidizes both DCA and propene, the presence of a mixture of these substrates would lead to lower reaction rates. Since DCA degradation could be measured more accurately than propene depletion, the effect of different concentrations of propene on DCA conversion rates was determined. The results of this competition experiment, performed with whole cells of strain DCA1, clearly show that at higher propene concentrations the DCA degradation rates are lower (Fig. 4).

Influence of protein content on monooxygenase activity. Many monooxygenases are multicomponent enzymes. This can often be illustrated by determining the enzyme activity as a function of the protein content in the assay. Figure 5 shows that the relation between protein content and DCA monooxygenase activity is nonlinear, resulting in lower specific activities at protein concentrations below approximately 0.4 mg/ml. The maximum specific DCA monooxygenase activity in cell
study the DCA degradation pathway, Pseudomonas nase activity was found in cells grown on all of the tested cells, whereas negligible activities were found in cells grown on enzyme. DCA monooxygenase activity was found in DCA-grown extracts, since DCA monooxygenase is a rather unstable en-
terprises were determined in washed whole cells instead of cell

Strain DCA1 could also grow on 2-chloroethanol. The

DCA was inhibited in the presence of 1 mM chloroacetaldehyde, probably due to the toxicity of this compound, since growth on

drogenase, and chloroacetic acid dehalogenase was deter-

FIG. 6. Different pathways of DCA degradation by Pseudomonas sp. strain

extract, calculated from this experiment, is about 7 nmol min⁻¹

mg⁻¹ of protein. When NADPH was used instead of NADH,

this activity was threefold lower.

Enzymes involved in DCA degradation pathway. To further study the DCA degradation pathway, Pseudomonas sp. strain DCA1 was tested to see if it could grow on the chlorinated intermediates in the proposed DCA degradation pathway shown in Fig. 6. Strain DCA1 could grow on chloroacetic acid, but no growth was observed on chloroacetaldehyde. This is probably due to the toxicity of this compound, since growth on DCA was inhibited in the presence of 1 mM chloroacetaldehyde. Strain DCA1 could also grow on 2-chloroethanol. The expression of DCA monoxygenase, chloroacetaldehyde dehydrogenase, and chloroacetic acid dehalogenase was determined in cells of strain DCA1, grown on DCA, chloroacetic acid, and ethanol as a control (Table 1). DCA monoxygenase activities were determined in washed whole cells instead of cell extracts, since DCA monoxygenase is a rather unstable enzyme. DCA monoxygenase activity was found in DCA-grown cells, whereas negligible activities were found in cells grown on chloroacetic acid or ethanol. Chloroacetaldehyde dehydrogenase activity was found in cells grown on all of the tested substrates, with the lowest activity in cells grown on chloroa-

TABLE 1. Enzyme activities in Pseudomonas sp. strain DCA1 grown on various carbon sources

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>DCA monoxygenase</th>
<th>Chloroacetaldehyde dehydrogenase</th>
<th>Chloroacetic acid dehalogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCA</td>
<td>62</td>
<td>21</td>
<td>1,008</td>
</tr>
<tr>
<td>Chloroacetic acid</td>
<td>&lt;0.5</td>
<td>8</td>
<td>907</td>
</tr>
<tr>
<td>Ethanol</td>
<td>&lt;0.5</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

a Specific activities of DCA monoxygenase were determined in washed whole cells and are expressed as nanomoles per minute per milligram (dry weight) of cells.

b Activities were measured in cell extracts and are expressed as nanomoles per minute per milligram of protein.

tic acid. Cells grown on DCA and chloroacetic acid expressed high chloroacetic acid dehalogenase activities, whereas no de-
halogenase activity was present in ethanol-grown cells of strain DCA1.

DISCUSSION

To obtain new DCA-degrading microorganisms, we set up batch enrichment cultures with 1 mM DCA at different temperatures with various inocula. However, no DCA degradation was observed in any of these cultures. Apparently, DCA-degrading bacteria are not widespread in the environment.

Stucki and Thüer described the treatment of groundwater that contained DCA as the sole contaminant (17). The ground-

water treatment plant consisted of activated carbon filters and a rotating biological contactor (RBC) and was inoculated with X. autotrophicus GJ10 and Pseudomonas sp. strain DE2. Initially, the RBC had little effect on DCA removal. However, during the first year of operation, DCA elimination by the RBC increased continuously. During the fifth year, most of the DCA was removed by the RBC, resulting in effluent concentrations of DCA which were usually much lower than 10 μM. The initially black surface of the RBC had by then changed to a yellow color, suggesting the presence of X. autotrophicus GJ10 as the predominant DCA degrader. Reisolation of DCA-degrading microorganisms confirmed the presence of Xanthobacter sp. with an identical gene encoding for the DCA dehalogenase (17). The average DCA concentration in the effluent, however, was much lower than could be expected, based on the kinetic parameters determined for suspended cells of X. autotrophicus GJ10 (20). We therefore anticipated that another DCA-degrading microorganism with a high affinity for DCA must be present in the RBC biofilm. To isolate this strain, a sample of the RBC biofilm was used to inoculate a fermentor. Enrichment in a continuous culture under DCA limitation was used to select the microorganism with the highest affinity for DCA (lowest Kₚ value). The isolated Pseudomonas sp. strain DCA1 did indeed exhibit a much higher affinity for DCA than strain GJ10, as was shown by the DCA depletion curves determined in closed cultures (Fig. 2). Considering the high affinity of strain DCA1 and also the relatively high growth rate with DCA as a carbon source, it is somewhat surprising that strain GJ10 was still present in the RBC biofilm. However, besides these kinetic differences, other factors such as decay rates and biofilm-forming capabilities, will affect the performance of the microorganisms in the biofilm.

The first DCA-utilizing pseudomonad described in the literature was Pseudomonas sp. strain DE2 (16), which was also used to inoculate the RBC (17). The physiological similarities between strains DE2 and DCA1 are striking; however, there are some distinctions. Strain DE2 does not form colonies on nutrient agar or other solidified media (16). Strain DCA1 does not grow on mineral salt agar plates either, but it does form small colonies on rich-medium agar or other solidified media (16). Strain DCA1 does not require any additional organic nutrients for optimal growth. Further studies, e.g., 16S sequence analysis, are required to establish whether these two strains are intrinsically different.

In contrast to the hydrolytic dehalogenation of DCA in X. autotrophicus GJ10 and A. aquaticus AD25 (12, 19), an oxidation reaction seems to be the first step in DCA degradation in both Pseudomonas strains DE2 and DCA1. No DCA degradation was observed in the cell extracts of strain DE2, and a hydroxylation of DCA, yielding the unstable intermediate 1,2-
dichloroethanol, was suggested as the first step in DCA metabolism (16). We also did not observe degradative activity of DCA in cell extracts of strain DCA1 unless NAD(P)H was added to the incubation mixture. In the presence of NAD(P)H, DCA degradation in cell extract was oxygen dependent, suggesting the involvement of a monoxygenase (Fig. 3). Oxygen-dependent dehalogenation of long-chain (C₃ to C₁₂) α,ω-dichloroalkanes was recently observed in Pseudomonas sp. strain 273 (22), and hexadecane-grown cells of Rhodococcus erythropolis Y2 showed oxidase-type dehalogenation activity towards C₂ to C₁₂ α-chloroalkanes (2). Conversion of DCA by a monoxygenase was suggested by Yokota et al. (24). They described the conversion of DCA by resting cells of two different methanotrophic strains.

Additional proof that a monoxygenase activity was responsible for DCA degradation in our strain was provided by conversion of propene to 1,2-epoxypropane and the results of competition experiments (Fig. 4). Propene competitively inhibited DCA degradation in DCA-grown cells of strain DCA1, suggesting that both substrates compete for the same active site. In general, alkane monoxygenases yield racemic mixtures of epoxides (18). Therefore, the observed conversion of propene to both enantiomers of 1,2-epoxypropane was expected. Interestingly, at a DCA concentration of 250 μM, the presence of 8 μM propene already inhibited DCA degradation by the monoxygenase for more than 50%, indicating an even higher affinity of the monoxygenase for propene than for DCA.

The nonlinear relationship between the protein content in the enzyme assay and the specific activity of DCA monoxygenase (Fig. 5) could indicate that DCA monoxygenase is a multicomponent enzyme, which is not uncommon for monoxygenases (9). Significant levels of DCA monoxygenase activity were only present in DCA-grown cells of strain DCA1 (Table 1). Apparently, DCA monoxygenase is an inducible enzyme, and DCA itself or chloroacetalddehyde could be the inducer.

The next enzymatic step in DCA degradation is probably the dehydrogenation of chloroacetalddehyde. Chloroacetalddehyde dehydrogenases are reported to have broad substrate specificities and are also induced during growth with ethanol (13, 15). Chloroacetalddehyde dehydrogenase activities in cell extracts of strain DCA1 were rather low, probably due to nonoptimal assay conditions. The activity in cell extracts was higher in DCA- or ethanol-grown cells of strain DCA1 than in cells grown on chloroacetic acid (Table 1).

The product of chloroacetalddehyde dehydrogenase is chloroacetic acid, which subsequently has to be dehalogenated. Dehalogenation of chloroacetic acid by halidohydrolases was observed in all DCA-utilizing bacteria described in the literature (12, 16, 19). Chloroacetic acid dehalogenase in strain DCA1 is obviously an inducible enzyme, since no activity was found in ethanol-grown cells. Dehalogenation of chloroacetic acid will most likely result in the formation of glycolic acid (15).

The complete proposed DCA degradation pathway of Pseudomonas sp. strain DCA1 is shown in Fig. 6. Only the initial attack of the DCA molecule seems to be different from the DCA degradation pathway in X. autotrophicus GJ10 and Ancylobacter aquaticus AD25 (12, 19).

The isolation of a DCA-degrading bacterial strain with a high affinity for DCA offers promising opportunities for the efficient biological removal of this compound from groundwater.

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REFERENCES