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Characterization of the requirements and substrates for reductive dehalogenation by strain DCB-1

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SUMMARY

An obligately anaerobic bacterium known as strain DCB-1 was grown under a variety of conditions to determine the requirements for dehalogenation as well as factors which stimulated or inhibited the process. Dechlorination was obligately anaerobic since introduction of O_2 immediately inhibited the reaction. Sulfuroxy anions, which also serve as electron acceptors for DCB-1, inhibited dechlorination but NO_3^- and fumarate did not. The optimum growth medium for dechlorination was 0.2% Na pyruvate and 20% rumen fluid in basal salts. Media with either pyruvate or rumen fluid alone did not support dechlorination. DCB-1 also consumed H_2 but typical substrate concentrations of H_2 (80 kPa) delayed dechlorination. Once the H_2 concentration was reduced to < 20 μM (2.67 kPa), dechlorination resumed. Dehalogenation by DCB-1 was restricted to the meta substituted benzoates as halogens in other positions and chloroaromatic compounds with other functional groups were not dechlorinated.

INTRODUCTION

Dechlorination of pollutant compounds in anaerobic environments, is becoming more commonly observed [1–6] but little is known about reductive dechlorination of aromatic compounds by isolated microorganisms. Only one anaerobic microorganism has been isolated that is capable of reductive dehalogenation of aryl compounds; this organism is a Gram-negative, obligately anaerobic rod and is known as strain DCB-1 [7]. The enrichment from which DCB-1 was isolated was used to determine the substrate specificity [8,9] and kinetics [10] of dehalogenation. However, more basic studies on the aryl dechlorination process are dependent on obtaining reproducible and more rapid dechlorination rates in pure culture. The purpose of this work was to identify media and culture conditions for improved dechlorination by DCB-1, and to determine

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whether the halo substrate specificity of DCB-1 was the same as for the enrichment from which it was isolated.

DCB-1 dehalogenates 3-Cl-benzoate forming benzoate [7] and chloride [11]. It is a sulfidogen and shows increased growth with thiosulfate and sulfite but not sulfate as electron acceptors [12]. Pyruvate is the only substrate on which DCB-1 can be easily maintained in culture [7,12]. DCB-1 fixes CO_2 and exhibits a mixotrophic metabolism [13]. DCB-1 can also be readily grown in coculture with the two other members of the original consortium which are needed to oxidize the benzoate produced by DCB-1 [11]. In this reconstructed consortium the H_2 produced from benzoate oxidation is apparently recycled as the reductant for dechlorination [11]. Also the reconstructed consortium produced more protein and ATP when the electron-accepting dechlorination step was available than when it was not [13], suggesting that dechlorination may contribute useful energy to DCB-1.

MATERIALS AND METHODS

Media and cultures

Strain DCB-1 was grown on the basal salt medium as described previously [7], but modified by the addition of clarified rumen fluid, trypticase or other nutrients as indicated. The standard growth medium was basal salts, 20% rumen fluid and 0.2% Na pyruvate (20.2 medium). Potential electron acceptors were added at concentrations ranging from 5 to 20 mM. The gas phase was 80% N_2–20% CO_2 (O_2-free). The media were reduced with either 0.5 mM cysteine hydrochloride and 0.5 mM $Na_2S \cdot 9H_2O$ (cysteine-SH) or 0.5 mM $Na_2S \cdot 9H_2O$.

Cultures were grown at 37°C in the dark in 160-ml serum bottles capped with butyl rubber stoppers. Inoculation was by transfer of a 10% inoculum from 14-day old cultures grown on the same medium used in the experiment. Dechlorination of the haloaromatic substrate was monitored following inoculation of the medium. Unless otherwise noted each treatment was performed in triplicate. For zero time samples the mean coefficient of variation for halo substrate concentrations was 6% for five experiments comprised of triplicate bottles. For samples in the process of dehalogenation, the mean coefficient of variation was 14%.

Halogenated benzoates and phenoxyacetates were prepared in aqueous solutions as the Na^+ salt while phenols were dissolved in water. Quantities of these stock solutions were added to individual serum bottles after filtration through syringe-mounted 0.22-μm Millipore filter units to yield the desired concentrations. Chlorinated anilines and benzonitriles were dissolved in ethanol and added to the serum bottles without sterilization. The initial medium concentration of the halo substrates was approximately 400 μM. Chloramphenicol and sulfanilamide were filter-sterilized and added to the medium to final concentrations of 20 μg/ml and 2.5 mg/ml, respectively.

For experiments where washed cells were used as inoculum, 200 to 400 ml of medium containing freshly grown cells was centrifuged anaerobically under N_2; the pellet was rinsed in fresh medium and resuspended in 60 to 120 ml of that same fresh medium. Experimental bottles received a 10% inoculum from this washed culture.

Analysis of halo substrates

Samples were taken aseptically by syringe from the serum bottle, filtered through Millipore 0.45-μm filters and frozen. Some samples were filtered through Pall filters, type Nylon-66, 0.45 μm, which were then washed with acetonitrile. Dehalogenation was monitored by a Varian Model 5000 HPLC coupled to a Hitachi 100-40 spectrophotometer equipped with an Altex 155-00 spectrophotometer flow cell. Substrate and product peaks were identified by comparison to authentic standards. Data were collected on a computing integrator.

The analytical column was a Hibar LiChrosorb, 10-μm particle size, RP-18, and the mobile phases were either 50 mM sodium acetate pH 4.5 diluted with acetonitrile or methanol, or a 70:40:13 (H_2O:CH_3OH:CH_3COOH) diluted as required with methanol. Flow rates were varied to obtain optimum quantification of the particular compound. Hydrogen concentrations were monitored

on a Carle AGC-111 gas chromatograph equipped with a 3-ml gas sampling loop as described previously [15].

RESULTS

General growth and dehalogenation conditions

Of the three temperatures tested, only 37°C supported growth and dechlorination by DCB-1 (Fig. 1). This isolate was from a sludge digestor where temperature is usually maintained at 35°C perhaps explaining the negative result at 20°C. Opening the culture to air immediately stopped dechlorination (Fig. 1) confirming that this is an obligately anaerobic process. Cultures with gas phases of 80:20 N_2:CO_2 and H_2:CO_2 that were autoclaved after inoculation all failed to dechlorinate 3-Cl-benzoate (Fig. 1); thus H_2 added to medium containing nonviable cells did not catalyze dechlorination.

When washed and concentrated thiosulfate-grown cells were used to inoculate fresh 20.2 medium and 20.2 medium containing sulfanilamide or chloramphenicol, no growth or dehalogenation was noted by either set of cultures with inhibitors, but both occurred in cultures without antibiotics. The addition of chloramphenicol to growing and actively dehalogenating cultures did not affect dechlorination until 3 days and then dechlorination ceased (Fig. 2). To test if physical support for bacterial growth such as might exist due to the presence of

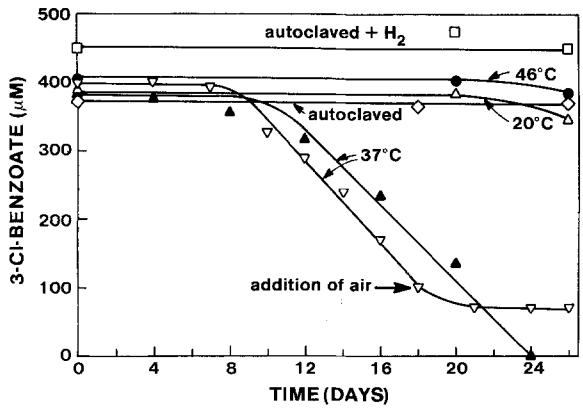


Fig. 1. The effects of autoclaving (+ or − H_2), different temperatures, and air addition (on day 18, arrow) on dehalogenation of 3-Cl-benzoate by strain DCB-1 growing in 20.2 medium.

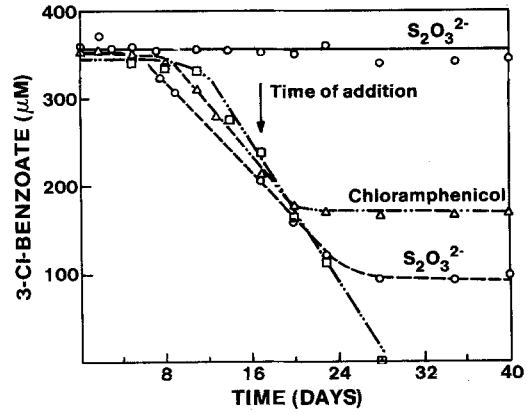


Fig. 2. The influence of thiosulfate on dehalogenation of 3-Cl-benzoate by DCB-1 growing in 20.2 medium. (—○—), 20 mM $S_2O_3^{2-}$ present at day zero; (□) no additions. At the arrow sets of cultures were amended with 20 mM $S_2O_3^{2-}$ (--○--) or with chloramphenicol (△).

organic matter flocs in digester sludge would help dehalogenation, DCB-1 was grown in the presence of glass beads (Glasperlen, 1.00 to 1.05 mm diameter). However, no difference in dehalogenation rates or lag time were noted.

Effect of thiosulfate and other electron acceptors on dechlorination

While thiosulfate increased the growth rate and yield of DCB-1 [12], it was a strong inhibitor of dehalogenation whether present in the original medium or added to actively dehalogenating cultures that were growing on pyruvate (20.2 medium) (Fig. 2). In the latter case the inhibition of dechlorination was not noted until 5 days after thiosulfate addition. Thiosulfate at concentrations of 5.0 mM or greater completely inhibited dechlorination (Fig. 3). Cells grown in the presence of 20 mM thiosulfate and exhibiting no reductive dehalogenation during growth regained the capacity to dehalogenate when washed free of thiosulfate and resuspended in new 20.2 medium.

To determine whether inhibition by thiosulfate was a general effect of electron acceptors, other electron acceptors were used to assess whether dehalogenation was also inhibited in their presence. Of the five electron acceptors studied, only the three sulfur compounds inhibited dehalogenation (Table 1).

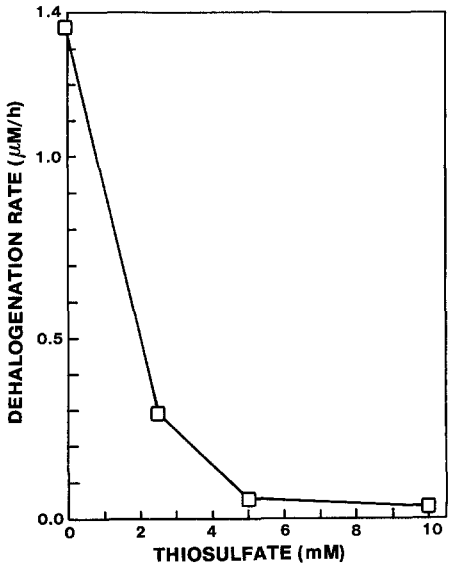


Fig. 3. Effect of thiosulfate concentration on rates of dehalogenation of 3-chlorobenzoate by DCB-1 growing in 20.2 medium.

Effects of organic and inorganic growth factors on dehalogenation

Carbon source and growth factors were altered to determine if changes would enhance or inhibit dehalogenation. Rumen fluid in concentrations of up to 20% (v/v) was the most effective medium supplement aiding both growth and dechlorination (Table 2). Neither characteristic was further enhanced by trypticase (0.1%) or casamino acid (0.1%) additions. Trypticase was only partially effective, compared to rumen fluid, in stimulating dechlorination (Table 2) and dechlorination was not as reproduc-

Table 1

Influence of several potential electron acceptors on dehalogenation of 400 μM of 3-Cl-benzoate by DCB-1 growing in 20.2 medium

Electron acceptor (5 mM)	3-Cl-benzoate remaining (%)[a] on	
	Day 20	Day 30
None	21	0
$S_2O_3^{2-}$	71	64
SO_3^{2-}	75	20
SO_4^{2-}	57	17
NO_3^-	21	0
Fumarate	14	0

[a] Values are means of six bottles from two experiments of three serum bottles each.

Table 2

Effect of rumen fluid and trypticase on the dehalogenation of 3-Cl-benzoate in a 0.2% pyruvate and basal salts medium[a]

Rumen fluid concn. and batch[b]	Trypticase	Order of lag[c]	Dehalogenation rate (μM h^{-1})	Growth[d]
20%-A	0	2	0.93	++++
10%-A	0	2	0.92	++++
5%-A	0	3	0.60	+++
1%-A	0	4	0.60	++
20%-B	0	1	1.43	++++
1%-B	0.001%	2	0.81	+++
0	0.001%	3	0.07	+
0	0.01%	3	0.78	++
0	0.1%	3	0.27	+++
20%-C	0.1%[e]	1	1.09	++++

[a] Data are means of two experiments; three serum bottles per experiment.

[b] Different batches (collection dates) of rumen fluid are designated A, B, C.

[c] Numerical value indicates the relative length of time before dehalogenation started; 1 represents the shortest lag, ~10 days.

[d] Growth was assessed by a visual estimate of turbidity with + barely more dense than the inoculum and ++++ an O.D. of approximately 0.15.

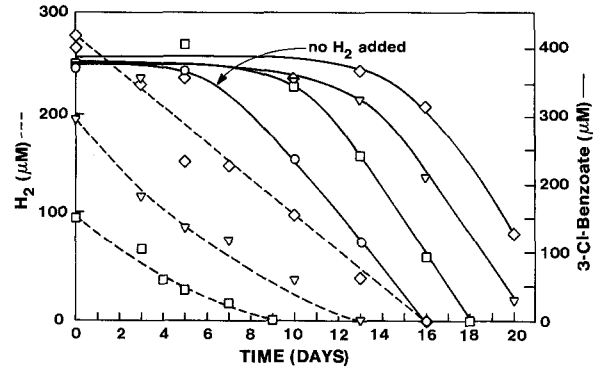
[e] Identical results were obtained if 0.1% casamino acids were substituted for trypticase.

ible as it was in the presence of rumen fluid. Also, trypticase concentrations above 0.01% inhibited dechlorination but not growth (Table 2). In the presence of rumen fluid, 0.1% trypticase did not inhibit dechlorination. No dehalogenation was observed in cultures of rumen fluid minus pyruvate (20.0 medium) or pyruvate minus rumen fluid (0.2 medium) when inoculated with thiosulfate grown cells that had been centrifuged, washed and concentrated. The batch of rumen fluid altered the rate of dehalogenation (Table 2) and the time needed to dehalogenate a given quantity of 3-Cl-benzoate. For five different batches of rumen fluid, the range of time necessary for the complete conversion of 400 μM of 3-Cl-benzoate to benzoate was 17 to 29 days. This indicates the uncertainty associated with using an undefined supplement. Nonetheless, 20% rumen fluid added to pyruvate medium was the most consistent medium for guaranteeing dehalogenation, and thus rumen fluid was used routinely in all media.

We also varied pyruvate and ammonium by a factor of 10 below that used in 20.2 medium to determine if dehalogenation of 3-Cl-benzoate was related to carbon or nitrogen nutrition. Little difference was seen in the time needed to dehalogenate 400 μm 3-Cl-benzoate (19 to 23 days) or the rate of dehalogenation (0.63 to 0.74 μmol l^{-1} h^{-1}) by altering the carbon to nitrogen ratio. In similar experiments the concentration of salts (Fe^{2+}, Mg^{2+}, Ca^{2+}) in the medium was increased 10-fold, but no consistent effect on dehalogenation was noted.

Dehalogenation in the presence of hydrogen

Since dehalogenation requires reductant and DCB-1 appears to use H_2 as its reductant [11], the role of H_2 in affecting dehalogenation was examined. Experiments comparing headspace gas composition indicated that hydrogen inhibited dehalogenation; the dehalogenation rate was 1.21 μmol l^{-1} h^{-1} under N_2:CO_2 (80:20) but only 0.63 μmol l^{-1} h^{-1} under H_2:CO_2 (80:20). In order to determine if hydrogen concentration was important to the dechlorination process, we examined the relationship between lower hydrogen concentrations and dechlorination. A direct relationship between dehalogenation and hydrogen consumption was found regardless of the initial H_2 concentration (Fig. 4). At all three H_2 concentrations, initiation of dehalogenation did not occur until the H_2 concentration dropped below 20 μM.



Dig. 4. Consumption of three concentrations (100, 200 and 280 μM) of H_2 (---) by DCB-1 growing in 20.2 medium and the correlation of H_2 removal with the onset of dechlorination (—). Lines with the same symbols are from the same H_2 addition treatment. The pattern of dechlorination in the absence of H_2 (○) is the control.

Substrate range and rate of dehalogenation

Other halogenated benzoates were dehalogenated by DCB-1, but activity was confined to the meta position. When the activity is expressed per mg protein, the rates were in the same order of magnitude as the rates found for the 3-Cl-benzoate enrichment [9]. The specificity for chemical and position (Table 3) was also similar to that of the enrichment [9].

Several non-benzoate aromatic compounds were also tested: 2-Cl-, 3-Cl- and 4-Cl-phenol; 2-Cl- and 4-Cl-phenoxyacetic acid; 2-Cl-, 3-Cl-, and 4-Cl-aniline; 3-Cl-acetophenone; 2-Cl-, 3-Cl-, and 4-Cl-benzonitrile; and 2,4,5,-trichlorophenoxyacetic acid (2,4,5-T). Also the other two isomers (ortho and para of chloro-, bromo-, and iodobenzoate) and 3-F and 4-F-benzoate were examined. At 400 μM concentrations none of these aromatic compounds was significantly dehalogenated as determined by substrate disappearance. While approximately 20% of the 2,4,5-T disappeared over 30 days, no product was found.

Table 3

Dehalogenation rate of various halogenated benzoates (BZ) by DCB-1 grown on 20.2 medium

Substrate	Product	Dehalogenation rate (μmol h^{-1} mg^{-1} protein)
3-F-BZ	none	0
3-Cl-BZ	BZ	0.093
3-Br-BZ	BZ	0.063
3-I-BZ	BZ	0.075
3,5-diCl-BZ	3-Cl-BZ	0.107
3,4-diCl-BZ	4-Cl-BZ	0.118
2,5-diCl-BZ	2-Cl-BZ	0.074
2,4-d iCl-BZ	none	0
2,6-diCl-BZ	none	0
2,3,6-triCl-BZ	2,6-diCl-BZ	0.024
5-Br-2-Cl-BZ	2-Cl-BZ	0.154
4-NH_2-3,5-diCl-BZ	4-NH_2-3-Cl-BZ	0.098

DISCUSSION

Na pyruvate (0.2%) plus rumen fluid (20%) proved to be the most reliable medium to obtain the highest rates of dechlorination. Because of the inconvenience of obtaining rumen fluid, we attempted to use trypticase as a substitute but with only partial success (only 50% of the activity found with rumen fluid at the optimum concentrations of each). Higher concentrations of trypticase (0.1%) resulted in more growth of DCB-1, but less dechlorination than with 0.01% trypticase. We note with this example and others (unpublished) a tendancy of DCB-1 to dechlorinate more readily when media are not too rich, much as if the organism needs to be forced to use a less favorable metabolic pathway. This trait is consistent with a scavenging niche for DCB-1 in nature, which has been suggested because of its mixotrophic use of carbon compounds [12,13] and its slow growth rate [7,11,12].

Finding that sulfuroxy anions inhibit dechlorination could have important ecological implications if sulfate-containing habitats such as marine environments and certain landfills and waste streams are inhibited in their capacity to dechlorinate. This implication is supported by the finding of Gibson and Suflita [16] that landfill sites containing sulfate did not exhibit dechlorination while those that were methanogenic (and thus low in sulfate) did. Since DCB-1 is a sulfidogen [12], the presence of a more favorable electron acceptor may divert the flow of electrons from dechlorination to sulfur anion reduction. If this is the mechanism it is not immediate since it took 5 days (equivalent to two doublings) before dechlorination was inhibited. Even though DCB-1 can reduce NO_3^- [7], it did not have a similar inhibitory effect on dechlorination.

DCB-1 has the ability to consume H_2 (Fig. 4) which is not unusual for a sulfidogen, but the relationship with dechlorination was surprising. Even though this reductant is apparently used for dechlorination [4], the higher concentrations used here inhibited this reaction. Perhaps this is because the production of lactate and other non-productive reduced products found when DCB-1 is grown in the presence of H_2 [13] has diverted electrons from the dechlorination process as well as restricted the organism's growth [12]. However, at the lower H_2 concentrations that would be expected in nature, H_2 should not inhibit dechlorination.

The halo aromatic substrate range known at this time for DCB-1 is limited to the benzoates. Compounds with functional groups other than COOH have been tested under the following conditions: 20.2 medium, pyruvate medium with trypticase with and without electron acceptors, added to cultures actively dehalogenating 3-Cl-benzoate (two compounds), and in medium inoculated from cultures that had just previously dehalogenated 3-Cl-benzoate; none was dehalogenated. This specificity further indicates that dehalogenation is an enzyme catalyzed process. Why the organism did not dehalogenate 2,4,5-T is surprising since the parent sludge enrichment did [17].

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