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Bacterial growth based on reductive dechlorination of trichlorobenzenes

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Abstract

An anaerobic mixed bacterial culture was enriched for bacteria dechlorinating 1,2,3- and 1,2,4-trichlorobenzene (TCB) to dichlorobenzenes by exclusive use of non-fermentable substrates and the application of vancomycin. Growth and dechlorination occurred in a purely synthetic medium with formate or hydrogen, acetate, and TCB. Neither acetogenesis nor methanogenesis was detected in the culture. Repeated subculturing maintaining high dechlorinating activities was also achieved when only hydrogen and TCB were supplied. This indicated that reductive dechlorination of TCB was the primary energy conservating process. The number of dechlorinating bacteria was strictly limited by the amount of TCB supplied in the medium. In addition, the dechlorinating activity could be maintained only in the presence of TCB. A most probable number analysis showed that the dechlorinating species amounted to at least 6×10^5 cells per ml at a total cell number of about 2×10^6 cells per ml. Vitamin B_{12} significantly stimulated the dechlorinating activity.

Abbreviations: DCB – dichlorobenzene(s); NTA – nitrilotriacetate; TCB – trichlorobenzene(s)

Introduction

Microbial reductive dehalogenation of a variety of halogenated organic compounds has been recognized as a wide-spread potential in anoxic environments. Several bacteria coupling growth to reductive dechlorination of chlorinated aromatic compounds have been isolated: *Desulfomonile tiedjei* grows well on formate plus acetate with 3-chlorobenzoate as the electron acceptor. Elevated levels of ATP were found within cells of *D. tiedjei* when 3-chlorobenzoate was added to starved cells (Dolfing 1990). *Desulfitobacterium* sp. strain PCE1 grows by reductive dechlorination of 3-chloro-4-hydroxy-phenylacetate or tetrachloroethene using formate as the electron donor (Gerritse et al. 1996). Growth of strain 2CP-1 by dechlorination of 2-chlorophenol using acetate as a source of electrons and carbon was reported by Cole et al. (1994). *Desulfitobacterium chlororespirans* grows by dechlorination of 3-chloro-4-hydroxybenzoate with formate or hydrogen as electron donor (Sanford et al. 1996). Because in these studies substrate level phosphorylation was excluded as the primary process of energy conservation, growth was ascribed to a respiratory process. With *Desulfitobacterium dehalogenans* and *Desulfitobacterium hafniense* a growth yield increase was detected after the addition of 3-chloro-4-hydroxyphenylacetate to a medium containing yeast extract and pyruvate (Utkin et al. 1994; Christiansen & Ahring 1996). Growth by reductive dechlorination of tetrachloroethene with hydrogen, formate or acetate as the electron donor has been shown for several bacteria (Holliger et al. 1999). The isolation of a pure culture dechlorinating chlorobenzenes at high rates has not been described yet. It has been reported that facultatively anaerobic *Staphylococcus* spp. isolated from intestinal contents of rats dechlorinated TCB in complex medium at very low rates (Tsuchiya & Yamaha 1984), however, with no apparent benefit for the or-

** Dedicated to F. Lingens on the occasion of his 75th birthday.

ganisms. Growth based on reductive dechlorination as an energy yielding process has also been demonstrated with mixed cultures. An increase of the cell number of *Desulfomonile tiedjei* DCB-1 was detected in a mixed consortium after 3-chlorobenzoate was added (Dolfing & Tiedje 1987). In a highly enriched culture strain PER-K23 (*Dehalobacter restrictus*) only grew in the presence of tetrachloroethene as an electron acceptor (Holliger et al. 1993). Another mixed culture dechlorinating tetrachloroethene to ethene could be transferred indefinitely only when hydrogen and tetrachloroethene were supplied, indicating that growth of the dechlorinating bacteria depended on the reductive dechlorination reaction (Maymó-Gatell et al. 1995). From this culture *Dehalococcoides ethenogenes* could be isolated (Maymó-Gatell et al. 1997). Hydrogen thresholds and determination of the fraction of electrons from substrate oxidation used for chloride release were recently described as accurate indicators for dehalorespiration in pure and mixed cultures (Löffler et al. 1999).

Reductive dechlorination by mixed cultures from a variety of anaerobic environments was reported for all chlorinated benzenes from monochlorobenzene to hexachlorobenzene (Adrian et al. 1998; Beurskens et al. 1994; Bosma et al. 1988; Fathepure et al. 1988; Fathepure & Vogel 1991; Holliger et al. 1992; Middeldorp et al. 1997; Nowak et al. 1996; Ramanand et al. 1993). Some physiological characteristics of chlorobenzene dechlorinating bacteria were derived from the study of mixed cultures (Adrian et al. 1998; Holliger et al. 1992; Middeldorp et al. 1997). However, it has not been demonstrated so far that bacteria can benefit from the reduction of chlorobenzenes.

The intention of the present study was to investigate whether bacteria can use reductive dechlorination of chlorobenzenes as an energy source for growth. Because a pure culture was not available, a well-established non-methanogenic, mixed bacterial culture growing on a completely defined medium was used (Adrian et al. 1998). Previously the culture was maintained in the presence of pyruvate and citrate which did not allow to distinguish between growth based on fermentation and growth based on reductive dechlorination. In the present study the enrichment of dechlorinating bacteria is described in a purely synthetic medium without substrates that could allow substrate level phosphorylation.

Material and methods

Chemicals and gases

All analytical grade chemicals were purchased from Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany) or Aldrich (Steinhofen, Germany). Titanium(III) chloride was obtained from Merck-Schuchard (Hohenbrunn, Germany) as a technical grade solution. Nitrogen was of 99.999% (vol/vol), carbon dioxide of 99.8% (vol/vol) quality. The gas mixture (80% N_2–20% CO_2, Linde, Berlin, Germany) used for bacterial cultures was further purified by removing traces of oxygen with a reduction column (Ochs, Göttingen, Germany). Ti(III)citrate was prepared from Ti(III) chloride and sodium citrate (Zehnder & Wuhrmann 1976) using solid sodium carbonate for neutralization. Ti(III)-NTA was prepared accordingly using sodium NTA instead of sodium citrate.

Inoculum and cultivation conditions

A stable dechlorinating, mixed bacterial culture (Adrian et al. 1998) was used as inoculum for the present study. The medium was a defined, bicarbonate buffered mineral medium amended with 15 μM 1,2,3-TCB, 15 μM 1,2,4-TCB, a trace element solution (SL9), a selenite-tungstate solution, a six-vitamin solution ("vitamin solution 1"), and a vitamin B_{12} solution as described previously (Adrian et al. 1998). In addition 0.2% (vol/vol) of a second vitamin solution ("vitamin solution 2") consisting of 1,4-naphthoquinone (100 mg/l), riboflavin (25 mg/l), folic acid (10 mg/l), thioctic acid (25 mg/l) and hemin (25 mg/l) was added. The medium was stored anoxically without reducing agents in 310 or 610 ml flasks with Teflon lined screw caps. For the preparation of cultures 30 ml medium from storage flasks were filled into 60 ml serum bottles. When lower concentrations of TCB were needed, medium with TCB was mixed at a calculated ratio with medium not containing TCB. Ti(III)citrate solution was added to a final concentration of 0.8 mM Ti(III) and 1.6 mM citrate to each serum bottle below the liquid surface from where it quickly dropped to the bottom. Here, the reducing agent was protected against oxidation by air during the gas exchange procedure. Ti(III)-NTA was added by the same procedure. The bottles were carefully closed with Teflon lined butyl rubber septa and aluminum crimp caps. Air was exchanged against a 80%–20%

N_2/CO_2-atmosphere by 7 cycles of under- and overpressure of 14.4 kPa and 110 kPa absolute pressure, respectively. Finally the bottles were gently shaken to distribute the Ti(III)citrate solution throughout the medium. After waiting for at least 1 hour, to allow redox equilibration, inoculation was carried out with glass syringes that had been flushed with sterile water. The standard inoculation volume was 0.5 ml per bottle. Cultures were incubated at 29 °C in the dark without shaking. Following this procedure, dechlorinating cultures could be transferred with excellent reliability and reproducibility.

Sterile supernatant from cultures was prepared by centrifugation (10 000 g, 15 min) and consecutive sterile filtration of the supernatant. Alternatively to sterile filtration the supernatant was autoclaved leading to formation of a precipitate that was removed by a second centrifugation step (10 000 g, 5 min).

To estimate the cell number of dechlorinating bacteria most probable number analyses were done in cultures with 18 ml of medium and 2 ml of inoculum. The medium contained 30 μM TCB, hydrogen (nominal concentration of 7.5 mM) and 5 mM acetate. After 7 weeks of incubation cultures were analyzed for DCB formation. Cultures were considered negative if no formation of DCB could be detected. In positive cultures the conversion of TCB to DCB was always 95% or above. For each dilution step 5 parallel cultures were prepared.

Isolations were done in deep agar shakes as described by Pfennig (1978) using the medium that was used for liquid cultures with 1% (wt/vol) agar (Difco, Detroit, USA). Gram staining was done with a kit by Merck (Darmstadt, Germany).

Analytical procedures

DCB and TCB were analyzed by gas chromatography using a capillary column and flame ionization detection as described earlier (Adrian et al. 1998). Formate, acetate, propionate, butyrate, lactate, pyruvate, and citrate were determined by HPLC with a HPX-87H column, 300 × 7.8 mm (Biorad, München, Germany) with 5 mM sulfuric acid at a flow rate of 0.5 ml/min and a temperature of 20 °C. The effluent was monitored photometrically at 210 nm. The detection limit for acetate was 30 μM. Methane was measured by gas chromatography and thermal conductivity detection as described previously (Adrian et al. 1998). Total cell numbers were determined by direct counting with an epifluorescence microscope (Axioskop, Zeiss, Jena, Germany) after filtration of 100 μl fresh bacterial suspension diluted with 900 μl of water on a 0.2 μm polycarbonate filter and subsequent staining with 4′,6-diamidino-2-phenyl-indole-dihydrochloride (DAPI, 100 μg/ml). At least 1000 cells or 10 visual fields of 100 × 100 μm in length were counted and averaged. Protein concentrations were determined by the bicinchoninic acid method (Smith et al. 1985) using bovine serum albumin as standard.

Results

Citrate-butyrate culture

A TCB dechlorinating mixed culture growing over many transfers in a medium with pyruvate was transferred to a pyruvate free medium (Table 1, enrichment stage 1). With 10 mM butyrate and Ti(III)citrate as reducing agent, resulting in a final citrate concentration of 1.6 mM, the highly active dechlorinating culture was maintained for more than 20 successive transfers. Under these conditions 1,2,3-TCB and 1,2,4-TCB (15 μM each) were completely dechlorinated to a mixture of 1,4- and 1,3-DCB within 7 to 9 days after the injection of a 1.7% (vol/vol) inoculum. Total cell numbers after complete dechlorination in the presence of butyrate and citrate were between 9.7×10^6 and 2×10^7 per ml. When butyrate was omitted at this stage, dechlorinating activity decreased (Table 1, enrichment stage 1). Citrate was essential to maintain cultures with high dechlorinating activity. The addition of acetate, however, had a positive effect on DCB formation in cultures without citrate. Methane formation was not observed in any of the enrichment stages.

To demonstrate that growth of the dechlorinating bacteria was limited by the concentration of TCB available an experiment involving two successive transfers was performed (Figure 1). All cultures in this experiment were grown on medium with Ti(III)citrate and 10 mM butyrate. In the first transfer, 5 triplicates of serum bottles with total TCB concentrations from 0 to 40 μM (series A cultures) were inoculated using a single actively dechlorinating culture. After 7 days of incubation TCB was completely dechlorinated to DCB in those cultures initially containing 10 or 20 μM TCB. After 14 days in all cultures TCB was completely dechlorinated (Figure 2). From each series A culture 0.5 ml were transferred to fresh medium containing 30 μM TCB (series B cultures, 5 triplicates). At the same time, another group of 5 triplicates

Table 1. DCB formation in cultures of successive enrichment stages with 30 μM of TCB

Enrichment stage	Media used		DCB formation[a] 7 days after inoculation		Infinite cultivation
	Reducing agent[b]	Further additions[c]	First transfer	Second transfer	
0	Ti(III)citrate	pyruvate[d]	43 ± 6	55 ± 4	+
1	Ti(III)citrate	butyrate	46 ± 3	48 ± 6	+
	Ti(III)citrate	none	17 ± 10	15 ± 5	+
	Ti(III)-NTA	butyrate	10 ± 3	0 ± 0	−
	Ti(III)-NTA	butyrate + acetate[e]	18 ± 4	9 ± 2	−
	Ti(III)-NTA	butyrate + citrate	51 ± 3	66 ± 4	+
2	Ti(III)citrate	butyrate + vancomycin	3 ± 2	0 ± 0	−
	Ti(III)citrate	butyrate + vancomycin + sterile supernatant[f]	64 ± 4	62 ± 5	+
3	Ti(III)citrate	butyrate + vancomycin + formate + acetate[g]	61 ± 2	54 ± 3	+
4	Ti(III)-NTA	formate + acetate[g]	48 ± 7	66 ± 5	+
	Ti(III)-NTA	hydrogen + acetate[g]	57 ± 9	71 ± 15	+
	Ti(III)-NTA	hydrogen	13 ± 11	55 ± 17	+
	Ti(III)-NTA	formate	4 ± 0	2 ± 4	−
	Ti(III)-NTA	acetate[f]	24 ± 2	7 ± 2	−

[a] In percent of total chlorobenzenes; mean of triplicate cultures ± standard deviation. [b] Concentrations: Ti(III) 0.8 mM; citrate 1.6 mM; NTA 1.6 mM. [c] Concentrations: butyrate 10 mM, pyruvate 10 mM; citrate 2 mM; vancomycin 5 mg/l, formate 5 mM, hydrogen 7.5 mM (nominal). [d] Data from Adrian et al. 1998. [e] Concentration 4 mM. [f] 20% (vol/vol) sterile supernatant from a vancomycin free culture. [g] Concentration 5 mM.

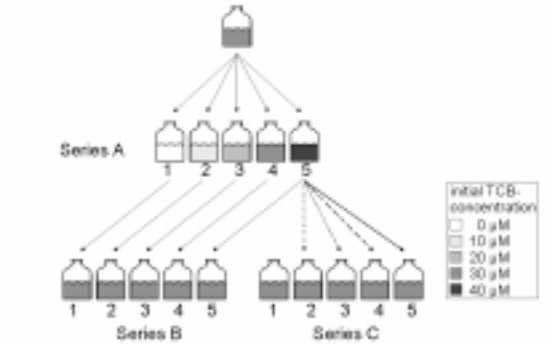

Figure 1. Experimental setup to test for growth linked to reductive dechlorination of TCB. Each bottle depicted in series A, B and C represents a triplicate of cultures. The series B cultures were inoculated with 0.5 ml of the various series A cultures. Series C cultures were inoculated with 0, 0.125, 0.25, 0.375, or 0.5 ml of a series A culture grown on 40 μM TCB.

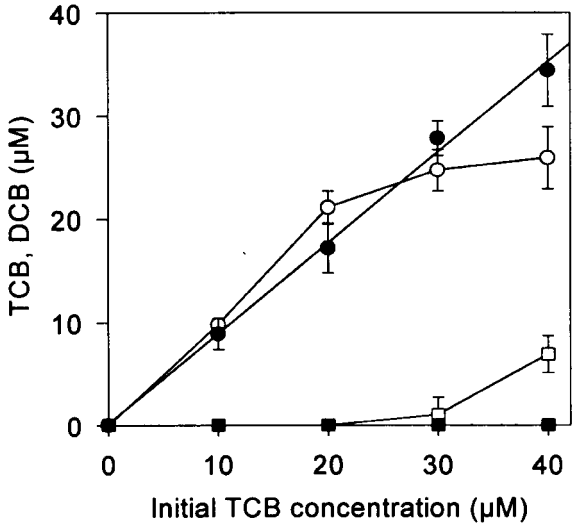

Figure 2. DCB formation from TCB in series A cultures. The medium contained 10 mM butyrate, Ti(III)citrate and increasing initial concentrations of TCB as indicated. Symbols: □ sum of TCB after 7 days; ○ sum of DCB after 7 days; ■ sum of TCB after 14 days; ● sum of DCB after 14 days. The results are means of triplicate cultures ± standard deviation.

of cultures containing 30 μM TCB was inoculated with increasing volumes from 0 to 0.5 ml of series A cultures, grown on 40 μM TCB (series C cultures). If growth of dechlorinating bacteria is limited by the amount of TCB available, 0.5 ml of series A flask no. 2 (10 μM TCB) will contain about the same number of dechlorinating bacteria as 0.125 ml of series A flask no. 5 (40 μM TCB); 0.5 ml of series A flask no. 3 (20 μM TCB) will contain about the same number as 0.25 ml of series A flask no. 5 (40 μM TCB), and so on. Therefore, dechlorinating activity determined after 6 days in series B and series C cultures is expected to increase linearly from flask 1 to flask 5 and the amount

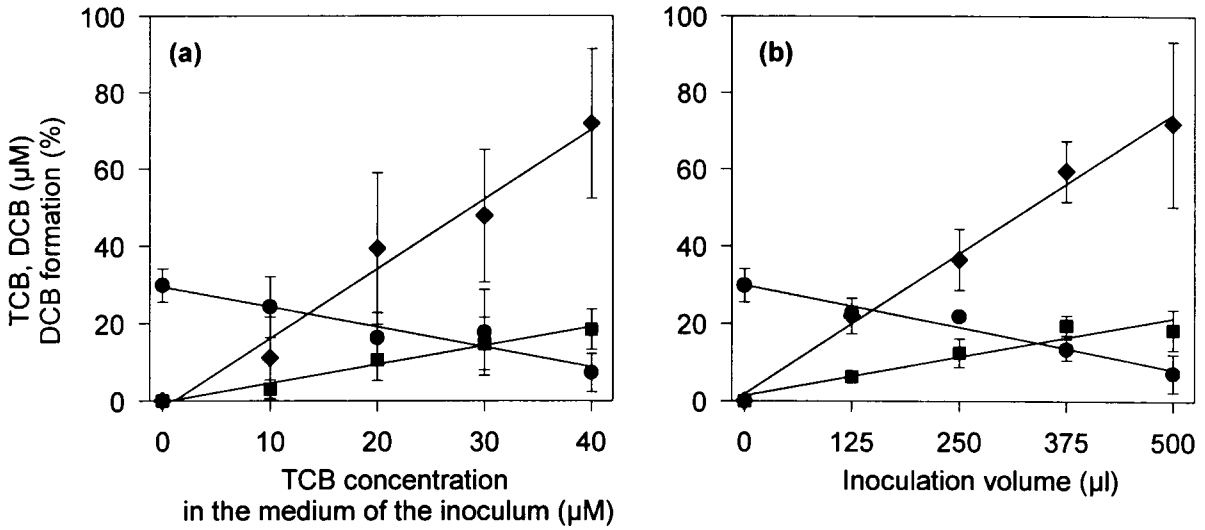

Figure 3. DCB formation in cultures of series B (a) and series C (b) after 6 days of incubation. All cultures contained initially 30 μM TCB. Symbols: ● sum of TCB, ■ sum of DCB, ♦ DCB formation expressed as percentage of the total amount of chlorobenzenes. Results are means of triplicate cultures ± standard deviation. The lines are calculated by linear regression.

of TCB converted to DCB should be the same in corresponding flasks of both series. This was indeed the case as shown in Figures 3a and 3b. The experiment was repeated using 21 days old cultures of series A as inoculum confirming the results described above (data not shown).

Bacterial isolates

From enrichment stages 0 and 1 (Table 1) several bacterial strains were isolated. However, none of these pure cultures reductively dechlorinated TCB. One of the isolates was a Gram-positive vancomycin-sensitive coccus growing on glucose by fermentation to lactate. The coccus grew in chains of about 10 single cells and was therefore easily to differentiate from other bacteria in the culture by phase contrast microscopy. These streptococcoidal bacteria were found in all cultures of enrichment stages 0 and 1 (Table 1) regardless if the cultures were actively dechlorinating or if the capability to dechlorinate was lost after exposure of the inoculum to air for 30 s.

Enrichment by vancomycin

In cultures containing 5 mM butyrate, 1.6 mM citrate added as a Ti(III) complex, and 30 μM TCB full dechlorinating activity could be detected after the addition of penicillin G up to a concentration of 10 mg/l, suggesting that the dechlorinating bacteria were Gram-negative. Therefore various antibiotics targeted predominantly against Gram-positive bacteria were applied. The addition of 5 mg/l vancomycin (Table 1, enrichment stage 2) or 5 mg/l fusidinic acid (data not shown) to cultures containing 10 mM butyrate, Ti(III)citrate, and TCB resulted in a drastic drop of dechlorinating activity and dechlorinating subcultures could not be established. However, high dechlorinating activities were maintained in cultures with 10 mM butyrate, Ti(III) citrate, TCB and 5 mg/l vancomycin, when 10 to 20% (vol/vol) of a sterile supernatant from cultures without vancomycin was added. Cultures with butyrate, Ti(III)citrate, TCB, vancomycin, and the sterile supernatant could be transferred indefinitely. Supernatant obtained from cultures grown in synthetic mineral medium with Ti(III)citrate as the sole organic addition also supported reductive dechlorination in cultures containing butyrate, Ti(III)citrate, TCB and vancomycin. In contrast, supernatant from cultures that did not contain citrate, because they were reduced with Ti(III)-NTA, did not support dechlorinating activity in cultures with butyrate, Ti(III)citrate, TCB and vancomycin.

The physiological activities in cultures with Ti(III)citrate and TCB but without vancomycin were analyzed in more detail. The initial concentration of citrate added as Ti(III)-complex was 1.6 mM. The medium was clear but showed a slight olive color due to Ti(III)-salts (Zehnder & Wuhrmann 1976). After

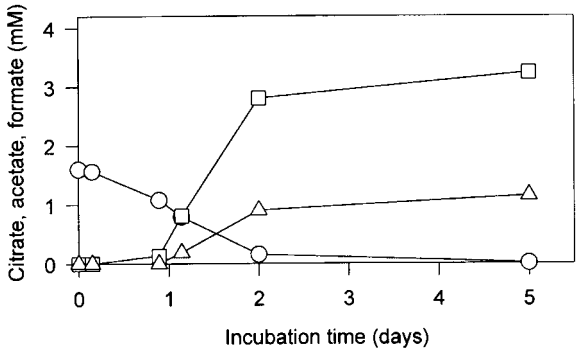

Figure 4. Fermentation of citrate by cultures without vancomycin. Cultures contained initially 1.6 mM citrate as a Ti(III)-complex, and 30 μM TCB. Symbols: ○ citrate, □ acetate, △ formate.

two days of incubation a voluminous olive precipitate appeared, indicating that the chelating agent citrate was metabolized. HPLC-analysis confirmed that citrate concentrations decreased rapidly during the first two days of incubation and formate and acetate were produced concomitantly (Figure 4). After 5 days of incubation citrate was completely converted to acetate (3.2 mM) and formate (1.0 mM). Other products transiently found in low concentrations were pyruvate (20 μM) and lactate (50 μM). A conversion of butyrate in cultures with butyrate, Ti(III)citrate, and TCB was not detected within the first 7 days of incubation.

The streptococcoidal isolate from enrichment stage 1 produced acetate and formate in similar concentrations after two days of incubation growing in a Ti(III)citrate reduced medium without further organic additions. Sterile supernatant from such cultures supported fast dechlorination in the mixed culture growing with butyrate, Ti(III)citrate, and TCB in the presence of vancomycin. In contrast to the mixed culture in medium with butyrate, Ti(III)citrate and TCB without vancomycin and to the isolated streptococcoidal bacterium, mixed cultures that contained butyrate, Ti(III)citrate, TCB and 5 mg/l vancomycin did not form acetate nor formate. Microscopic inspection revealed that indeed the streptococcoidal bacteria did not grow in the presence of vancomycin. Hence, cultures were set up containing butyrate (10 mM), TCB (30 μM), formate, acetate (5 mM each) and vancomycin (5 mg/l) in Ti(III)citrate reduced medium (Table 1, enrichment stage 3). These cultures showed high dechlorinating activities without the addition of sterile supernatant and could be maintained indefinitely with high dechlorinating activity.

Growth with hydrogen as electron donor

After several transfers of enrichment stage 3 cultures, butyrate and vancomycin could be omitted and Ti(III)citrate could be replaced by Ti(III)-NTA maintaining high dechlorinating activities in the presence of TCB, formate and acetate (Table 1, enrichment stage 4). Many successive transfers in medium with TCB (30 μM), acetate, formate (5 mM each), and either Ti(III)-NTA or Ti(III)citrate as a reducing agent were achieved maintaining high dechlorinating activity. The culture could also be maintained indefinitely by biweekly transfers in Ti(III)-NTA reduced medium with TCB, acetate and hydrogen (7.5 mM nominal concentration). In Ti(III)-NTA reduced medium with hydrogen and TCB but without acetate the culture was transferred 4 consecutive times without losing the dechlorinating activity. In these cultures no acetate was detected after one or three weeks of incubation at a detection limit of 30 μM. Acetogenesis also was not observed in cultures supplied with formate or hydrogen but not with TCB. DCB formation was generally higher in TCB cultures with hydrogen and acetate than with hydrogen alone (Table 1, enrichment stage 4). Cultivation with TCB and acetate or TCB and formate resulted in a drop of dechlorinating activity (Table 1, enrichment stage 4).

Total cell numbers determined by direct cell counting and dechlorinating activities of the successive enrichment stages are summarized in Figure 5. By exclusion of fermentable substrates and application of vancomycin the total cell number was reduced from 10^8 to 2×10^6 cells per ml but high dechlorinating activity was maintained. Protein concentrations in cultures with Ti(III)-NTA, formate (5 mM), acetate (5 mM) and TCB were below 1 mg/l after 7 days of incubation. Most probable number analyses, based on DCB formation after 7 weeks of incubation, revealed that in cultures with high dechlorinating activities the minimum cell number of dechlorinating bacteria was 6×10^5 per ml.

The standard medium contained three different vitamin solutions and a selenite-tungstate solution. The effect of these additions on dechlorination of TCB was studied by preparing a medium without the additions and amending the medium with none, one, two or all four of the solutions. Cultures further contained Ti(III)citrate, vancomycin, acetate (5 mM), formate (5 mM), and TCB. DCB formation was increased significantly only in the presence of vitamin B_{12} (Figure 6). Abiotic reduction of TCB by Ti(III) and vitamin

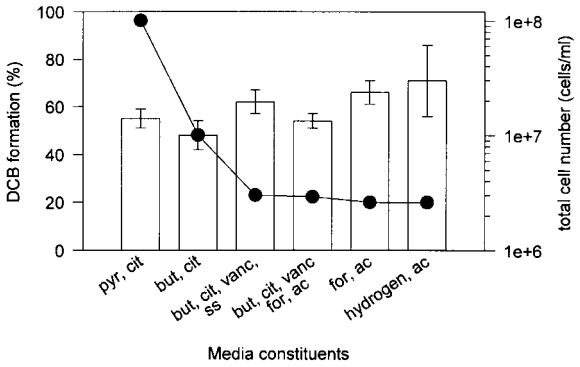

Figure 5. Summary of the dechlorinating activities and total cell numbers of the successive enrichment stages. The media contained 30 μM TCB and additions: ac – acetate (5 mM); but – butyrate (10 mM); cit – citrate (1.6 mM); for – formate (5 mM); pyr – pyruvate (10 mM); ss – sterile supernatant (20% vol/vol); vanc - vancomycin. Symbols: ● total cell number. Bars show the amount of DCB formed after 7 days, expressed as percent of the total amount of chlorobenzenes. Experiments were performed in triplicate.

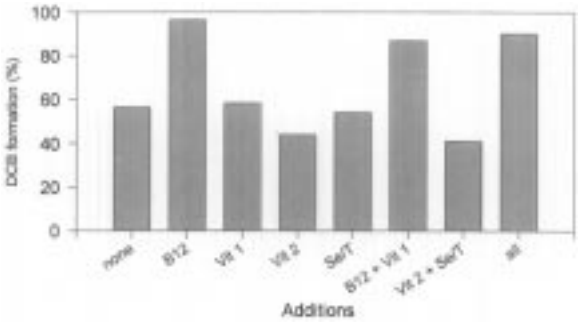

Figure 6. Effect of vitamin and selenite-tungstate solutions on DCB formation. All cultures contained Ti(III)citrate, vancomycin, acetate (5 mM) and formate (5 mM). Bars show the amount of DCB formed after 7 days expressed as percent of the total amount of chlorobenzenes. Abbreviations: B12 – vitamin B_{12} solution; Se/T – selenite-tungstate solution; vit 1 – vitamin solution 1; vit 2 – vitamin solution 2.

B_{12} was not observed under the cultivation conditions used.

Discussion

The present communication reports on a highly enriched bacterial culture forming DCB from TCB in a Ti(III)-NTA reduced medium containing acetate, hydrogen and carbonate. Light or electron acceptors other than TCB and carbonate were not available. Because neither methanogenesis nor acetogenesis was observed, reductive dechlorination of TCB is the only primary energy yielding process possible. The mixed culture could be transferred indefinitely using inocula of 1.7% (vol/vol) without losing the dechlorinating activity, indicating that the dechlorinating bacteria can grow using dehalorespiration with TCB as an energy yielding process. It is not yet known, if the dechlorinating bacteria grow autotrophically, or if other autotrophs in the mixed culture provide a carbon source for the dechlorinating bacteria. However, with hydrogen as electron donor the addition of acetate showed a positive effect on DCB formation, indicating that the dechlorinating bacteria can use acetate as a carbon source, which has been shown also for other dechlorinating bacteria (Holliger et al. 1999).

In contrast to findings with previous enrichment stages (Adrian et al. 1998), the dechlorinating culture now uses hydrogen as an electron donor. This discrepancy cannot be explained at present and will be subject to further investigation. In its current composition the mixed culture also uses formate as an electron donor. The observation that dechlorinating activity could be maintained with formate only when also acetate was supplied, can be explained by assuming that formate is converted to hydrogen by other bacteria of the culture, which require acetate for growth.

Our dechlorinating mixed culture did not use TCB as a carbon source. Instead, stoichiometric amounts of DCB were formed, demonstrating the exclusive use of the chloroaromatic compound as an electron acceptor in the energy yielding process. That indeed growth of the dechlorinating bacteria is limited by the amount of TCB available was demonstrated by a two stage transfer experiment showing that growth of dechlorinating bacteria is a linear function of the TCB concentration supplied. Complete loss of dechlorinating activity occurred after one passage in a medium with 10 mM butyrate and Ti(III)citrate but without TCB. The only vitamin that was found to increase DCB formation in the mixed culture was vitamin B_{12}. This result is noteworthy because corrinoids have been detected as cofactors in most reductive dehalogenases isolated so far (Holliger et al. 1999).

For many dehalorespiring bacteria growth yields between 1 and 5 g of cell protein per mole chloride released have been reported (Mohn & Tiedje 1990; Holliger et al. 1993; Cole et al. 1994; Scholz-Muramatsu et al. 1995; Gerritse et al. 1996; Maymó-Gatell et al. 1997 – and calculated from cell dry weight yield – Mackiewicz & Wiegel 1998). Löffler et al. (1999) determined that the fraction of electrons that were used for dehalorespiration by pure and mixed cultures was between 0.63 and 0.67, leading to calculated yields of 2.8 to 3.3 g protein per mol Cl^- released. Assuming

a similar growth yield of the dechlorinating bacterium in our mixed culture (3 g of cell protein per mole of chloride released) and assuming a number of 3.3×10^{13} cells per g of cell protein as determined for *Dehalospirillum multivorans* (Scholz-Muramatsu et al. 1995), the total concentration of 30 μM TCB used in our experiments is sufficient to allow growth of 3×10^6 dechlorinating bacteria per ml. If a number of 6.5×10^{12} cells per g of cell protein is assumed, as described for *E. coli*, the supplied amount of TCB supports growth of 5.9×10^5 bacteria. By a most probable number experiment a minimum number of 6×10^5 TCB dechlorinating bacteria per ml was found in our mixed culture, indicating that indeed TCB dehalorespiration supplies enough energy to account for the observed growth of the dechlorinating bacteria.

While dehalorespiration with chlorobenzoates, chlorophenols or tetrachloroethene is well established, it has not been previously demonstrated that bacteria can derive energy by using chlorinated benzenes as an electron acceptor. Indeed, chlorobenzenes represent a separate group of chlorinated aromatic compounds which do not contain oxygen or oxygenated substituents. Pure cultures of facultative anaerobic *Staphylococcus* spp., isolated from intestinal contents of rats, reductively dechlorinated 1,2,4-TCB in a complex medium, but no specific energy yielding process was demonstrated. The rate of DCB formation from TCB with this organism was about 6.8 nmol DCB formed per mg cell dry weight and day (Tsuchiya & Yamaha 1984). The highly enriched culture described in the present study dechlorinated 30 μM TCB to DCB within 7–9 days. Protein concentrations in the bacterial suspensions were below the detection limit of 1 mg/l. Assuming that the protein content accounts for 50% of the cell dry weight (Mackiewicz & Wiegel 1998) a maximum of 2 mg/l cell dry weight was estimated. This allows to convert the minimal overall rate of dechlorination found in our culture (30 μM/9 days/1 mg protein l^{-1}) to 1.7 μmol DCB per day and mg dry weight. Thus the minimal overall rate of dechlorination was at least 245 times higher than that found with *Staphylococcus* spp. With the highly active culture described in the present communication, we demonstrate for the first time that reductive dechlorination of chlorobenzenes is used as an energy yielding process for bacterial growth.

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