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PHYSIOLOGY OF DESULFOMONILE TIEDJEI, A STRICTLY ANAEROBIC BACTERIUM CAPABLE OF REDUCTIVE DEHALOGENATION

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PHYSIOLOGY OF DESULFOMONILE TIEDJEI, A STRICTLY ANAEROBIC BACTERIUM CAPABLE OF REDUCTIVE DEHALOGENATION

By

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William W. Mohn

Desulfomonile tiedjei (formerly strain DCB-1) is a strictly anaerobic bacterium which is capable of reductive dehalogenation of benzoates and ethylenes. This reaction is of possible significance for bioremediation of toxic wastes and pollutants. The organism is not closely related to any other known bacterium. General physiological studies were undertaken for the following purposes: (1) to better classify *D. tiedjei*, (2) to understand what physiological significance reductive dehalogenation has for this organism, (3) to devise strategies for the isolation of other dehalogenating anaerobes and (4) to better understand ecological principles which might affect reductively dehalogenating organisms in natural environments. *D. tiedjei* was determined to be a sulfate-reducing bacterium capable of reducing sulfate and thiosulfate stoichiometrically to sulfide with the following electron donors: H_2, formate, CO, lactate, pyruvate, butyrate and 3-methoxybenzoate. In the absence of an electron donor, thiosulfate was instead fermented to sulfide plus sulfate. *D. tiedjei* grew by a novel fermentation of pyruvate plus CO_2, the latter serving as an electron acceptor and being reduced to acetate. Carbon monoxide dehydrogenase activity indicated that CO_2 reduction was probably via the acetyl coenzyme A pathway. Autotrophic and diazotrophic growth were possible. 3-chlorobenzoate, which was reductively dehalogenated to benzoate, also served as an electron acceptor for energy metabolism. This reaction was stoichiometrically coupled to oxidation of formate to CO_2. *D. tiedjei* was grown on formate plus 3-chlorobenzoate in

defined medium. Resuspended cells catalyzed dehalogenation-dependent ATP synthesis. The effects of respiratory inhibitors suggest that dehalogenation and ATP synthesis are coupled via a chemiosmotic mechanism. The effects of an ATPase inhibitor and of imposed pH gradients suggest that a proton-driven ATPase is involved in the above chemiosmotic process. Thus, reductive dehalogenation appears to be a novel mode of anaerobic respiration. Isolation of other dehalogenating anaerobes was attempted using strategies based on the above findings. One isolate very similar to *D. tiedjei* was obtained, but the dehalogenating agents in the majority of enrichment cultures used were not isolated.

in the hope that our curiosity (science)
does more good than harm

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TABLE OF CONTENTS

LIST OF TABLES

Table Page

Chapter 5

LIST OF FIGURES

LIST OF FIGURES

Figure Page

Chapter 4

Chapter 5

Appendix

Chapter 1:

Reductive dehalogenation of aromatic compounds, a review

Introduction

Reductive dehalogenation is the replacement of a halogen substituent of a molecule with an hydrogen atom. In all known biological examples of this activity, the halogen is released as a halide anion. This process makes many xenobiotic compounds less toxic and more readily degradable, and appears to be the essential primary step in anaerobic degradation of halogenated aromatic compounds. Since it was first reported by Suflita et al. (1982), reductive dehalogenation by anaerobic bacteria has justifiably generated great interest due to its potential application to bioremediation of hazardous wastes. The study of reductive dehalogenation has also made significant contributions to basic microbiology in the areas of ecology, physiology and phylogeny. The study of reductively dehalogenating bacteria is essential for their exploitation by humans, but is also intriguing from a more fundamental scientific perspective.

Recent studies have shown that many of our most problematic pollutants are susceptible to biological reductive dehalogenation. Anaerobic reductive dehalogenation is the only known biodegradation mechanism of certain significant environmental pollutants (e.g., highly chlorinated biphenyls, hexachlorobenzene and tetrachloroethylene). This process appears to occur within mutualistic anaerobic microbial communities. Such communities frequently are able to adapt to growth on halogenated aromatic compounds. Using undefined cultures, researchers are beginning to understand the ecological factors which influence reductive

dehalogenation. However, the individual organisms which catalyze the process remain elusive, and only a single anaerobe capable of reductive dehalogenation of aromatic compounds is currently in pure culture. This organism, *Desulfomonile tiedjei* (formerly strain DCB-1), represents a new genus, with a morphology and mode of division singular among described bacteria, and with unusual catabolic abilities. *D. tiedjei* has the novel ability to gain energy for growth from reductive dechlorination. The uniqueness of *D. tiedjei* and the difficulty in isolating other anaerobes capable of reductive dehalogenation suggest that continued study of this process may lead to the discovery of other novel and interesting organisms. Finally, certain aerobic bacteria are now also known to employ reductive dehalogenation steps in degradation pathways for halogenated aromatic compounds.

Scope

Tiedje et al. (1987) have reviewed literature on the subject of anaerobic reductive dechlorination of aromatic compounds prior to 1986. Reductive dehalogenation has also been included as a part of general reviews on biodegradation (Reineke & Knackmuss, 1988; Sahm et al., 1986). The recent review by Kuhn and Suflita (1989) extensively covers anaerobic degradation, including reductive dehalogenation, of pesticides in soils and groundwater. These previous reviews have thoroughly described the range of substrates for this activity and habitats where the activity is found, and here only the most important and new information on these subjects will be summarized. The primary purpose of this review is to examine the ecological and

physiological principles which are beginning to emerge from the study of reductive dehalogenation of aromatic compounds. Where possible, studies with undefined anaerobic communities are related to others using pure cultures. Reductive dehalogenation of volatile alkyl solvents (e.g., tetrachloroethylene) is not addressed, although this activity has not been proven to be distinct from reductive dehalogenation of aromatic compounds.

Range of activity

Microbes from a variety of anaerobic habitats reductively dehalogenate a great variety of aromatic compounds (Table 1). Initial studies focused on relatively simple model compounds (e.g., halobenzoates and halophenols) and are described in the above reviews. Recent studies have found that the same activity can transform compounds which are considered more significant as pollutants (e.g., polychlorinated phenols, polychlorinated benzenes and polychlorinated biphenyls). The latter compounds tend to be more complex, less water-soluble and more toxic. These findings greatly increase the significance of reductive dehalogenation from an applied perspective.

Recently reported substrates

Polychlorinated biphenyls (PCBs) are currently of great concern due to their recalcitrance and toxicity. Laboratory studies have now demonstrated that the more highly chlorinated PCB congeners in mixtures such as Aroclors 1242, 1248, 1254 and 1260 can be reductively dechlorinated by anaerobic microorganisms from PCB-

TABLE 1. Aromatic substrates known to be reductively dehalogenated by organisms from anaerobic environments

Substrate	Inoculum	First report
Benthiocarb[1]	Paddy soils	Moon & Kuwatsuka, 1984
Bromacil[2]	Aquifer slurry	Adrian & Suflita, 1990
Bromophenol	Marine sediment	King, 1988
Chloroanilines	Aquifer slurry	Kuhn & Suflita, 1989
Chloroanilines	Pond sediment	Strijs & Rogers, 1989
Chlorocatechols	Reactor column	Hakulinen et al., 1982
Chloroguaiacols	Reactor column	Hakulinen et al., 1982
Chloronitrofen[3]	Paddy soil	Yamada & Suzuki, 1983
Chlorophenols	Sewage sludge	Boyd et al., 1983
Chlorophenoxyacetates	Sewage sludge	Mikesell & Boyd, 1985
Chlorophenoxyacetates	Aquifer slurry	Gibson & Suflita, 1986
Chlorophenoxyacetates	Pond sediment	Gibson & Suflita, 1986
Chlororesorcinol	Sewage sludge	Fathepure et al., 1987
Diuron[4]	Pond sediment	Attaway et al., 1982
Halobenzoates	Lake sediment	Suflita et al., 1982
Halobenzoates	Sewage sludge	Suflita et al., 1982
Hexachlrobenzene	Sewage sludge	Fathepure et al., 1988
Pentachlorophenol	Paddy soil	Ide et al., 1972
Polybrominated biphenyls	River sediment	Quensen et al., 1990
Polychlorinated bipyenyls	River sediment	Quensen et al., 1988
Propanil[5]	Pond sediment	Stepp et al., 1985
Techloftham[6]	Paddy soil	Kirkpatrick et al, 1981
TPN[7]	Flooded soil	Sato & Tamaka, 1987
Trichlorobenzene	Rat gut	Tsuchiya & Yamaha, 1984
Trichlorobenzene	River sediment	Bosma et al., 1988

[1]S-4-chlorobenzyl-N,N-diethyl thiocarbamate
[2]5-bromo-3-*sec*-butyl-6-methyl uracil
[3]4-nitrophenyl-2,4,6-trichlorophenyl ether
[4]3-(3,4-dichlorophenyl)-1,1-dimethyl urea
[5]N-(3,4-dichlorophenyl) propanamide
[6]N-(2,3-dichlorophenyl)-3,4,5,6-thtrachlorophthalamic acid
[7]2,4,5,6-tetrachloroisophthalonitrile

contaminated river sediments (Alder et al., 1990; Quensen et al., 1988, Quensen et al., 1990a). These studies support previous evidence for *in situ* activity in anaerobic sediments (Brown et al., 1984, Brown et al., 1987a, Brown et al., 1987b). Reductive dehalogenation activity was nearly entirely at the *meta* and *para* positions, and the major products were mono- and dichlorobiphenyls. Significant detoxication of the Aroclors resulted since the more highly chlorinated congeners are more toxic (Safe et al., 1982). Different patterns of dechlorination were observed in various cultures (Quensen et al., 1990a), similar to patterns previously elucidated for activity in natural sediments (Brown et al., 1984, Brown et al., 1987a, Brown et al., 1987b). The observation of these patterns has led to speculation that distinct organisms may exist having individual dehalogenation activities. The products of anaerobic PCB degradation can be mineralized aerobically (Bedard et al., 1987), suggesting that a combination of anaerobic and aerobic microbial communities might mineralize all PCB congeners. Biological reductive dehalogenation of polybrominated biphenyls has also been demonstrated very recently (Quensen et al., 1990b).

Hexachlorobenzene (HCB) and other chlorobenzene congeners are also widespread pollutants of very low water solubility. Fathepure et al. (1988) have shown reductive dechlorination of HCB to 1,3,5-trichlorobenzene and small amounts of dichlorobenzenes in stationary incubations of anaerobic sewage sludge. Dehalogenation of HCB in incubations of sediments has also been reported (Mousa and Rogers, 1990). In the latter study, different inoculum sources exhibited

different dehalogenation patterns, one resembling that of the former study and another yielding penta-, 1,2,3,4- and 1,2,3,5-tetra-, 1,2,3-tri- and 1,2-dichlorobenzene. Using the three trichlorobenzene isomers as substrates, Bosma et al. (1988) showed that anaerobic river sediment in upflow columns could reductively dechlorinate all tri- and dichlorobenzene isomers, yielding di- and monochlorobenzenes. Di- and monochlorobenzenes can be mineralized aerobically (De Bont et al., 1986; Schraa et al, 1986; Spain and Nishino, 1987; Van der Meer et al., 1987); thus, as in the case of PCBs, the proper sequence of conditions may allow biological mineralization of all chlorobenzene congeners.

Recent reports indicate that chloroanilines, which are used in industrial syntheses, can be reductively dechlorinated by organisms from aquifer material and pond sediment (Kuhn & Suflita, 1989; Strijs & Rogers, 1989). In both reports, more highly chlorinated anilines were dechlorinated, but monochloroanilines persisted. Chlororesorcinols are possible by-products of industrial syntheses, and 4-chlororesorcinol has been shown to be reductively dechlorinated in anaerobic sewage sludge (Fathepure et al., 1987a). The resorcinol product subsequently disappeared from enrichment cultures from the sludge, suggesting that anaerobic communities may be capable of mineralizing 4-chlororesorcinol.

In the first report of biological reductive dehalogenation of a heterocyclic compound, Adrian and Suflita (1990) clearly demonstrated removal of bromine from the herbicide, bromacil, by aquifer slurries

(Figure 1). No further evidence concerning the fate of the debrominated product was given.

Not all haloaromatic compounds are xenobiotic. The burrows of a hemichordate inhabiting marine sediment were found to contain 2,4-dibromophenol (King, 1986). The worm apparently synthesizes the compound which inhibits growth of aerobic bacteria in the burrow. Subsequently it was shown that an anaerobic microbial community from the sediment could first debrominate and then mineralize this compound (King, 1988). These important studies are the first to examine anaerobic metabolism of numerous naturally occurring haloaromatic compounds. The existence of such compounds suggests that selective pressure for reductive dechlorination may have existed during the evolution of anaerobic bacteria. Further examination of the metabolism of such natural compounds will likely contribute greatly to our understanding of the metabolism of xenobiotic compounds.

Undefined cultures

Like most anaerobic processes, anaerobic reductive dehalogenation has typically been found to occur in syntrophic communities. It has proved very difficult to obtain pure cultures with the activity. Ecological understanding of these communities is thus critical for any applied use of the activity or investigation of the individual organisms responsible for the activity. Laboratory investigations with undefined anaerobic communities have been employed in the study of reductive dehalogenation out of necessity.

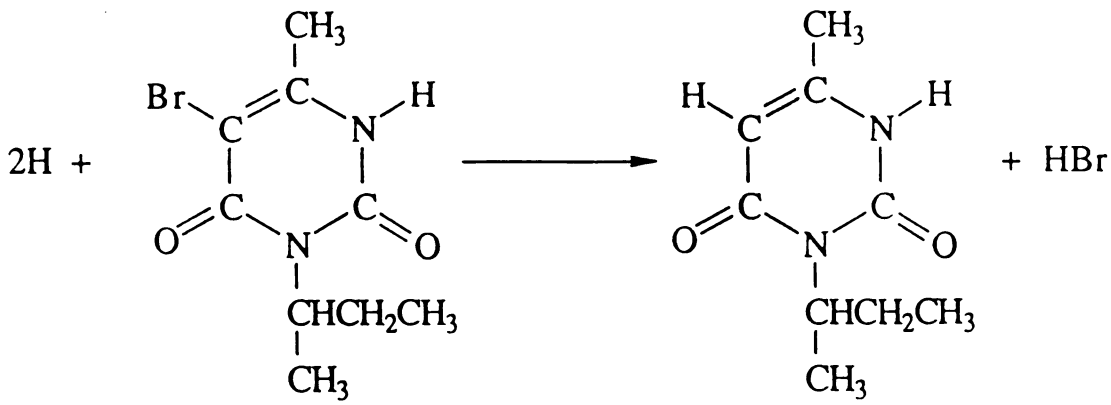


FIGURE 1. Reductive dehalogenation of the heterocyclic compound, bromacil.

This approach can identify ecological factors affecting reductive dehalogenation, but is of limited value in determining the mechanisms of these effects. This approach does have predictive value, since undefined cultures are more likely than pure cultures to behave like populations in natural habitats or habitats which may be derived for bioremediation. Recent contributions to our understanding of the ecology of anaerobic reductive dehalogenation are described below.

UNIFYING PRINCIPLES

Several general principles concerning anaerobic reductive dehalogenation were established by early work with chlorobenzoates and chlorophenols (Boyd and Shelton, 1984; Boyd et al., 1983; Horowitz et al., 1983; Suflita et al., 1982), and, as will be shown, these principles have been corroborated by more recent studies. Almost all activity is biological and can be killed by autoclaving. Horowitz et al. (1983) also showed that activity could be killed by gamma radiation, formaldehyde and oxygen. Reductive dehalogenation has always been found to be the primary step in anaerobic degradation of haloaromatic compounds with the dehalogenated product persisting or appearing as a transient intermediate. Natural samples often exhibit acclimation periods of months before dehalogenation occurs. Substrate specificity, both for the type of aromatic compound (i.e., benzoate, phenol) and for the halogen position, is often observed for acclimation. Enrichment of the activity is frequently possible, and often the haloaromatic compound can serve as a sole carbon and energy source for such a culture. The above phenomena have led to the

hypothesis that the reductive dehalogenation of aromatic compounds is catalyzed by individual organisms with enzymatic specificities for certain haloaromatic compounds; however, until the individual organisms are isolated, other possible explanations remain. Specificities could reside at the level of enzymes, organisms or broad physiological groups.

ENRICHMENT

All anaerobic communities are not equal with respect to potential for reductive dechlorination. Certain communities, often in polluted habitats, are adapted to certain xenobiotic compounds. Thus, inocula from PCB-contaminated river sediments dechlorinated PCBs while inocula from uncontaminated sediments did not during prolonged incubations (Quensen et al., 1988). Polluted river and estuary sediments had greater potential than unpolluted ones for dehalogenation of monochlorophenols and monochlorobenzoates (Sharak Genthner et al., 1989a). Sewage sludges also showed varied potentials for those activities (Shelton and Tiedje, 1984a). Additionally, by serial transfer of laboratory cultures, it has been possible to enrich for dehalogenation of a number of substrates, including halobenzoates (Sharak Genthner et al. 1989a; Shelton and Tiedje, 1984b), halophenols (Sharak Genthner et al. 1989a; Zhang and Wiegel, 1990), 4-chlororesorcinol (Fathepure et al., 1987a), dichloroanilines (Struijs and Rogers, 1989) and PCBs (unpublished data). Except for dichloroanilines and PCBs, these substrates apparently served as sole carbon and energy sources. Adaptation and enrichment are common observations in microbial cultures, but they

are not trivial observations for xenobiotic substrates. One would not necessarily expect such substrates to support growth, especially in cases, such as PCBs, HCB and chloroanilines, where reductive dehalogenation is not followed by degradation of the remaining hydrocarbon compound. In these cases, adaptation and enrichment clearly indicate selective pressure for reductive dehalogenation *per se*. Such selective pressure could be positive (e.g., for use as a substrate) or negative (e.g., for detoxication).

ACCLIMATION

Linkfield et al. (1989) have examined the acclimation period preceding reductive dehalogenation of halobenzoates. The periods of acclimation were reproducible for various compounds and inoculum sources. These authors identify the following possible explanations for the periods of acclimation (1) genetic change, (2) induction, (3) exhaustion of a preferred substrate (diauxy) or (4) growth of the active population from very low initial numbers. It was suggested that induction best explains the observed patterns. Genetic change was considered unlikely due to the reproducibility of the acclimation periods. The acclimation periods were determined to be too long for growth of the active population; although, this determination was based on the questionable assumption that the specific growth rate of the dechlorinating population would be approximately that of strain the isolate, *D. tiedjei*, in pure culture. A diauxy response was considered unlikely because sediment stored for two years at 4°C exhibited the same acclimation period as fresh sediment. However, Kohring et al. (1989a) found that storage of sediment for two months

at 12°C increased acclimation time. The latter finding would also not be consistent with a diauxy response, as storage should then decrease acclimation time, but would be consistent with an acclimation period due to growth of a dehalogenating population which decreased in viability during storage. Such an effect on viability might not occur during storage at 4°C as in the former study (viability of anaerobic cultures can be remarkably stable during storage at low temperatures). It is not clear that the acclimation periods do not have different causes in the different sediments studied or combinations of the above mentioned causes. Acclimation periods required for anaerobic reductive dehalogenation may be very long (6 mo or longer) and must be allowed for in studies of this activity. Our present understanding of the acclimation periods preceding reductive dehalogenation by natural communities is highly speculative.

ELECTRON ACCEPTORS

Electron acceptors are frequently the limiting nutrient for anaerobic communities, typically being a major determinant of the structure of these communities; therefore, the presence of reductively dechlorinating organisms in a community may be affected by electron acceptors. Furthermore, it might be expected that the availability of electron acceptors might affect the flow of electrons required for reductive dehalogenation. This effect might occur via intracellular channelling of electrons or via interspecific competition for electron donors. Accordingly, evidence is accumulating which indicates that electron acceptors do affect

dehalogenation activity in anaerobic communities. However, this relationship appears to be complex.

The laboratory of J. M. Suflita has examined two closely located sites within an aquifer contaminated by landfill leachate (Beeman and Suflita, 1987; Gibson and Suflita, 1986; Suflita and Miller, 1985). The sites differed in being dominated by either methanogenesis or sulfate reduction. Only samples from the methanogenic site demonstrated the ability to dechlorinate and mineralize chlorobenzoates, chlorophenols and chlorophenoxyacetates. The potential for dehalogenation existed at both sites with sulfate apparently inhibiting dechlorination in samples of the sulfate-reducing site, since addition of sulfate inhibited dehalogenation of 2,4,5-trichlorophenoxyacetate by samples from the methanogenic site, and since depletion of sulfate by addition of acetate (as an electron donor) allowed this activity by samples from the sulfate-reducing site. The occurrence of methanogenesis did not insure the activity, since stimulation of methanogenesis in samples from the sulfate-reducing site by addition of methanol (as a substrate only used by methanogens) did not allow the activity. This does not prove that methanogenesis from other substrates would not cause dehalogenation. Additionally, amendment of aquifer slurries with nitrate or sulfate was found to inhibit debromination of the heterocyclic ring of bromacil (Adrian and Suflita, 1990). These data clearly indicate inhibition of dehalogenation by sulfate in this aquifer. However, this conclusion should be extrapolated to other habitats or xenobiotic compounds with caution.

Kohring et al. (1989b) examined dechlorination of phenols by samples from freshwater sediments. Addition of nitrate to samples completely inhibited dechlorination of 2,4-dichlorophenol. Sulfate increased the adaptation time, decreased the rate of the initial dechlorination at the *ortho* position, and prevented dechlorination of the 4-chlorophenol product. When samples were first acclimated to 4-chlorophenol without sulfate, subsequently added sulfate was reduced to sulfide and had little effect on dechlorination of 4-chlorophenol. In this case, it appears that sulfate does not inhibit reductive dehalogenation directly, but rather, sulfate inhibits enrichment of the activity.

In a broader survey, Sharak Genthner et al. (1989a and 1989b) tested the effects of sulfate and nitrate on degradation of monochlorobenzoates and monochlorophenols by samples from a variety of sediments taken from river and estuary locations. Added sulfate and nitrate, generally inhibited degradation by the samples, but there were several exceptions. An inhibitor of methanogenesis, bromoethane sulfonate (BES), also generally inhibited degradation by the samples. This finding may imply that methanogenesis is required for degradation for various possible reasons including (1) methanogens simultaneously metabolize natural substrates and the xenobiotic compounds, or (2) the dehalogenating organisms are dependent on methanogens. However, the effect of BES on methanogenesis was not verified, and a direct effect on dehalogenation was not excluded. Sulfate and nitrate more consistently inhibited degradation in transfers of the original

samples, but there were still notable exceptions. Transfers of one sample required nitrate for degradation of 3- and 4-chlorobenzoate, another transfer was stimulated by sulfate in the degradation of 4-chlorophenol. Thus, electron acceptors have variable effects on reductive dechlorination which are subject to biological and chemical variables.

In river sediments incubated under sulfate reducing conditions, all three monochlorophenols and 2,4-dichlorophenol were degraded (Haggblom et al., 1990). In these cultures sulfate reduction appeared to be required for degradation, as an inhibitor of sulfate reduction, molybdate, inhibited degradation.

In the previously mentioned study of 2,4-dibromophenol in marine sediment (King, 1988), molybdate did not affect debromination but did prevent degradation of the phenol product. The effect of the inhibitor on sulfate reduction was not verified, but the effect on phenol degradation probably indicates that sulfate reduction was blocked. It is not clear that, as the author suggests, sulfate reducers were not responsible for debromination, since the specific blockage of ATP sulfurylase by molybdate would not stop all other activities of these organisms.

The evidence available suggests that nitrate and sulfate most often inhibit dehalogenation by anaerobic communities, but the nature of this inhibition varies. Direct inhibition of the dehalogenation process by electron acceptors appears to occur in some communities; while in others, electron acceptors apparently select for a nondehalogenating population. A nondehalogenating population might

outcompete dechlorinators by virtue of a higher growth rate, but once established, certain dechlorinating populations apparently can compete successfully for electron donors. In a minority of cases tested, sulfate or nitrate did not inhibit dehalogenation or were even required for activity. The effects on dehalogenation of other electron acceptors such as iron, manganese and carbon dioxide have yet to be examined.

Other nutrients

In contrast to the effects of electron acceptors, the effects of other nutrients on reductive dehalogenation have not been examined extensively. In most studies of dehalogenation using undefined communities, specific electron donors and carbon sources were not added but were presumably supplied by the source material (i.e., sediment, sludge, aquifer solids) in complex and rather recalcitrant forms. When halogenated compounds were oxidized after dehalogenation, they also provided electrons and carbon. In several of the studies detailed above, enrichments were serially transferred, mineralizing haloaromatic compounds and using them as sole carbon and energy sources. Added nutrients can stimulate dehalogenation activity or enrichment of that activity as demonstrated for 4-chlororesorcinol (Fathepure et al., 1987a) and PCBs (unpublished data). In the case of 4-chlororesorcinol dehalogenation in sewage sludge enrichments, yeast extract, trypticase, rumen fluid, glucose, sludge supernatant and resorcinol, in order of decreasing effect, stimulated dehalogenation activity. Thus, the enrichments appeared to be limited by some or all of the following: carbon, electron

donor, amino acids and micronutrients. Of course, certain nutrients may also select against dehalogenating populations. It would be of value to determine nutrients which limit dehalogenation; however, studies with undefined cultures are severely limited in such determinations.

Temperature

Temperature might be expected to affect reductive dehalogenation by a direct effect on reaction rates and by selective pressure on populations. Both effects were indicated in a study of lake sediment samples to which 2,4-dichlorophenol was added (Kohring et al., 1989a). Temperature affected both the acclimation period and the rate of dechlorination activity. Activity was found between 5 and 50°C with distinct rate peaks at 30°C and 43°C, suggesting selection for two distinct dehalogenating populations. A direct correlation of temperature and dehalogenation rate was found only from 15 to 30°C. Thus, it may be difficult to extrapolate laboratory rate measurements at higher temperatures to natural habitats which are below 15°C. Clearly temperature severely limits dehalogenation rates in many habitats.

Substrate availability

The hydrophobicity of many haloaromatic compounds certainly affects their biological dehalogenation. The availability of the substrates to dehalogenating organisms will have a direct effect on dehalogenation rates. In addition, because of the toxicity of many of these substrates, availability may also have an inhibitory effect on dehalogenation rates. A large body of literature describes the

various nonbiological fates of haloaromatic compounds in natural habitats (e.g., Chiou, 1989; Hassett and Banwart, 1989). Here it will simply be pointed out that fates such as sorption and volatilization will affect the aqueous concentration of these compounds. Additionally, physical surfaces may juxtapose sorbed hydrophobic compounds and microbes attached to the surfaces. Studies of the dehalogenation of very hydrophobic aromatic compounds in liquid cultures have employed various carriers for these compounds, including a liquid organic phase (Holliger et al., 1989,) or sediments (Quensen et al., 1988). In the above studies, the carriers were probably required to enhance availability. Dispersants have been developed for delivery of hydrophobic antibiotics. These are molecules designed to be water-soluble but to have a hydrophobic interior region which may contain a hydrophobic compound. Such compounds may have potential for use in studies of aerobic biodegradation of hydrophobic compounds. Studies of reductive dehalogenation have not yet addressed optimization of the availability of haloaromatic compounds.

Conclusion

Studies using undefined cultures clearly indicate the potential of anaerobes to reductively dehalogenate aromatic compounds. Such studies also indicate ecological factors detailed above which significantly affect activity. Often results did not distinguish between direct effects on activity and effects on population selection. Results were not always consistent with different inocula and different haloaromatic substrates; therefore, in these cases it

is not yet reasonable to generalize conclusions. It seems likely that reductive dechlorination is catalyzed by physiologically diverse organisms in diverse anaerobic communities. In many cases a better understanding of ecological factors will require pure culture studies. Paradoxically, isolation of more reductively dehalogenating anaerobes probably depends on better understanding of their ecology.

The majority of the studies described above involved sealing natural samples in serum bottles and incubating them in the laboratory. These cultures thus differed from *in situ* conditions in input of soluble nutrients, removal of soluble products, temperature and other aspects. Such experiments have logistical advantages and adequately address certain questions, but empirical knowledge of predictive value for natural and derived habitats requires experiments which more closely approximate those habitats. The latter experiments have yet to be undertaken.

Desulfomonile tiedjei

While reductive dehalogenation appears to be most favored in syntrophic communities, the above studies using undefined cultures indicate that certain aspects of the process will only be understood when the responsible organisms are studied in pure culture. Pure culture studies may answer essential questions including (1) which organisms have dehalogenation activity? (2) which enzymes and cofactors are responsible for the activity? (3) what is the chemical mechanism involved in activity? (4) how do various factors directly affect activity? and (5) how does the activity benefit (or harm) the responsible organisms? In addition to satisfying basic scientific

interest, answers to such questions are of obvious importance in applications of reductive dehalogenation.

At present *D. tiedjei* (formerly strain DCB-1) represents the only opportunity to study anaerobic reductive dehalogenation of aromatic compounds in pure culture. This organism is able to dehalogenate benzoate or certain of its analogs, with activity preferentially directed to the *meta* position (Figure 2; J.R. Cole, 1990, personal communication; DeWeerd et al., 1986; Shelton and Tiedje, 1984b). *D. tiedjei* also dechlorinates tetrachlorethylene (Fathepure et al., 1987b). In addition to dehalogenation activity, this organism has several unique morphological and metabolic characteristics which are of more general microbiological interest. The uniqueness of *D. tiedjei* suggests that further study of reductive dehalogenation will involve the challenges as well as the rewards of studying novel organisms.

Morphology/cell division

A unique morphological feature of *D. tiedjei* is a collar which girdles each cell (see Appendix, Figures 1-4). This collar consists of a region where the cell wall folds over itself (Shelton and Tiedje, 1984b). The collar is the origin of polar cell growth and cell division (Mohn et al., 1990).

General physiology

Initially *D. tiedjei* could only be cultured on an undefined medium including rumen fluid, and pyruvate was the only substrate found to significantly support growth (Shelton and Tiedje, 1984b).

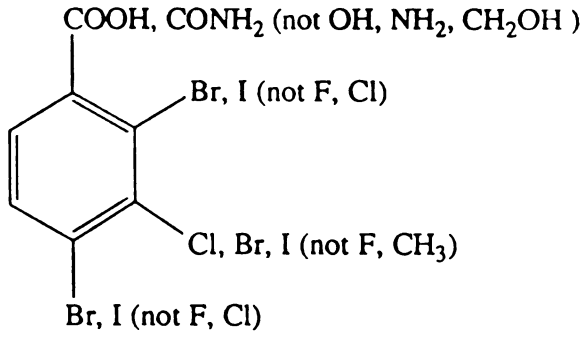


FIGURE 2. Aromatic substrates for reductive dehalogenation by *Desulfomonile tiedjei*: one of the substituents shown in the one position is required, and the halogen substituents shown can be removed.

Thiosulfate was latter found to stimulate growth, suggesting that the organism might be a sulfate-reducing bacterium (Stevens et al., 1988). Growth on pyruvate was found to be mixotrophic, involving CO_2 fixation (Stevens and Tiedje, 1988). Identification of vitamins stimulatory to *D. tiedjei* (Apajalahti et al., 1989; DeWeerd et al., 1990a) has permitted studies using defined media which have greatly improved knowledge of the metabolic characteristics of this organism. *D. tiedjei* exploits several catabolic electron acceptors. The organism is a *bona fide* sulfate-reducing bacterium (Mohn and Tiedje, 1990b), having the usual ability to reduce sulfate or thiosulfate to sulfide (Equations 1 and 2) as well as the ability, in the absence of a suitable electron donor, to gain energy from the disproportionation of thiosulfate to sulfide plus sulfate (Equation 3). Growth by the latter lithotrophic fermentation is apparently possible for only a few of the sulfate reducers presently in pure culture (Kramer & Cypionka, 1989). *D. tiedjei*, like *Desulfotomaculum* spp. is more sensitive to sulfide than other sulfate reducers. Sulfide typically limits growth of *D. tiedjei* on sulfur compounds in batch cultures and sulfide production is usually less than 3 mM (Mohn & Tiedje, 1990b). This sensitivity would select against *D. tiedjei* during routine enrichment and isolation of sulfate reducers and may contribute to the lack of similar isolates.

$$4\ HCOOH + SO_4^{=} + H^+ \rightarrow 4\ CO_2 + HS^- + 4\ H_2O \quad (1)$$

$$4\ HCOOH + S_2O_3^{=} \rightarrow 4\ CO_2 + 2\ HS^- + 3\ H_2O \quad (2)$$

$$S_2O_3^{=} + H_2O \rightarrow HS^- + SO_4^{=} + H^+ \quad (3)$$

D. tiedjei also grows by an unusual fermentation of pyruvate plus CO_2 (Mohn & Tiedje, 1990b). The fermentation involves the oxidation of pyruvate to acetate plus CO_2 (Equation 4) and the reduction of CO_2 to acetate (Equation 5), with the oxidative and reductive processes balancing one another (Equation 6). In many respects this resembles the terminal steps in homoacetogenic fermentation of sugars; however, *D. tiedjei* is unable to use sugars. Like homoacetogens, *D. tiedjei* has carbon monoxide dehydrogenase activity and is believed to employ the acetyl-CoA pathway for CO_2 reduction. Unlike many homoacetogens, *D. tiedjei* cannot grow on H_2 plus CO_2 or formate plus CO_2.

$$4\ CH_3COCOOH + 4\ H_2O \rightarrow 4\ CH_3COOH + 4\ CO_2 + 8\ H \quad (4)$$

$$8\ H + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O \quad (5)$$

$$4\ CH_3COCOOH + 2\ H_2O \rightarrow 5\ CH_3COOH + 2\ CO_2 \quad (6)$$

Reductive dehalogenation is yet another reaction exploited by *D. tiedjei* for energy metabolism. Dolfing and Tiedje (1986) constructed a defined consortium including *D. tiedjei* which used 3-chlorobenzoate (3CB) as a sole substrate. Growth of the consortium was stimulated by dechlorination (Dolfing and Tiedje, 1987). Subsequently, dechlorination was found to stimulate growth of *D. tiedjei* in pure culture (Dolfing, 1990; Mohn and Tiedje, 1990a). Dechlorination was coupled to formate oxidation (Figure 3), or, probably, H_2 oxidation (Mohn and Tiedje, 1990a). If other dehalogenating anaerobes can also employ this novel form of chemotrophy, it may support the previously mentioned enrichment of

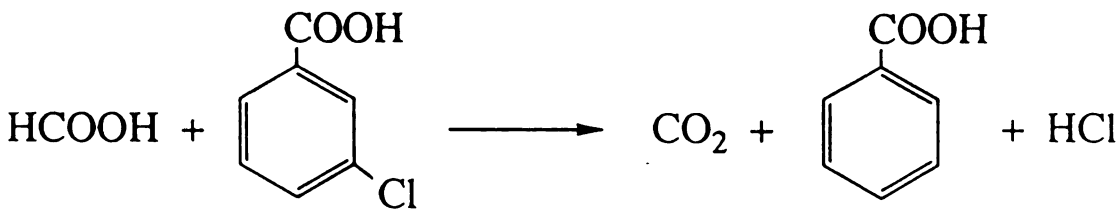


FIGURE 3. Reductive dehalogenation of 3-chlorobenzoate coupled to formate oxidation by *Desulfomonile tiedjei*.

cultures which reductively dehalogenate substrates which are not further degraded (e.g., PCBs or HCB).

The coupling of dehalogenation to formate or H_2 oxidation suggests that energy conservation is via a respiratory process, since neither formate or H_2 is known to support substrate-level phosphorylation. Like other sulfate reducers, *D. tiedjei* appears to have several respiratory electron carriers. Desulfoviridin and cytochrome c_3 were isolated from *D. tiedjei* (DeWeerd et al., 1990a). Naphthoquinone (or menadione) is required by *D. tiedjei* for dehalogenation but not for fermentative growth (J. Apajalahti, 1989, personal communication). It is not reported whether the vitamin is also required by *D. tiedjei* for sulfoxy anion metabolism; although the vitamin stimulates growth on pyruvate plus thiosulfate (DeWeerd et al., 1990a). In addition to their probable role in sulfate reduction, some or all of the above electron carriers may participate directly in dehalogenation or in respiratory energy conservation from dehalogenation. Dechlorination directly supported ATP synthesis in stationary phase cultures which were limited by 3CB (Dolfing, 1990) and in cell suspensions (see Chapter 4). In the latter system, the effects respiratory inhibitors and imposed pH gradients suggest that dechlorination and ATP synthesis are coupled via a chemiosmotic mechanism involving a proton-driven ATPase. Thus, reductive dechlorination may support a novel mode of anaerobic respiration.

D. tiedjei appears to have a relatively limited range of electron donors. In addition to pyruvate, formate and H_2 mentioned above, *D. tiedjei* oxidizes CO, lactate, butyrate (Mohn and Tiedje,

1990b) 3- and 4-methoxy benzoates and their derivatives, and benzoate (DeWeerd et al., 1990a). Acetate appears to be used, but only slowly (DeWeerd et al.,1990a; Mohn and Tiedje, 1990b). Oxidation of methoxy benzoates is via O-demethylation to corresponding hydroxy benzoates (DeWeerd et al., 1986) which are not further degraded (Mohn and Tiedje, 1990b). The latter activity is characteristic of organisms with the acetyl-CoA pathway. The electron donor range of *D. tiedjei* is typical of sulfate reducing bacteria, and could allow *D. tiedjei* a terminal position in anaerobic food chains, using products of fermentative organisms.

INDUCTION OF DEHALOGENATION

Initially it was reported that dehalogenation activity in *D. tiedjei* was dependent on growth in the presence of 3CB (DeWeerd and Suflita, 1989). More recently *m*-halobenzoates or analogs were also found to specifically induce activity (Cole and Tiedje, 1990). There are a number of inducers which are not substrates (gratuitous) as well as a number of substrates which are not inducers (Figures 2 and 4; J.R. Cole, 1990, personal communication). Inducers must be *meta*-substituted, but dehalogenation activity can also act at the *ortho* and *para* positions. Inducers can have certain *meta*-substituents which are not transformed (e.g., F, CH_3, CF_3). If the lack of induction by certain substrates is common, the failure to detect activity is not proof that organisms having activity are not present. Thus, dehalogenating organisms could possibly be undetected in experiments using current methodology.

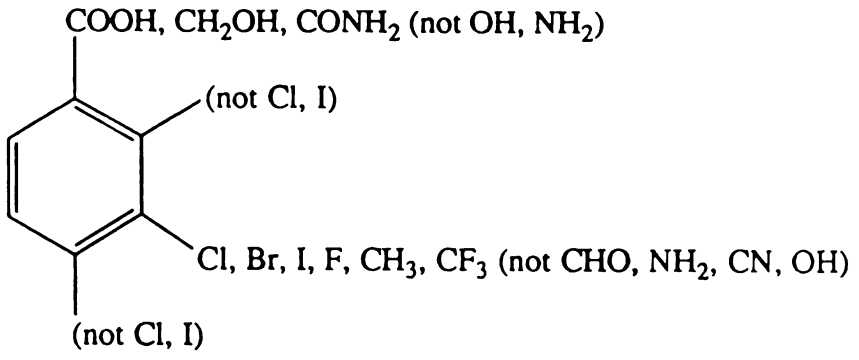


FIGURE 4. Inducers of reductive dehalogenation activity in *Desulfomonile tiedjei*: one of the substituents shown in both the one and three positions is required.

Inhibition of dehalogenation by sulfoxy anions

The relationship between dehalogenation and metabolism of sulfoxy anions by *D. tiedjei* is not simple. Thiosulfate and sulfite inhibit dechlorination of 3CB by growing cells (DeWeerd et al, 1986; Linkfield and Tiedje, 1990) and by resuspended cells (DeWeerd et al., 1990b). Sulfate inhibited dechlorination of 3CB by cells growing on one medium (Linkfield and Tiedje, 1990) but not by cells growing on a different medium (J.R. Cole, 1989, personal communication) or by resuspended cells (DeWeerd et al., 1990b). The latter authors found that both 3CB and sulfoxy anions could support H_2 consumption by resuspended cells, the latter at a higher rate. With both electron acceptors present an intermediate rate of H_2 consumption was observed, suggesting to these authors that dechlorination and sulfoxy anion reduction are enzymatically distinct pathways which compete for limited electron donors. The above results suggest that inhibition of dehalogenation by thiosulfate and sulfite observed in undefined cultures may occur via intraspecific channelling of electrons to electron acceptors. However, if dehalogenating organisms in such undefined cultures resemble *D. tiedjei*, inhibition by sulfate must involve interspecific competition for electron donors.

Taxonomy

DeWeerd et al. (1990a) determined the 16S rRNA sequence of *Desulfomonile tiedjei* which clearly indicates that the organism is a member of the delta subdivision of the class Proteobacteria (purple bacteria). The degree of distance between *Desulfomonile tiedjei* and *Desulfovibrio desulfuricans* was greater than between *Desulfomonile*

tiedjei and *Desulfuromonas acetoxidans* (an elemental sulfur-reducing bacterium) or *Desulfobacter* spp. It was concluded from the 16S rRNA sequence that *Desulfomonile tiedjei* represents a new genus among the sulfate-reducing bacteria, although comparisons were only made with three other species of that group. The unique physiological characteristics of *D. tiedjei* (above) and the sequence analysis led DeWeerd et al. (1990b) to assign the name, *Desulfomonile tiedjei* gen. nov. and sp. nov., to the organism, formerly strain DCB-1.

SYNTROPHY

Studies of *D. tiedjei* suggest possible reasons why reductive dehalogenation is favored in undefined communities. In natural habitats *D. tiedjei* appears to obtain a number of nutrients and other factors from other organisms. First, as an obligate anaerobe, *D. tiedjei* requires a reduced, oxygen-free environment created by other organisms. As mentioned above, *D. tiedjei* uses electron donors which probably originate as end products of other anaerobes. Products of *D. tiedjei* which are toxic to the organism, such as sulfide and benzoate, may be removed by other organisms. Five vitamins are stimulatory or, possibly, required by *D. tiedjei* (DeWeerd et al., 1990a). Initially, *D. tiedjei* required rumen fluid for growth and dehalogenation activity (Shelton and Tiedje, 1984b). It was latter found that fermentative growth could occur in a defined medium but that dehalogenation activity required a factor which could be provided by rumen fluid, a *Propionibacterium* sp. in coculture or the culture fluid of the *Propionibacterium* sp. (Apajalahati et al., 1989). The factor in the culture fluid was extractable and its

chemical properties suggested that it was a quinoid compound. The factor could be replaced by 1,4-naphthoquinone or menadione (vitamin K_3). Thus, *D. tiedjei* conforms to the general rule of syntrophy in anaerobic ecosystems. A consequence of such interdependence may be the observed difficulty of isolating other dehalogenating organisms. Successful applications of biological reductive dehalogenation will likely require the use and understanding of these complex anaerobic communities.

Conclusion

A fundamental question eluded to above is whether dehalogenation activity *per se* was independently selected for (evolved) or whether the activity is coincidentally catalyzed by an enzyme(s) evolved for a different activity (fortuitous). Natural selection for this activity is possible because of the existence of naturally occurring haloaromatic compounds. The ability of *D. tiedjei* to use dehalogenation for energy metabolism indicates one selective advantage of this activity; detoxication might be another. The specific induction of dehalogenation by *D. tiedjei* suggests that at least the regulation of this activity is evolved in *D. tiedjei*. The apparent distinction between the enzymatic pathways of dehalogenation and sulfoxy anion metabolism by *D. tiedjei* indicate that the former activity is not a fortuitous consequence of the latter. The evidence is not conclusive, but it suggests the possibility that, in the case of *D. tiedjei*, dehalogenation may be an evolved activity.

Attempts to find other dehalogenators

Sulfate-Reducing Bacteria

After *Desulfomonile tiedjei* was identified as a sulfate-reducing bacterium, a variety of other sulfate reducers were tested for the ability to reductively dehalogenate haloaromatic compounds. Linkfield (1985) tested three *Desulfovibrio* spp. for dechlorination of 3-chlorobenzoate under sulfate-reducing conditions. Later, ten species of the following genera were tested: *Desulfovibrio*, *Desulfobacter*, *Desulfobacterium* and *Desulfococcus* (see Chapter 5). In the latter test, conditions used were based on optimal conditions for *D. tiedjei* (i.e., in the presence of required electron donors and vitamins and in the absence of competitive electron acceptors) and substrates tested included halobenzoates and halophenols. No dehalogenation activity was detected. Thus, dehalogenation activity does not appear to be a general property of sulfate reducers; although, it should be noted that a major group, gram-positive sulfate reducers, was not tested.

Isolates

The difficulty in isolating anaerobes capable of reductive dehalogenation of aromatic compounds has been mentioned above. Only one such strain other than *D. tiedjei* has been isolated (see Chapter 5). The new strain is from the same sewage sludge sample as *D. tiedjei* and closely resembles *D. tiedjei* morphologically and physiologically. Medium selecting for the new isolate was based on the ability of *D. tiedjei* to grow diazotrophically on pyruvate plus thiosulfate (Mohn & Tiedje, 1990b). This isolation strategy has

failed to obtain dehalogenating organisms from other enrichments, suggesting that in these enrichments the dehalogenating organisms are physiologically distinct from *D. tiedjei*.

Zhang and Wiegel (1990) have recently reported the establishment of stable culture which reductively dechlorinates 2,4-dichlorophenol to 4-chlorophenol without further degrading this product. The dechlorinating organism is believed to be a spore-forming rod, since the *ortho*-dehalogenation activity was separated from a 2,4-dichlorophenol-mineralizing enrichment by pasteurization. However, the rod has not yet been purified.

Cell-free activity

Cofactors

In the presence of the strong reductant, titanium (III) citrate, cofacters common to a variety of bacteria (e.g., cobalamin, factor F_{430} and hematin) have recently been found to reductively dehalogenate a variety of compounds. This activity has been observed with alkyl solvents such as polychloromethanes and polychloroethylenes (Holliger, 1990) as well as with hexachlorobenzene (L. Wackett, 1990, personal communication). Not all organisms with these cofactors have dehalogenation activity; thus, if these cofactors are involved in dehalogenation by whole cells, they interact with other cell components. The activity of the cofactors suggests the possibility that lysed cells might catalyze some dehalogenation activities observed in reduced environments; although, activity is typically not found in killed control cultures.

CRUDE EXTRACT OF *Desulfomonile tiedjei*

DeWeerd and Suflita (1989) have obtained cell-free extract from *D. tiedjei* which dechlorinates 3CB. Activity is heat-labile; thus, it differs from the above activity of heat-stable cofactors. Dehalogenation by the crude extract is dependent on methyl viologen. A number of biological electron carriers tested were unable to replace methyl viologen. Activity appears to be membrane associated (K.A. DeWeerd, 1989, personal communication). As in whole cells (DeWeerd et al., 1990b), thiosulfate and sulfite inhibit cell-free activity while sulfate does not. Kinetic experiments determining the nature of this inhibition might be very useful in resolving the relationship between sulfoxy anion metabolism and dehalogenation, especially if the dehalogenase can be purified.

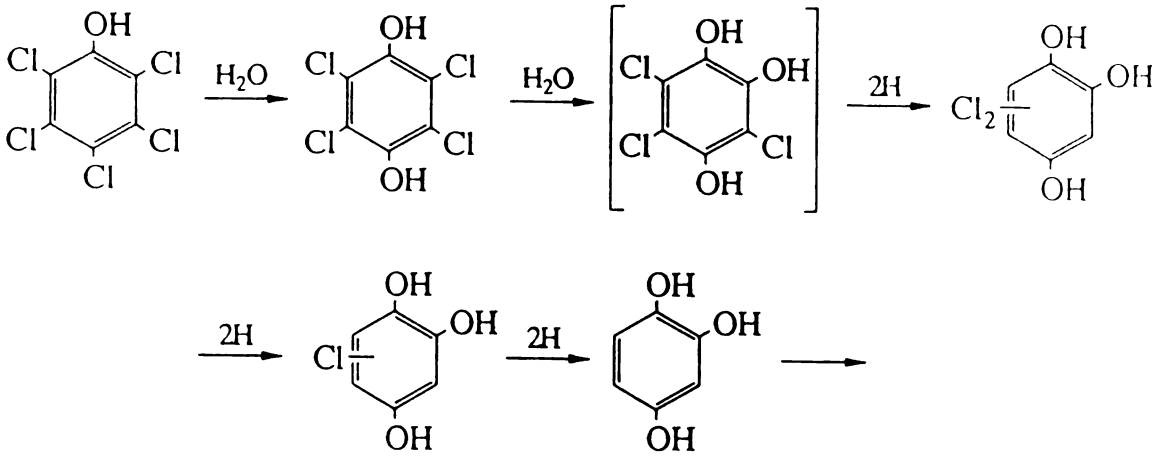
Reductive dehalogenation by aerobes

Reductive dehalogenation also appears to be employed by aerobic bacteria in the degradation of highly chlorinated aromatic rings which are invulnerable to ring-cleaving oxygenases. Even when such organisms are in oxidized environments, their cytoplasm probably has a low redox potential and is favorable for reductive reactions. The pathways of aerobic mineralization of pentachlorophenol by two different organisms, a *Flavobacterium* sp. (Steiert & Crawford, 1986) and *Rhodococcus chlorophenolicus* (Apajalahti & Salkinoja-Salonen, 1987) have been shown to involve reductive dehalogenation steps. The degradation pathways are different for the two organisms, but they both commence with hydrolytic dechlorination(s) followed by reductive dechlorinations (Figure 5). For both aerobes, chlorophenols

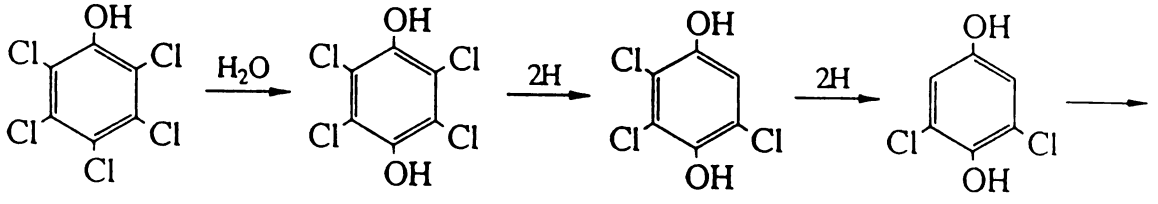
specifically induced their own dechlorination, but it is not clear whether only the hydrolytic activities are inducible or whether the reductive activities are also inducible. The dechlorination substrate specificity varied for the above aerobes, but again it is not clear whether this is a property of only the hydrolytic activities or also of the reductive activities. Whole cells of the *Flavobacterium* required O_2 for the first reductive dechlorination, while cell extracts of *R. chlorophenolicus* did not require O_2 for the reductive dechlorinations.

A facultative anaerobe, *Alcaligenes denitrificans*, also employs both hydrolytic and reductive dechlorination reactions for the aerobic mineralization of 2,4-dichlorobenzoate (Van den Tweel et al., 1987). It was proposed that reductive dechlorination preceded hydrolytic dechlorination (Figure 5); although, the evidence leaves the possibility that the order is reversed. The consumption of 2,4-dichlorobenzoate, presumably including the reductive dechlorination reaction, was catalyzed by cells grown on 4-iodo-, 4-bromo- and 4-chlorobenzoates. Thus, the reductive dechlorination activity was not specifically induced by its substrate. Similarly to the *Flavobacterium* above, whole cells of *A. denitrificans* required O_2 for the dechlorination reactions. In both cases it is possible that energy is required for the activity, but other explanations are also possible. Until the activities of the *Flavobacterium* and *A. denitrificans* are tested in cell extracts, it will not be known whether they differ from that of *R. chlorophenolicus* in requirement

Rhodococcus chlorophenolicus:



Flavobacterium sp.:



Alcaligenes denitrificans:

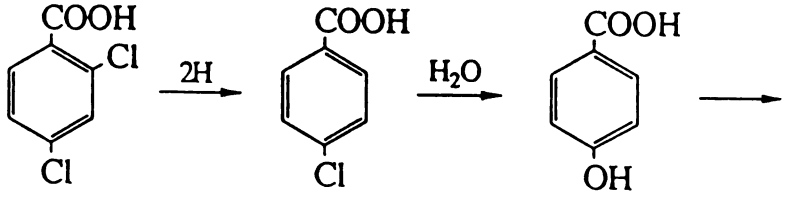


FIGURE 5. Initial steps in aerobic degradation pathways which include reductive dehalogenation reactions.

of O_2. Regardless, these aerobes experience a net gain of energy after oxidation of the resulting aromatic rings.

Conclusions

1. In agreement with early studies, recent studies indicate that reductive dehalogenation is the primary step in the biological anaerobic degradation of haloaromatic compounds, including compounds which are extremely toxic and are not known to be biodegraded aerobically.
2. This activity most readily occurs in undefined anaerobic communities suggesting that the responsible organisms may be obligate syntrophs.
3. Such communities may vary fundamentally in composition, as they respond differently to environmental factors, notably including the availability of various electron acceptors.
4. At least one anaerobe, *Desulfomonile tiedjei*, can gain energy from reductive dechlorination, and the ability of others to do so is consistent with the observed enrichment of this activity.
5. Reductive dehalogenation can be catalyzed by common cofactors in the presence of a strong reductant, but this activity appears distinct from reductive dehalogenation activities in most anaerobic cultures.
6. Reductive dehalogenation also appears to be employed by aerobes in the degradation of haloaromatic compounds which are not initially susceptible to oxidative degradation.
7. Studies of *Desulfomonile tiedjei* as well as the difficulty of isolating other dehalogenating anaerobes suggest that further study

of these environmentally and economically significant organisms may be of fundamental microbiological interest.

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Chapter 2:

Catabolic thiosulfate disproportionation and carbon dioxide reduction in strain DCB-1, a reductively dechlorinating anaerobe

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Catabolic Thiosulfate Disproportionation and Carbon Dioxide Reduction in Strain DCB-1, a Reductively Dechlorinating Anaerobe

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Strain DCB-1 is a strict anaerobe capable of reductive dehalogenation. We elucidated metabolic processes in DCB-1 which may be related to dehalogenation and which further characterize the organism physiologically. Sulfoxy anions and CO_2 were used by DCB-1 as catabolic electron acceptors. With suitable electron donors, sulfate and thiosulfate were reduced to sulfide. Sulfate and thiosulfate supported growth with formate or hydrogen as the electron donor and thus are probably respiratory electron acceptors. Other electron donors supporting growth with sulfate were CO, lactate, pyruvate, butyrate, and 3-methoxybenzoate. Thiosulfate also supported growth without an additional electron donor, being disproportionated to sulfide and sulfate. In the absence of other electron acceptors, CO_2 reduction to acetate plus cell material was coupled to pyruvate oxidation to acetate plus CO_2. Pyruvate could not be fermented without an electron acceptor. Carbon monoxide dehydrogenase activity was found in whole cells, indicating that CO_2 reduction probably occurred via the acetyl coenzyme A pathway. Autotrophic growth occurred on H_2 plus thiosulfate or sulfate. Diazotrophic growth occurred, and whole cells had nitrogenase activity. On the basis of these physiological characteristics, DCB-1 is a thiosulfate-disproportionating bacterium unlike those previously described.

The process of anaerobic reductive dechlorination of aromatic compounds is well documented (23, 28) and is of great interest for degradation of hazardous chemicals. However, very little is known of the specific organisms and the metabolic properties which are responsible for this process. At present only one isolate, strain DCB-1, is known to reductively dehalogenate aromatic compounds under anaerobic conditions. This organism provides an opportunity to identify metabolic processes which underlie reductive dehalogenation. Additionally, it would be useful to identify a physiological group to which DCB-1 belongs, as other organisms in such a group may be responsible for the reductive dehalogenation activities presently known in uncharacterized communities.

Strain DCB-1 is a gram-negative, strict anaerobe isolated from an enrichment mineralizing 3-chlorobenzoate (3CB) (25). DCB-1 is capable of reductive dehalogenation of 3CB (25), other halogenated benzoates (8), and tetrachloroethylene (12). Growth of DCB-1 is stimulated by thiosulfate, sulfate, and sulfite, and with these substrates, sulfide is produced; thus, the organism has been labeled a sulfidogen (26). Strain DCB-1 can fix CO_2, and this process was reported to occur simultaneously with fermentative growth on pyruvate (27). Dolfing and Tiedje (9) constructed a defined anaerobic consortium containing DCB-1, a benzoate fermentor, and a methanogen which grows on 3CB. Evidence was presented that reductive dechlorination of 3CB stimulated growth of the consortium (10). Recent evidence indicates that pure cultures of DCB-1 can conserve energy for growth from dechlorination of 3CB coupled to H_2 or formate oxidation (8a, 20a). It thus appears that reductive dechlorination of aromatic compounds can serve as a catabolic electron-accepting process.

This physiological study of DCB-1 was undertaken to elucidate metabolic activities which may be related to dehalogenation activities and which further classify the organism physiologically. Growth experiments with defined medium identified several previously unknown substrates. A balance of carbon and electrons was determined to investigate the role of CO_2 in the previously reported fermentation of pyruvate. Carbon monoxide dehydrogenase (CODH) activity was assayed to indicate whether the acetyl coenzyme A (acetyl-CoA) pathway is involved in CO_2 reduction. The metabolism of sulfoxy anions was more fully investigated by varying electron donors and quantifying products.

MATERIALS AND METHODS

Cultures. Strain DCB-1 and *Methanospirillum* sp. strain PM-1 were obtained from our laboratory culture collection. All cultures in this study were done in a previously described anaerobic mineral medium (25) with the following modifications. Naphthoquinone (0.6 μM) was added before autoclaving (K. A. DeWeerd and J. M. Suflita, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, I-108, p. 199). Unless otherwise stated, media were buffered with bicarbonate (pH 7) by addition of 28 mM $NaHCO_3$ and an N_2-CO_2 (80:20) gas phase before autoclaving. When CO_2 was not wanted as a potential carbon source or electron acceptor, media were buffered with 10 mM phosphate (pH 7) and an N_2 gas phase was used. Where indicated, H_2 was included in the gas phase after autoclaving as H_2-CO_2 (80:20), introduced with a vacuum/pressure-gassing manifold; CO and CH_4 were added to the gas phase by syringe. The sulfide reductant in the described medium was replaced with 1 mM cysteine, added before autoclaving, and 0.1 mM titanium citrate (30), added after autoclaving. These reductants were replaced with 0.5 mM dithionite, added after autoclaving, from freshly prepared, filter-sterilized, anaerobic stock solution when cysteine was not wanted as a potential carbon source. All other additions to the media were made after autoclaving from filter-sterilized, anaerobic stock solutions. Growth experiments used 10-ml cultures in 26-ml anaerobic tubes with 10% inocula. Products from pyruvate plus CO_2 were determined in cultures buffered by addition of 15 mM $NaHCO_3$ and an N_2-CO_2 (95:5) gas phase and reduced with only cysteine. Products from sulfoxy anions were determined in 25-ml cultures in the

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same tubes with 4% inocula. Cultures used for determination of metabolic products were inoculated with cells grown on homologous media. Whole-cell enzyme assays were done with 100-ml cultures in 160-ml serum bottles. All incubations were done at 37°C.

Analytical methods. Growth of cultures was measured as optical density at 660 nm (lightpath of culture tubes, 1.6 cm). Culture yields were determined as protein concentration by a modification (14) of the assay of Lowry et al. The gasses CO_2, H_2, and CH_4 were analyzed by gas chromatography with a Carle model AGC-111 gas chromatograph equipped with a 2-m Porapak Q column and a microthermister detector. The carrier for the gas analysis was Ar at 20 ml/min, and the column temperature was 40°C. For CO_2 determinations, cultures were acidified by addition of 3 drops of H_2SO_4 (pH < 3), shaken, and allowed to equilibrate for at least 1 h before analysis. Ethanol and ethylene were analyzed with a Varian model 3700 gas chromatograph equipped with a 2-m Porapak Q column, a flame ionization detector, and, as the carrier, N_2 at 9 ml/min. For the ethanol analysis, the column was at 170°C and the detector was at 200°C. For the ethylene analysis, the column was at 35°C and the detector was at 170°C. Organic acids were analyzed by the high-pressure liquid chromatography (HPLC) method of Stevens et al. (27), with the column at 60°C. Benzoates were analyzed by the HPLC method for benzoates of Stevens et al. (26), except that the eluent was water-methanol-phosphoric acid (55:45:0.05) and the UV detector was set at 230 nm. Stop-flow UV scanning was performed with a Hewlett Packard model 1050 variable-wavelength detector. Samples for sulfoxy anion analysis were flushed for 15 min with N_2 to remove H_2S and stabilized from oxidation by addition of 0.2 mM glycerol (19). Sulfoxy anions were analyzed with a Dionex model 2000i ion chromatograph equipped with a Dionex AS4A column and a conductivity detector. The eluent for sulfoxy anions was 3.0 mM $NaHCO_3$–2.5 mM Na_2CO_3 at 2.4 ml/min. Samples for sulfide assay were diluted 10-fold in 100 mM zinc acetate to precipitate sulfide and prevent loss of hydrogen sulfide (6). Sulfide was assayed by the colorimetric method of Cline (7).

Radioisotope methods. The CO_2 pool of cultures was labeled with 177 MBq (1.33 μCi) of sodium [^{14}C]bicarbonate (Research Products Inc., Mount Prospect, Ill.). Incorporation of $^{14}CO_2$ into cell material was determined by collecting cells on membrane filters (0.45 μm pore size), washing the cells with water, and dissolving the filters in Filter Solv (Beckman Instruments, Inc., Fullerton, Calif.). Incorporation of $^{14}CO_2$ into organic acids was determined by injection of 100 μl of culture fluid on the organic acid HPLC column and collection of effluent fractions in basic solution. A Packard model 1500 liquid scintillation analyzer was used to measure ^{14}C.

CODH assay. Cells used for the CODH assay were harvested by centrifugation (30 min at 1,500 × *g*) in the culture bottles. The cells were washed and suspended, concentrated 20-fold, in anaerobic 30 mM Tris hydrochloride buffer (pH 7) under a headspace of N_2. Cell suspensions were stored at −20°C until use. CODH activity was determined as CO-dependent reduction of methyl viologen by the procedure of Krzycki and Zeikus (18).

Nitrogenase assay. For ammonium-free medium, NH_4Cl in the previously described mineral medium was replaced with an equimolar amount of NaCl, leaving N_2 as the sole N source. Cells for the nitrogenase assay were harvested as described above and suspended, concentrated 10-fold, in homologous medium. The suspensions of 10 ml were placed in 23-ml serum bottles, 0.05 atm (ca. 50.5 kPa) acetylene was added to the headspace, and ethylene production in the bottles was measured during incubation at 37°C.

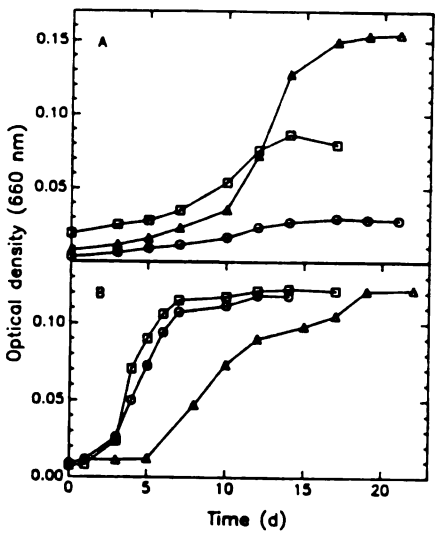

FIG. 1. Growth of strain DCB-1 on various media. (A) Requirement for electron acceptor. After two passages on phosphate-buffered medium containing 10 mM pyruvate and no electron acceptor, cells were used to inoculate homologous medium (circles), bicarbonate-buffered medium containing 10 mM pyruvate (squares), and phosphate-buffered medium containing 10 mM pyruvate plus 5 mM thiosulfate (triangles). (B) Effect of electron donors and carbon sources on growth on bicarbonate-buffered medium containing 5 mM thiosulfate. Cultures contained 20 mM formate (circles), no electron donor with 1 mM acetate as a carbon source (squares), or 1.6 atm of hydrogen with CO_2 as the sole carbon source (triangles).

RESULTS

Pyruvate metabolism. Fermentative growth of DCB-1 on pyruvate was tested by growing cultures on phosphate-buffered medium with 10 mM pyruvate. It was found that growth only occurred if a potential electron acceptor was present (Fig. 1A). Growth on pyruvate plus thiosulfate in phosphate-buffered medium indicates that CO_2 (other than that produced from pyruvate oxidation) is not required for anabolic purposes. Thus, DCB-1 appears to require an electron acceptor for growth on pyruvate, and added CO_2 appears to serve this function. In bicarbonate-buffered medium reduced with only cysteine, the consumption of 85 ± 7 μmol of pyruvate was accompanied by the production of 105 ± 1 μmol of acetate and 43 ± 5 μmol of total CO_2 (values here and below are means of triplicates ± standard error). Parallel replicate cultures with [^{14}C]bicarbonate added incorporated 4.1 ± 0.1 μmol of CO_2 into cell material and 23.8 ± 0.3 μmol of CO_2 into acetate. No other ^{14}C-labeled products were detected. The balances of carbon and electrons between substrates and products were near 100% (Table 1) and confirm the function of CO_2 as an electron acceptor. Lactate was not produced in the above cultures, but on similar media with stronger reductants (e.g., sodium sulfide or titanium citrate), lactate was found as a product of pyruvate in lesser amounts than acetate. Other organic acids, ethanol, and H_2 were never detected as products of pyruvate. Thus, on

TABLE 1. Carbon and electron balance for strain DCB-1 grown on pyruvate plus CO_2[a]

Compound	Amt used (μmol)	Carbon (μmol)	*O/R* value[b]	Balance (μmol × *O/R* value)
Substrates				
Pyruvate	85	255	+1	+85
CO_2	28	28	+2	+56
Total		283		+141
Products				
Acetate	105	210	0	0
CO_2[c]	71	71	+2	+142
Cell C	4	4	0	0
Total		285		+142

[a] Recovery was 101%, determined as amount of carbon or as carbon and electron balance.
[b] *O/R* value is the oxidation state of carbon, calculated by the method of Gottschalk (13).
[c] Product CO_2 is net CO_2 produced plus CO_2 consumed.

media with no electron acceptor other than CO_2, DCB-1 primarily oxidizes pyruvate while it reduces CO_2. Growth did not occur if pyruvate was omitted from the medium, indicating that cysteine could not support growth. Growth did not occur on bicarbonate-buffered medium with 10 mM lactate, either with or without 10 mM acetate.

Use of electron acceptors. Because DCB-1 could not grow on phosphate-buffered medium with pyruvate, apparently due to the lack of an electron acceptor, this medium was used to test potential electron acceptors. Furthermore, since pyruvate oxidation can support substrate-level phosphorylation (SLP), this medium indicated general (i.e., both respiratory and nonrespiratory) electron acceptors. Putatively respiratory electron acceptors were distinguished by substituting formate for pyruvate as an electron donor, since DCB-1 can oxidize formate (data shown below), but formate cannot support SLP. With formate, CO_2 was provided as an additional carbon source.

Sulfoxy anions served both as general and as putatively respiratory electron acceptors (Table 2). Dithionite appeared to serve as an electron acceptor, since growth was proportional to dithionite concentration, but because of the chemical instability of dithionite, other sulfur compounds may be partially or completely responsible for the growth observed. Fumarate supported relatively slow growth and low yields with pyruvate, the doubling time being 17 days. Fumarate did not support respiratory growth (Table 2), and succinate did not accumulate during growth on pyruvate plus fumarate.

TABLE 2. Use of general and putatively respiratory electron acceptors by strain DCB-1

Test compound	Concn (mM)	Use as electron acceptor[a]	
		General[b]	Respiratory[c]
Sulfate	5	+	+
Thiosulfate	5	+	+
Sulfite	3	+	+
Dithionite	0.5, 1, 2	+[d]	ND
Fumarate	5	+	−
Nitrate	5	−	ND
CO_2 (0.2 atm)		+	−

[a] Symbols: +, duplicate cultures were successfully grown through three serial transfers on the medium indicated; −, criterion for + not met; ND, not done.
[b] Phosphate-buffered medium containing 10 mM pyruvate.
[c] Bicarbonate-buffered medium containing 10 mM formate.
[d] We did not conduct a serial transfer experiment, but cell yield was correlated with dithionite concentration.

TABLE 3. Metabolism of sulfoxy anions by strain DCB-1 during 16 days of incubation on bicarbonate-buffered medium

Electron donor (20 mM)	Electron acceptor (3 mM)	Mean (mM) ± SE[a]		
		Electron acceptor consumed	Sulfide produced	Sulfate produced
Formate	Sulfate	2.5 ± 0.2	2.3 ± 0.1	
Formate	Thiosulfate	0.8 ± 0.1	1.5 ± 0.2	0.0 ± 0.0
None[b]	Thiosulfate	2.4 ± 0.0	2.5 ± 0.0	1.7 ± 0.1

[a] Data are means of triplicates ± standard error.
[b] None provided other than thiosulfate and acetate (1 mM), which was required, presumably as a carbon source.

Metabolism of sulfoxy anions. Both sulfate and thiosulfate were reduced stoichiometrically to sulfide when formate was provided as an electron donor (Table 3). Sulfide production from cysteine was negligible, as controls without sulfate or thiosulfate had less than 0.1 mM sulfide after autoclaving and less than 0.2 mM sulfide after inoculation and incubation. Sulfate and thiosulfate were stable in uninoculated controls, while 1.0 mM sulfide was partially oxidized during the 16-day incubation and formed 0.2 mM thiosulfate. There was a linear relationship between limiting amounts of sulfate or thiosulfate (i.e., 1 mM or less) and total growth. Thus, sulfate and thiosulfate clearly served as respiratory electron acceptors.

Thiosulfate was disproportionated to sulfide and to a lesser amount of sulfate when formate was omitted (Table 3). There was again a linear relationship between limiting amounts of thiosulfate and total growth. Growth occurred through at least five passages on medium containing 5 mM thiosulfate and 1 mM acetate, the latter substrate being required, presumably as a carbon source. Thiosulfate disproportionation therefore supports growth of DCB-1, although some acetate oxidation may have occurred, accounting for the production of more sulfide than sulfate. Apparent growth rates on thiosulfate were similar with and without formate (Fig. 1B). In the presence of 20 mM pyruvate, both sulfide and a smaller amount of sulfate were found as products of thiosulfate (data not shown). Together, these results indicate that the fate of thiosulfate may depend on thermodynamic conditions for growth of DCB-1 (i.e., the availability of electron donors and their strength as reductants).

The toxicity of the sulfide product apparently limited growth and consumption of sulfate or thiosulfate. Starting concentrations of 3 mM sulfate and thiosulfate were never completely consumed, even though electron donors were supplied in excess. Cultures never produced 3 mM or more sulfide. When cultures growing on 20 mM formate and 3.5 mM thiosulfate were flushed with N_2-CO_2 in late log phase, removing H_2S, growth continued longer than in unflushed control cultures. Flushing increased thiosulfate consumption from 30 to 88 μmol and final cell protein from 0.78 to 0.97 mg. Cultures incubated on medium containing 10 mM pyruvate, 0.05% yeast extract, and various initial sulfide concentrations grew only when the sulfide concentration was 2 mM or less.

Test of syntrophic growth. To determine whether an H_2-consuming organism could substitute for an electron acceptor, DCB-1 and *Methanospirillum* sp. strain PM-1 were cocultured on bicarbonate-buffered medium with pyruvate. Growth occurred, but there was no significant production of

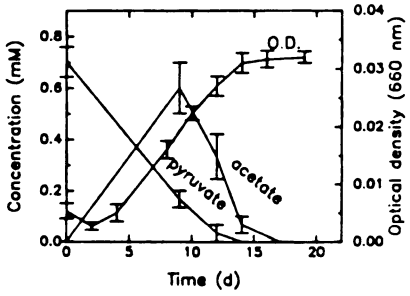

FIG. 2. Consumption of a limiting amount of pyruvate with transient accumulation of acetate during growth of strain DCB-1 on 5 mM sulfate as an electron acceptor. Data are means of triplicates with standard errors (bars). O.D., Optical density.

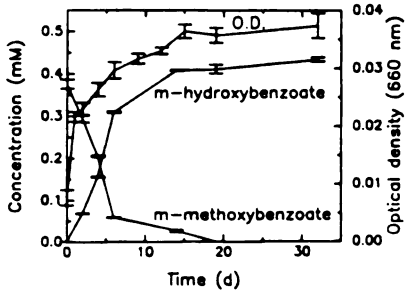

FIG. 3. Consumption of a limiting amount of 3-methoxybenzoate with accumulation of 3-hydroxybenzoate as an end product during growth of strain DCB-1 on 5 mM sulfate as an electron acceptor. Data are means of triplicates with standard errors (bars). O.D., Optical density.

methane. Microscopic observation of the coculture revealed only DCB-1 cells. Thus, even under favorable conditions (i.e., low H_2 partial pressure), DCB-1 did not produce H_2 from pyruvate.

Use of electron donors and carbon sources. The use of electron donors was tested with 5 mM sulfate as an electron acceptor. The criteria for use were significant (by the Student *t* test, $\alpha = 0.05$) stimulation of both growth (measured as optical density) and sulfide production relative to those in control cultures without electron donors. The following electron donors were used (initial concentration): H_2 (1.6 atm), formate (10 mM), CO (0.1 atm), lactate (10 mM), pyruvate (10 mM), butyrate (10 mM), and 3-methoxybenzoate (2.5 mM). The following were not used: methane (0.1 atm), methanol (2.5 mM), ethanol (2.5 mM), acetate (10 mM), propionate (10 mM), glycerol (10 mM), malate (10 mM), fructose (5 mM), or glucose (5 mM). Conditions used here to test electron donors differed from conditions used previously to test substrates (26) in several ways: (i) sulfate was provided here as an electron acceptor, (ii) naphthoquinone was provided here, and (iii) ruminal fluid was not provided here. Growth with CO was very slow relative to growth with other electron donors. An initial concentration of 10 mM pyruvate was incompletely oxidized to acetate during growth on sulfate thiosulfate, but at a lower initial concentration with excess sulfate, the acetate product subsequently disappeared (Fig. 2). Sulfide accumulation may have prevented complete oxidation of the higher concentration of pyruvate. Thus, with sulfate as an electron acceptor, DCB-1 may completely oxidize pyruvate despite its inability to grow when transferred to medium with sulfate and acetate as a sole electron donor (see above). Consumption of a low initial concentration of 3-methoxybenzoate with excess sulfate was accompanied by stoichiometric production of 3-hydroxybenzoate, which was not degraded further (Fig. 3), indicating that only the methoxyl group of that substrate was used. The identity of the 3-hydroxybenzoate end product was confirmed by comparison of its UV spectrum with that of an authentic standard. Both lactate and pyruvate served as sole carbon sources, supporting serial transfers on phosphate-buffered, dithionite-reduced medium with 5 mM thiosulfate.

Autotrophic growth was possible, as growth occurred through at least five serial transfers each on dithionite-reduced, bicarbonate-buffered medium containing 1.6 atm of H_2 plus either 5 mM thiosulfate or 5 mM sulfate. The apparent doubling time of autotrophic cultures with thiosulfate was longer than that of other cultures with thiosulfate (Fig. 1B). Autotrophic growth on thiosulfate did not occur without an additional electron donor.

CODH activity. CODH activity was assayed to determine whether the acetyl-CoA pathway could be responsible for CO_2 fixation by DCB-1. Activity was detected in cells grown on all media tested (Table 4). These activities are lower than those reported for some other sulfate reducers believed to be using the acetyl-CoA pathway (24), but the differences in most cases are less than an order of magnitude. Factors contributing to the differences may be the slower growth rate of DCB-1 and the testing of whole rather than permeabilized DCB-1 cells. The addition of 1 mM potassium cyanide to a suspension of cells grown on H_2, CO_2, and 5 mM thiosulfate caused an 86% inhibition of CODH activity. Growth of DCB-1 did not occur with 10 mM thiosulfate plus CO as a sole carbon source.

Test of homoacetogenic growth. On bicarbonate-buffered medium with no additional electron acceptor, growth did not occur with 1.6 atm of H_2 or 10 mM formate. Growth did occur on this medium with 5 mM 3-methoxybenzoate through three serial transfers but then stopped. Acetate was not produced from 3-methoxybenzoate. Since the carbon sources provided in these media were sufficient for growth with thiosulfate, it appears that DCB-1 did not gain sufficient energy for growth from these substrates by homoacetogenesis.

Diazotrophic growth. Growth of DCB-1 occurred on 10 mM pyruvate plus 5 mM thiosulfate through at least five serial transfers with N_2 as the sole N source. Serial transfers could not be maintained if N_2 was replaced by Ar. The growth rate was lower on N_2 than on ammonium. Cells grown on N_2 had nitrogenase activity, measured as production of 0.24 μmol of ethylene min^{-1} g of $protein^{-1}$ from acetylene. This activity is probably inducible, as cells grown on ammonium produced no ethylene.

TABLE 4. CODH activity in whole cells of DCB-1 grown on various bicarbonate-buffered media

Substrate (concn, mM)	Reductant	Activity[a]
Pyruvate (10)	Cysteine	81
H_2 (1.6 atm) + thiosulfate (5)	Dithionite	363
Formate (10) + thiosulfate (5)	Dithionite	479
Pyruvate (10) + thiosulfate (5)	Dithionite	721

[a] Activity in micromoles of CO per minute per gram of protein, values are means of triplicates.

DISCUSSION

Stevens et al. (26) reported that various sulfoxy anions stimulated the growth of DCB-1 on pyruvate with the formation of undetermined amounts of sulfide and so classified the organism as a sulfidogen. Here, we show that sulfate was reduced stoichiometrically to sulfide (Table 3); thus, DCB-1 is a sulfate-reducing bacterium. Since H_2 and formate are not known to support SLP, the ability of DCB-1 to grow with either of these electron donors and sulfate (Table 2) indicates a respiratory process, like that found in other sulfate reducers (2). Like most other sulfate reducers, DCB-1 will also grow on thiosulfate, sulfite, and, possibly, dithionite (Table 2). Use of dithionite as a reductant probably explains the previous finding that DCB-1 grew on lactate plus acetate (26), which we did not find on cysteine–titanium citrate-reduced medium. Growth of DCB-1 was also supported by disproportionation of thiosulfate to sulfate and sulfide (Table 3). Growth by this type of lithotrophic fermentation has been reported for *Desulfovibrio sulfodismutans*, *D. desulfuricans*, and two other strains of sulfate reducers (3, 4, 17). Sulfide appears to be relatively toxic to DCB-1 compared with other sulfate reducers. However, toxicity of 2 mM sulfide has also been reported for *Desulfotomaculum* spp. (16). In open natural systems, such sensitivity may not be a great disadvantage, as sulfide might be prevented from accumulating by precipitation, diffusion, and volatilization of H_2S. However, such sensitivity to sulfide could greatly bias traditional enrichment and isolation procedures against such organisms.

The apparent use of fumarate as an electron acceptor by DCB-1 (Table 2) seems not to be respiratory, as it is in many other organisms, including some sulfate reducers (5). Despite the fact that DCB-1 can reduce nitrate to nitrite (25), nitrate did not serve as a catabolic electron acceptor for DCB-1 (Table 2) as it can for some other sulfate reducers (20). *Desulfovibrio desulfuricans*, which can grow by sulfite disproportionation, can use both fumarate and nitrate as electron acceptors.

DCB-1 was previously reported to ferment pyruvate (26); however, the fermentation balance was not complete. The carbon recovery was near 100%, but the recovery of reducing equivalents was low. We found that DCB-1 is unable to grow on pyruvate without some electron acceptor (e.g., sulfoxy anions or CO_2). Specifically, DCB-1 did not ferment pyruvate to acetate, CO_2, and H_2 as can many sulfate reducers, and although lactate was formed by DCB-1 or by nonbiological reactions under certain conditions (i.e., in the presence of strong reductants), DCB-1 did not ferment pyruvate to equimolar amounts of lactate, acetate, and CO_2. Our results indicate that pyruvate oxidation (equation 1, below), in the absence of any other electron acceptor, is balanced by CO_2 reduction to acetate and cell material (equation 2). For simplification, we assume that cell carbon has the same oxidation state as that of acetate (*O*/*R* value = 0). Our data fit the net equation (equation 3) well and indicate a balance of carbon and electrons between substrates and products (Table 1). In agreement with these results, Stevens and Tiedje (27) reported that during metabolism of pyruvate by DCB-1, 20% of the acetate formed was from CO_2. The proposed catabolic mode for DCB-1 (equation 3) is not a fermentation in the strict sense of the term, as a substrate is not being simultaneously oxidized and reduced to yield a net balance of reducing equivalents in the substrate and the products. Like a fermentation, however, this mode may provide energy for growth entirely from SLP. Lactate could not be used in the preceding manner or be fermented by DCB-1 under the conditions provided.

$$4\ CH_3COCOOH + 4\ H_2O \rightarrow 4\ CH_3COOH + 4\ CO_2 + 8\ H \quad (1)$$

$$8\ H + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O \quad (2)$$

$$4\ CH_3COCOOH + 2\ H_2O \rightarrow 5\ CH_3COOH + 2\ CO_2 \quad (3)$$

The finding of CODH (acetyl-CoA synthase) activity suggests that CO_2 fixation by DCB-1 occurs via the acetyl-CoA pathway, as in *Desulfovibrio baarsii* (15). Operation of the reductive pentose phosphate pathway is unlikely, as DCB-1 was found to lack ribulose bisphosphate activity (T. O. Stevens, M.S. thesis, Michigan State University, East Lansing, 1987). The acetyl-CoA pathway probably accounts for several other activities which we identified in DCB-1. The pathway may allow DCB-1 to reduce CO_2 to acetate, as do homoacetogens and some *Desulfotomaculum* spp. (16); however, unlike homoacetogens and *Desulfotomaculum orientis*, DCB-1 apparently can only gain energy by coupling CO_2 reduction to oxidations which support SLP (i.e., pyruvate oxidation). The slow oxidation of CO and, possibly, acetate by DCB-1 may also be catalyzed by the acetyl-CoA pathway, as in other sulfate reducers (24), and is consistent with the reversible nature of the pathway. DeWeerd et al. (8) have shown that DCB-1 metabolizes 3-methoxybenzoate by O-demethylation, an activity found in acetogens (1) and *Desulfotomaculum* spp. (16) and also thought to be catalyzed by enzymes of the acetyl-CoA pathway. Finally, the ability of DCB-1 to dechlorinate tetrachloroethylene may be due to the acetyl-CoA pathway, as Egli et al. (11) have proposed a correlation of this pathway and the ability of anaerobes to dechlorinate volatile alkanes, although it should be noted that this correlation was based on dechlorination of tetrachloromethane. Thus, several of our findings can be explained by the acetyl-CoA pathway.

Diazotrophic growth is another property of DCB-1 common to many other sulfate reducers. This capacity is known among several members each of the genera *Desulfovibrio* (22), *Desulfotomaculum* (21), and *Desulfobacter* (29). The rate of acetylene reduction found for DCB-1 is lower than, but within an order of magnitude of, rates reported for members of the above genera.

Strain DCB-1 is a sulfate reducer which has physiological characteristics found in a broad range of members of this group but does not fit well into any known physiological subset of the group. The morphology of DCB-1 is unique by virtue of a collar structure (25). It remains to be determined whether DCB-1 is a representative of a physiological group of sulfate reducers yet to be characterized and, if so, whether other reductive dehalogenators are also in such a group. This study will hopefully facilitate attempts to find other organisms related to DCB-1. Recent evidence indicates that reductive dechlorination of benzoates by DCB-1 is a respiratory process yielding energy which supports growth (8a, 20a). Presumably DCB-1 did not evolve under natural selection for a respiratory system dedicated to chlorinated substrates. Since sulfoxy anions are the only other putatively respiratory substrates for DCB-1 presently known, it appears most likely that the same respiratory system may be coupled to sulfoxy anion reduction and reductive dehalogenation of benzoates. It is possible that other sulfate reducers are capable of reductive dehalogenation under certain conditions, especially sulfur compound disproportionaters, which appear to have the most catabolic similarity to DCB-1. Although some *Desulfovibrio* spp. have been tested for

dechlorination activity without success (T. G. Linkfield, Ph.D. thesis, Michigan State University, East Lansing, 1985), this possibility needs to be more thoroughly investigated.

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ADDENDUM IN PROOF

The name *Desulfomonile tiedjei* has recently been proposed for strain DCB-1 (K. A. DeWeerd, L. Mandelco, R. S. Tanner, C. R. Woese, and J. M. Suflita, Arch. Microbiol., in press). This new genus is indicated by physiological characterization as well as 16S rRNA sequence analysis of strain DCB-1.

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Chapter 3:

Strain DCB-1 conserves energy for growth from reductive dechlorination coupled to formate oxidation

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Strain DCB-1 conserves energy for growth from reductive dechlorination coupled to formate oxidation

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Abstract. Strain DCB-1 is a strict anaerobe capable of the reductive dechlorination of chlorobenzoates. The effect of dechlorination on the yield of pure cultures of DCB-1 was tested. Cultures were incubated with formate or H_2 as electron donors and CO_2 as a putative carbon source. Relative to control cultures with benzoate, cultures which dechlorinated 3-chlorobenzoate and 3,5-dichlorobenzoate had higher yields measured both as protein and cell density. On the media tested the apparent growth yield was 1.7 to 3.4 g cell protein per mole Cl^- removed. Dechlorination also stimulated formate oxidation by growing cultures. Resuspended cells required an electron donor for dechlorination activity, with either formate or elemental iron serving this function. Resuspended cells did not require an electron acceptor for formate consumption, but reductive dechlorination of 3CB to benzoate stoichiometrically stimulated oxidation of formate to CO_2. These results indicate that DCB-1 conserves energy for growth by coupling formate, and probably, H_2 oxidation to reductive dechlorination.

Key words: Anaerobic degradation – Reductive dehalogenation – Chlorobenzoate – Growth yield – Anaerobic respiration

Dolfing and Tiedje (1987) demonstrated that the cell yield of a defined anaerobic consortium was greater with 3-chlorobenzoate (3CB) as a substrate than with benzoate. This finding indicates that the initial reaction in the degradation of 3CB, reductive dechlorination to benzoate, stimulates growth of the consortium. Thermodynamic data were also presented in that study showing that the reductive dechlorination of 3CB coupled to H_2 oxidation is exergonic under the physicochemical conditions of the consortium ($\Delta G' = -112$ kJ per mole 3CB). Together this evidence suggests a novel mode of catabolism whereby energy is conserved during anaerobic reductive dechlorination. Because the consortium contained three organisms which were nutritionally interdependent, it could not be definitely concluded that the dechlorinating organism, strain DCB-1, conserved energy from reductive dechlorination.

Recently, we discovered that the addition of *Propionibacterium* sp. culture fluid (PCF) stimulates growth and dechlorination activity in pure cultures of DCB-1 with pyruvate and CO_2 as carbon and energy sources (Apajalahti J, Cole J and Tiedje JM, Abstr. Ann. meeting Amer. Soc. Microbiol., 1989). Strain DCB-1 was found to consume H_2 (Linkfield and Tiedje 1989), use thiosulfate for growth (Stevens et al. 1988) and fix CO_2 (Stevens and Tiedje 1988). We subsequently found that putatively autotrophic growth could occur on hydrogen or formate with a suitable electron acceptor such as thiosulfate, and that whole cells of DCB-1 have carbon monoxide dehydrogenase activity (Mohn and Tiedje, submitted). DeWeerd et al. (1986) found that 3,5-dichlorobenzoate (35DCB) could also be dechlorinated by DCB-1. These advances have allowed the following study in which DCB-1 was grown in pure culture with either H_2 or formate as an electron donor and 3CB or 35DCB as an electron acceptor. Resuspended cells were used to test various electron donors for dechlorination, and to determine the stoichiometry of formate oxidation coupled to 3CB dechlorination.

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Non-standard abbreviations: 3CB, 3-chlorobenzoate; 35DCB, 3,5-dichlorobenzoate; PCF, *Propionibacterium* sp. culture fluid

Materials and methods

Culture methods

Strain DCB-1 was isolated from a sewage sludge enrichment grown on 3-chlorobenzoate (Shelton and Tiedje 1984). All cultures were grown on reduced anaerobic mineral medium prepared as described by Shelton and Tiedje (1984), with the following modifications. The mineral salts included 0.5 mg/l $CoCl_2 \cdot 6\ H_2O$ and 0.05 mg/l Na_2SeO_3. Before autoclaving, the vitamin mixture of Wolin et al. (1963) plus 50 µg/l naphthoquinone (DeWeerd K, personal communication) were added. To reduce the medium, 1 mM cysteine was added before autoclaving, and 0.1 mM titanium citrate (Zehnder and Wuhrmann 1976) was added after autoclaving. All amendments after autoclaving were from anaerobic, filter-sterilized stock solutions. All cultures were 50 ml, incubated in 160-ml serum bottles at 37 C.

The medium for stock cultures had 0.01% yeast extract and 10% *Propionibacterium* sp. culture fluid (PFC), 10 mM pyruvate acid and 0.5 mM sodium 3-chlorobenzoate added before autoclaving. Dechlorination was monitored in stock cultures, and 0.5 mM 3CB was again added after dechlorination of the initial amount. After the total 1 mM 3CB was dechlorinated, cultures were in stationary phase and were used as inocula for experimental cultures or were resuspended for cell suspension experiments.

Propionibacterium sp. was grown on the same mineral medium with 0.05% yeast extract and 20 mM lactic acid added before autoclaving. The culture fluid was filtered

Table 1. The effect of reductive dechlorination on yield of DCB-1 cultures grown on several media[a, b]

PCF	Electron donor	Electron acceptor	Time (days)	DeCl[c] (mM)	Protein (μg/ml)	Cells/ml ($\times 10^{-5}$)	Growth yield[d]
−	Form	BA	72	−	6.9 ± 0.7	68 ± 16	−
−	Form	3CB	72	1.9	12.2 ± 1.0**	160 ± 10**	2.8
−	Form	35DCB	72	1.4	11.6 ± 0.4**	130 ± 10*	3.4
+	Form	BA	44	−	11.2 ± 0.5	86 ± 5	−
+	Form	3CB	44	3.8	17.5 ± 0.8**	300 ± 90*	1.7
+	H_2	BA	36	−	10.1 ± 0.1	110 ± 0	−
+	H_2	3CB	36	1.9	13.6 ± 0.6**	160 ± 10*	1.8

[a] *Abbreviations*: PCF, *Propionibacterium* sp. culture fluid; OD, optical density at 660 nm; Form, formate; BA, benzoate; 3CB, 3-chlorobenzoate; 35DCB, 3,5-dichlorobenzoate

[b] All values are means of triplicates ± standard error; except for duplicate 35DCB cultures

[c] Dechlorination as mM Cl^- removed

[d] Growth yield as the protein yield in g from dechlorination (dechlorinating cultures less corresponding controls) per mole Cl^- removed

* $P < 0.2$, ** $P < 0.02$, probabilities that dechlorinating cultures are not significantly different than corresponding controls by Student's *t*-test

(0.22 μm) under anaerobic conditions and stored at 4°C under N_2.

Experimental cultures also had the same mineral medium with the following amendments for the indicated treatments. PCF, 5%, was added before autoclaving. H_2 was added after autoclaving by changing the headspace to 2 atm $H_2 - CO_2$ (80:20) using a gassing manifold. Sodium formate, 10 mM, and sodium benzoate or sodium chlorobenzoates, 1 mM, were added after autoclaving. All experimental cultures were in triplicate. Because of the reported toxicity of 3CB to DCB-1 (Shelton and Tiedje 1984), chlorobenzoates were added incrementally to cultures. Concentrations of benzoates were monitored, and chlorobenzoate concentrations were maintained between 0.1 and 1 mM by addition from stock solutions. Cultures without PCF received 5% inocula, and those with PCF received 10% inocula.

Cell suspensions

Stock cultures were harvested by centrifugation ($1500 \times g$) in culture bottles. The cells were washed and resuspended, concentrated approximately fivefold, in 20 mM TRIS · HCl buffer pH 7.0 under N_2. This buffer was deoxygenated by boiling under N_2, but no reductant was added. All treatments with cell suspensions were done in triplicate and were incubated at 37°C.

Analytical methods

Organic acids were analyzed by HPLC using the method of Stevens et al. (1988) with the column temperature at 60°C. Benzoic acids were analyzed by HPLC using the method of Stevens et al. (1988) except that the eluent was methanol – 0.10% phosphoric acid (55:45), the column was at 40°C, the UV detector was set at 230 nm and 500 μM 2,4-dichlorobenzoate was added to samples before filtration as an internal standard. Total CO_2 was determined by acidifying cell suspensions with H_2SO_4 (pH < 2), allowing 30 min for equilibration, measuring CO_2 in the headspace and calculating dissolved CO_2. A 0.20-ml headspace sample was injected on a Carle model AGC-111 gas chromatograph equiped with 6 m Porapak Q column and microthermister detector; the carrier was Ar at 20 ml/min. Protein concentration was determined by the method of Lowry as described by Hanson and Phillips (1981). Cell density was determined with a Burker-Turk counting chamber and a Leitz Orthoplan 2 microscope using 640 × magnification and phase contrast illumination; samples for counting were fixed with 5% formalin and concentrated 10-fold by centrifugation. Examination of cultures for purity was with the same microscope using 1570 × magnification. Dry weight was determined by collecting 35 ml samples on tared membrane filters (0.45 μm) and drying to constant weight at 80°C.

Results

Experimental cultures

Reductive dechlorination of 3CB or 35DCB occurred on all media used, and there was complete recovery of the aromatic ring as benzoate and residual chlorobenzoates (93 – 105% recovery). Cultures were incubated until the turbidity of the dechlorinating cultures was clearly above that of the controls. The yields, as protein and cell density, of reductively dechlorinating cultures were significantly higher than those of corresponding control cultures with benzoate added, indicating both an increase in biomass and cell number due to dechlorination (Table 1). Comparison of dry weights, cell densities and protein concentrations indicate that the mean dry weight per cell was 1.8 pg, and protein was a mean of 49% of dry weight; these expected proportions support the accuracy of the protein and cell density determinations and are similar to values found for DCB-1 grown on other media. The stoichiometric recovery of benzoate from chlorobenzoates in the dechlorinating cultures, as well as the presence of benzoate in control cultures, indicate that reductive dechlorination, and not utilization of the benzoate product, is responsible for the differences in yield observed. The growth yield per mole Cl^- removed from 35DCB was similar to, and not lower than, that from 3CB in corresponding cultures (Table 1); thus, the molar growth yield from 35DCB is approximately double that from 3CB. This difference is consistent with conservation of energy for growth from the removal of each chlorine atom from 35DCB.

Syntrophic interactions, such as those with the other members of the defined consortium or those with a *Propionibacterium* sp., affect growth and dechlorination activity

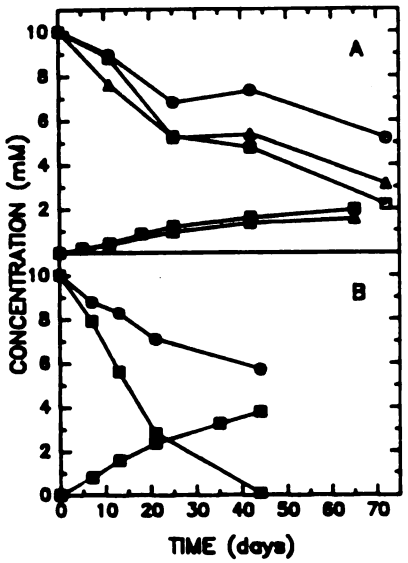

Fig. 1. Formate consumption and dechlorination by DCB-1 cultures. Panel A, without *Propionibacterium* sp. culture fluid, panel B, with culture fluid. *Open symbols*, formate consumption; *closed symbols*, dechlorination as Cl^- removed from 3CB or 35DCB. *Circles*, control cultures with benzoate, *squares*, cultures with 3CB; *triangles*, cultures with 35DCB

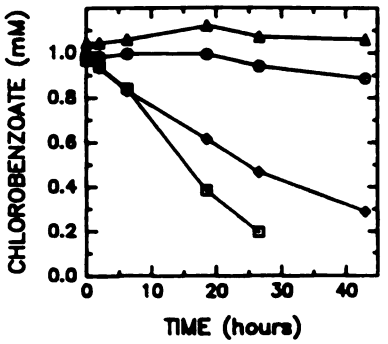

Fig. 2. Dechlorination of 3CB by resuspended DCB-1 cells (148 μg protein per ml) in the presence of various electron donors. *Circles*, control; *squares*, 10 mM formate; *triangles*, 1 atm H_2; *diamonds*, 5 mg/ml iron powder

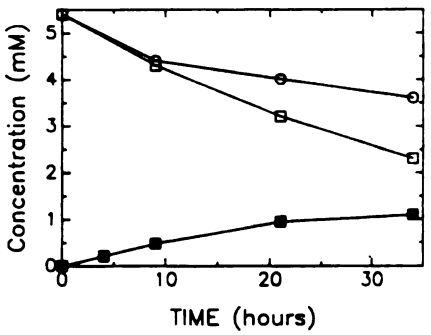

Fig. 3. Formate consumption and dechlorination of 3CB by resuspended DCB-1 cells. *Open symbols*, formate consumption; *closed symbols*, dechlorination measured as benzoate produced. *Circles*, control suspensions without 3CB; *squares*, suspensions with 3CB

of DCB-1. Because such effects would greatly complicate interpretation of these experiments, we carefully checked the purity of our cultures. The stock cultures used to inoculate experimental cultures were also used to inoculate the medium for *Propionibacterium* sp. After 2 weeks incubation growth was not apparent, and microscopic examination revealed only cells having the morphology characteristic of DCB-1, indicating that the stock cultures were not contaminated with *Propionibacterium* sp. or any other organism capable of lactate fermentation. At the end of incubation, experimental cultures were also examined, and only cells with the morphology of DCB-1 were found.

The initial protein concentrations of cultures supplemented with PCF and formate was 8.9 μg/ml, and that of cultures supplemented with PCF and H_2 was 7.9 μg/ml. Growth therefore occurred in both dechlorinating cultures and controls; although, growth was higher in dechlorinating cultures than in corresponding controls, the difference being approximately fourfold between those with formate, and threefold between those with H_2 (Table 1). Assuming growth was logarithmic, dechlorinating cultures with formate and H_2 had doubling times of 45 and 46 days, respectively.

To test the coupling of formate oxidation to reductive dechlorination, formate consumption was measured in experimental cultures. Formate consumption was markedly increased by the presence of 3CB or 35DCB (Fig. 1); although, control cultures also consumed formate. The molar difference in formate consumption between the dechlorinating cultures and controls approximated Cl^- removal, but was slightly higher (Fig. 1). Organic acids, including acetate, were not produced in amounts sufficient to balance formate consumption observed (i.e. less than 0.5 mM).

Cell suspensions

Cell suspensions were used to more directly test the coupling of formate oxidation to dechlorination. DCB-1 cells retained reductive dechlorination activity when washed and resuspended in deoxygenated, but unreduced buffer under N_2 (Fig. 2). An electron donor was required for activity, and either formate or elemental iron could serve this function, the latter indicating that the electron donor need not also be a proton donor. An electron acceptor was not required for formate consumption by resuspended cells; however, the addition of 3CB to such suspensions stimulated formate consumption (Fig. 3). These results are consistent with those from growing cultures. The stimulation of oxidation of formate to CO_2 was stoichiometrically equal to the reductive dechlorination of 3CB to benzoate (Table 2). Acetate was not produced by cell suspensions.

Discussion

The ability of formate to support reductive dechlorination in cell suspensions (Fig. 2) and the stimulation of formate consumption by dechlorination in growing cultures (Fig. 1)

Table 2. Substrate consumption and product formation after 34 h incubation of resuspended DCB-1 cells provided with 26.8 μmol formate with or without 5.2 μmol 3CB*

Treatment	Formate consumed	CO_2 produced	3CB consumed	Benzoate produced
Without 3CB	9.0 ± 0.2	8.1 ± 0.1	–	–
With 3CB	15.3 ± 0.3	13.4 ± 0.5	5.2 ± 0.0	5.8 ± 0.1
Difference due to dechlorination	5.7 ± 0.5	5.3 ± 0.6	5.2 ± 0.0	5.8 ± 0.1

* Values are means of triplicates in μmol ± standard error

and cell suspensions (Fig. 3) strongly support the coupling of formate oxidation and dechlorination by DCB-1. The differences in formate consumption between dechlorinating cells and non-dechlorinating controls, relative to dechlorination, in both growing cultures (Fig. 1) and cells suspensions (Table 2) suggest that the two processes are stoichiometrically related according to the following equations:

$$HCOO^- + 3CB^- + H_2O \rightarrow HCO_3^- + \text{benzoate}^- + H^+ + Cl^-$$

$$2\,HCOO^- + 35DCB^- + 2\,H_2O \rightarrow 2\,HCO_3^- + \text{benzoate}^- + 2\,H^+ + 2\,Cl^-.$$

Another oxidant than 3CB or 35DCB is required to explain the formate consumption in non-dechlorinating cultures and cell suspensions and the stimulation of formate consumption in dechlorinating cultures and cell suspensions somewhat in excess of that predicted by the above equations. Fixation of CO_2 probably accounts for at least some formate oxidation in the growing cultures. We have found that CO_2 fixation consumes a significant portion of the reducing equivalents produced by DCB-1 cultures oxidizing pyruvate (Mohn and Tiedje, submitted). Since acetate was not formed in cultures or cell suspensions, homoacetogenesis cannot account for formate consumption. Formate consumption by resuspended DCB-1 cells without 3CB resembles the ability of washed and starved *Desulfovibrio desulfuricans* cells to consume H_2 without an electron acceptor (Postgate 1949) and may indicate oxidation potential endogenous to the cells.

The failure of H_2 to serve as an electron donor in cell suspensions does not rule out the coupling of H_2 oxidation to reductive dechlorination in growing cultures, since a lack of induction, or conditions in the cell suspensions, may have prevented uptake hydrogenase activity. In growing cultures containing H_2 plus 3CB, no electron donor other than H_2 was present in quantities sufficient to support the dechlorination observed.

Results of this study indicate that, in pure culture, strain DCB-1 can couple oxidation of formate and, probably, of hydrogen to reductive dechlorination of 3CB or 35DCB, and can conserve energy for growth from these redox couples. Since oxidation of neither electron donor is thought to support substrate-level phosphorylation, it is likely that energy conservation is via electron transport phosphorylation. Because chlorobenzoates are not substrates normally available in natural habitats, the ability of DCB-1 to catabolically utilize chlorobenzoates is probably not due to natural selection for such utilization. Catabolism of chlorobenzoates probably is due to a respiratory system, either known or novel, having another terminal electron acceptor. Stevens et al. (1988) have shown that DCB-1 can utilize sulfoxy anions by what may be a respiratory process resembling that found in sulfate reducing bacteria. It remains to elaborate the respiratory system of DCB-1 and to demonstrate coupling of that system to reductive dechlorination.

In the accompanying paper, Dolfing also provides evidence that DCB-1 can conserve energy for growth from reductive dechlorination. The two studies are consistent, but as one would expect, the different culture conditions of the studies resulted in different growth rates and growth yields. Unique to Dolfing's medium were 5 mM acetate and the filter-sterilized vitamins, naphthoquinone, nicotinamide and thiamine. Unique to our medium was PCF. Also, Dolfing's medium was reduced with cysteine and sulfide, while ours was reduced with cysteine and titanium citrate. Dolfing reports a doubling time of 8 to 10 days for DCB-1 growing on 3CB; while in our study it was approximately 45 days. The faster dechlorination observed in our cultures with PCF relative to those without PCF (Fig. 1) indicates that some factor provided by PCF may be limiting dechlorination, and thereby, growth on our media. A 45-day doubling time is long relative to most laboratory batch cultures, but is not long relative to those experienced by bacteria in many natural systems. Components provided by Dolfing's medium may have decreased the energy requirement for growth (Y_{ATP}) relative to our cultures. Also, the slower growth of our cultures would have increased the ratio of energy requirement for maintenance functions to energy requirement for growth relative to Dolfing's cultures. These two factors may contribute to the difference in growth yield values reported by Dolfing (6.0 g protein per mole 3CB) and by us (1.7 – 3.4 g protein per mole 3CB).

Dolfing and Tiedje (1986) reported a doubling time of 3 days for the defined consortium growing on 3CB. The shorter doubling time of the consortium may indicate that syntrophic conditions are more favorable; one possibly significant factor is the removal of the benzoate product in syntrophic cultures. The growth yields reported here are close to that of 1.9 g protein per mole 3CB reported for the defined consortium (Dolfing and Tiedje 1987).

Recent work in this laboratory (Quensen et al. 1988) found biological dechlorination of polychlorinated biphenyls (PCBs) added to river sediments with a history of PCB contamination, but found no activity in sediments upstream of the contamination. Thus, natural selection for dechlorinating populations seems possible. The finding that dechlorination can support growth identifies a possible mechanism for such selection. An improved understanding of this phenomenon may prove very useful both in understanding the response of natural systems to chlorinated pollutants and in developing methods for biological remediation of chlorinated wastes.

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Chapter 4:

Evidence for chemiosmotic coupling of reductive dechlorination and ATP synthesis in *Desulfomonile tiedjei*

Introduction

Desulfomonile tiedjei (formerly strain DCB-1) is a sulfate reducing bacterium (DeWeerd et al., 1990; Mohn and Tiedje, 1990a) which is capable of the reductive dehalogenation of chlorobenzoates (Shelton and Tiedje, 1984; DeWeerd et al., 1986; Cole, 1990) and chloroethylenes (Fathepure et al., 1987). This organism was shown to conserve energy for growth from reductive dechlorination of 3-chlorobenzoate (3CB) and 3,5-dichlorobenzoate (Dolfing, 1990; Mohn and Tiedje, 1990b). The addition of 3CB also supported ATP production in stationary phase cultures which had been limited by 3CB (Dolfing, 1990). Dechlorination of 3CB was shown to be coupled to formate and, probably, to H_2 oxidation (Mohn and Tiedje, 1990b). Since those electron donors are not known to support substrate-level phosphorylation, it seems likely that a chemiosmotic (respiratory) mechanism might couple dechlorination and ATP synthesis. Such a process would represent a novel mode of anaerobic respiration. Of possible significance to such a mechanism, the electron carrier cytochrome c_3 has been identified in *D. tiedjei* (DeWeerd et al., 1990) and dechlorination activity appears to be membrane associated in this organism (K.A. DeWeerd, 1989, personal communication).

Respiratory inhibitors originally used in mitochondrial systems have been successfully used to study anaerobic chemiosmotic mechanisms in a phylogenetically diverse range of bacteria. Extensive work of this nature on methanogenic bacteria has been reviewed by Daniels et al. (1984). Respiratory inhibitors have also been used with sulfate-reducing bacteria (Barton et al., 1970; Kramer

and Cypionka, 1989; Steenkamp and Peck, 1981), acetogenic bacteria (Ivey and Ljungdahl, 1986), dissimilatory iron reducing bacteria (Arnold et al., 1986) and fermentative bacteria (Cox and Henick-Kling, 1989; Russell and Strobel, 1989). The following inhibitors have been found broadly effective in various of the above studies and were employed in this study: the uncouplers, pentachlorophenol (PCP), 2,4-dinitrophenol (DNP), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP); the ionophores, monensin and gramicidin; and the proton-driven ATPase inhibitor, N,N'-dicyclohexylcarbodiimide (DCCD).

This study examined the effects of respiratory inhibitors and imposed pH gradients on resuspended *D. tiedjei* cells in order to test the hypothesis that a chemiosmotic mechanism couples reductive dechlorination and ATP synthesis. *D. tiedjei* was also maintained through serial transfers on defined medium with formate plus 3CB as growth substrates, providing further evidence reductive dechlorination supports energy metabolism.

Materials and Methods

MEDIA. Cultures were grown on formate plus 3CB in a mineral medium plus the trace element solution for *Desulfobulbus* (Widdel and Pfennig, 1984) with the following modification and additions: $CaCl_2$ was reduced to 0.1 mM, 10 mM HEPS buffer (hemisodium salt), 1 mg/l resazurin, 12 mM sodium formate, 6 mM sodium acetate, 1 mM 3CB, 3 μg/l Na_2SeO_3, 8 μg/l Na_2WO_4, 30 mM $NaHCO_3$, 1 mM cysteine, vitamins and 0.1 mM titanium (III) citrate. The gas phase was N_2-CO_2 (95:5). The pH was adjusted to 7.5 before autoclaving. The vitamins included 500

μg/l nicotinamide, 200 μg/l naphthoquinone, 50 μg/l thiamine and 50 μg/l lipoic acid (DeWeerd et al., 1990) added from 1000-fold concentrated, filter-sterilized stock solution dissolved in NaOH. Titanium citrate was prepared according to Zehnder and Wuhrmann (1976) and added from filter-sterilized stock solution. When depleted, 3CB was replenished from filter-sterilized stock solution. Stock cultures used to provide resuspended cells were grown on the mineral medium of Shelton and Tiedje (1984) with the following modifications and additions: the reductant was changed to 1 mM cysteine plus 0.1 mM titanium citrate, the vitamins were changed to the above mixture, 10 mM HEPS buffer (hemisodium salt), 20 mM sodium pyruvate and 1 mM 3CB. Pyruvate was added from filter-sterilized stock solution. The pH, gas phase and 3CB additions were as above. All inocula were 10%, except for cultures used for the growth curve (Fig. 1) which had 5% inocula. All incubations of cultures and cell suspensions were at 37°C.

CELL SUSPENSIONS. Cells in log phase and actively dechlorinating 3CB were harvested by centrifugation in the culture vessels (160-ml serum bottles) for 60 min at 2500 rpm at 4°C. The pellet was washed in 4°C buffer containing, 20 mM HEPS or 20 mM Tris-HCl buffer, 1 mg/l resazurin, 1 mM Na_2S and 0.5 mM titanium citrate, pH 7.5 under a gas phase of N_2. Cells were centrifuged again for 30 min and resuspended in buffer, concentrated 3- to 5-fold (final protein concentration 90 to 186 μg/l). 3CB was added from a neutralized stock solution (pH 7.5). When used, inhibitors were added 20 min prior to 3CB addition from ethanol stock solutions and all treatments, including controls

received the same amount (0.5%) of ethanol. HCl was added from a 5 M stock solution.

ANALYSES. Benzoates were analyzed by high pressure liquid chromatography as described previously (Stevens et al., 1988) except the eluent consisted of water-acetone-phosphoric acid (66:33:0.1) and the UV detector was set at 230 nm. Organic acids were analyzed by high pressure liquid chromatography as described previously (Stevens et al., 1988) with the column at 60°C. ATP was extracted as follows: a 0.20-ml sample was added to 0.80 ml 95°C 20 mM Tris-HCl pH 7.8, incubated 5 min at that temperature and filtered (0.45 μm). ATP was assayed with luciferin-luciferase reagent in Tris-aspartate buffer (Sigma Chemical Co., St. Louis, MO) using a Chem-Glo photometer. A linear standard curve was obtained over the range of ATP concentrations assayed. Protein concentration was determined by a modification of the Lowry assay (Hanson and Phillips, 1981).

Results

D. tiedjei grew through at least five serial transfers on defined medium with formate plus 3CB. Growth was dependent on 3CB and benzoate accumulated as a product (Fig. 1). The final protein concentrations in control cultures and in cultures with 3CB were 9 and 68 μg/ml, respectively. Cultures with 3CB consumed 12 mM formate and produced 1.6 mM acetate.

Upon addition of 3CB to resuspended cells, dechlorination immediately began and the cellular ATP pool rapidly increased approximately 3-fold (Fig. 2). Benzoate accumulated stoichiometrically as a product of 3CB. The dechlorination rate and

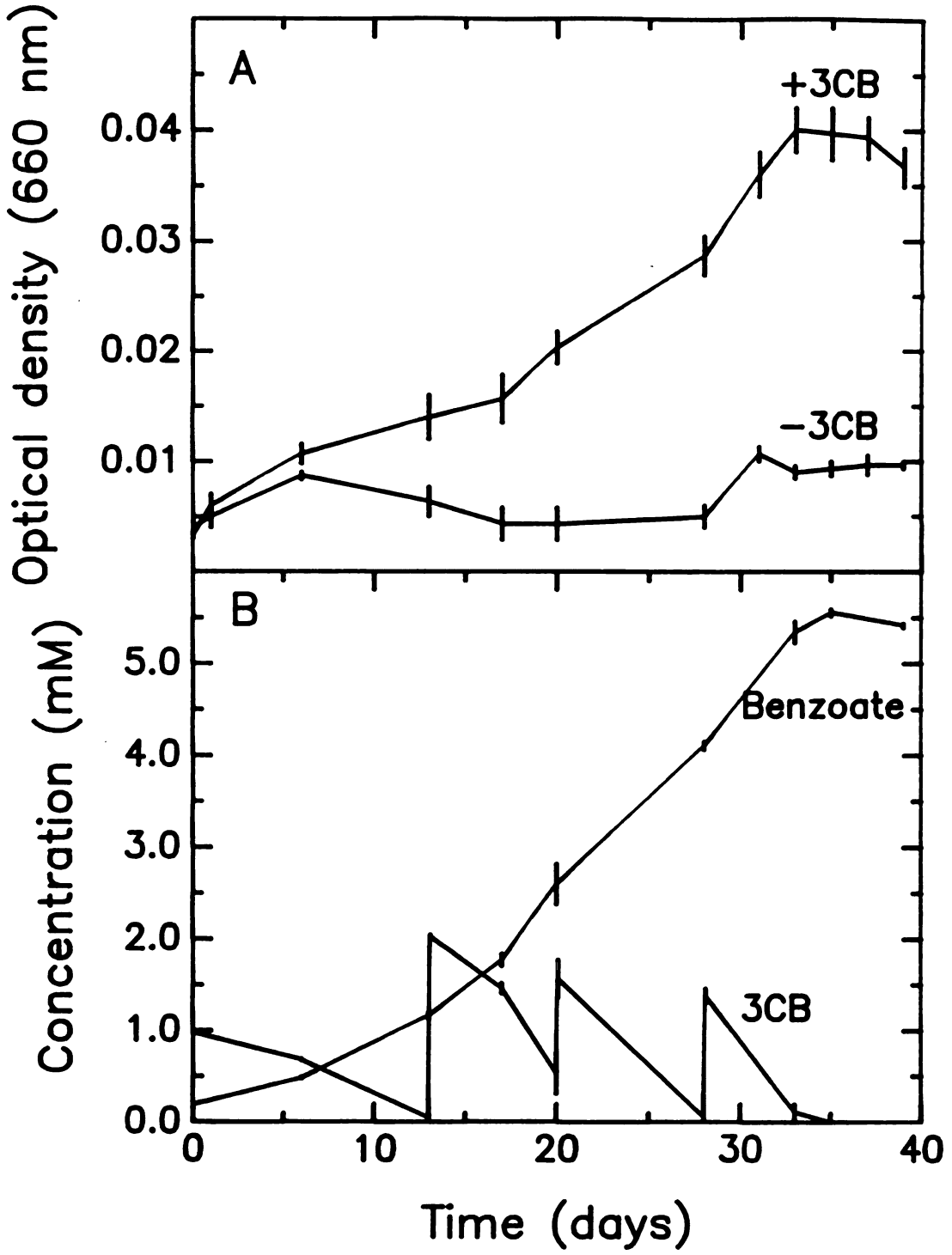

FIGURE 1. *Desulfomonile tiedjei* dependence on 3CB for growth (A) and metabolism of 3CB (B) on defined medium with formate plus 3CB as substrates. Data are means of triplicates with standard error bars.

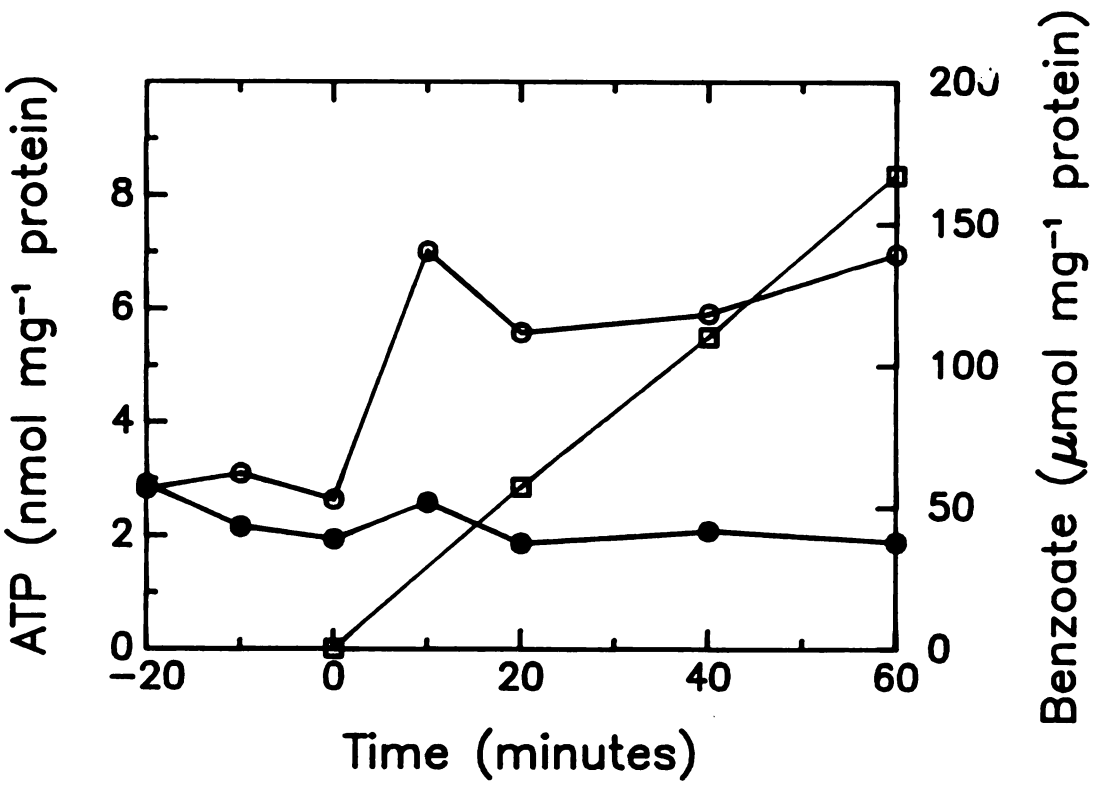

FIGURE 2. Reductive dechlorination and consequent ATP pool increase in *Desulfomonile tiedjei*. Cells were resuspended in Tris buffer. At 0 min 3CB was added to one suspension (open symbols) but not to the control (closed circles). Circles, ATP; squares, dechlorination measured as benzoate accumulation.

the ATP pool remained constant for at least one hour; thus, dechlorination and ATP pools were measured 1 h after addition of 3CB to similar suspensions in order to determine the effects of respiratory inhibitors.

Uncouplers including PCP, DNP and CCCP inhibited both dechlorination and the ATP increase, the latter being extremely potent and causing complete inhibition of both at a concentration of 5 μM. At a relatively low concentration, which caused only partial inhibition, PCP had a greater effect on ATP synthesis than on dechlorination (Table 1). The ionophore, monensin, had an effect similar to PCP; another ionophore, gramicidin, increased the dechlorination rate and had little effect on the ATP pool increase.

The proton-driven ATPase inhibitor DCCD inhibited both dechlorination and the ATP increase at high concentrations, but only inhibited the ATP increase at low concentrations (Fig. 3). At the lowest concentration tested (50 μM), DCCD reduced the increase in ATP due to 3CB but actually increased the dechlorination rate (Table 1).

An imposed pH gradient caused an immediate increase in the ATP pool by approximately half in resuspended cells (Fig. 4). Preincubation of the cells for 20 min with 1 mM KSCN, a permeant ion, had no apparent effect.

Discussion

The serial transfer of *D. tiedjei* on defined medium with formate plus 3CB indicates that all growth requirements were provided, and the dependence of growth on 3CB which was converted to benzoate (Fig. 1) leaves little doubt that 3CB functions as a

TABLE 1. Effects of respiratory inhibitors on dechlorination and ATP concentration in resuspended cells of Desulfomonile tiedjei.

Treatment[1]	Dechlorination (umol/h /mg protein)	ATP (nmol/mg protein)	ATP increase (nmol/mg protein)	Ratio ATP increase: dechlorination
No 3CB added	-	1.4 ± .06[2]	0.0	-
Control	1.00 ± .01[2]	4.9 ± .23	3.5	3.5
DCCD, 50 uM	1.27 ± .02	3.4 ± .23	2.0	1.6
PCP, 10 uM	0.89 ± .10	3.4 ± .30	2.0	2.2
Monensin, 100 uM	0.80 ± .08	3.4 ± .62	2.0	2.5
Gramicidin, 100 uM	1.19 ± .08	4.6 ± .29	3.2	2.7

[1]Cells were resuspended in HEPS buffer.
[2]Values are means of triplicates ± standard error.

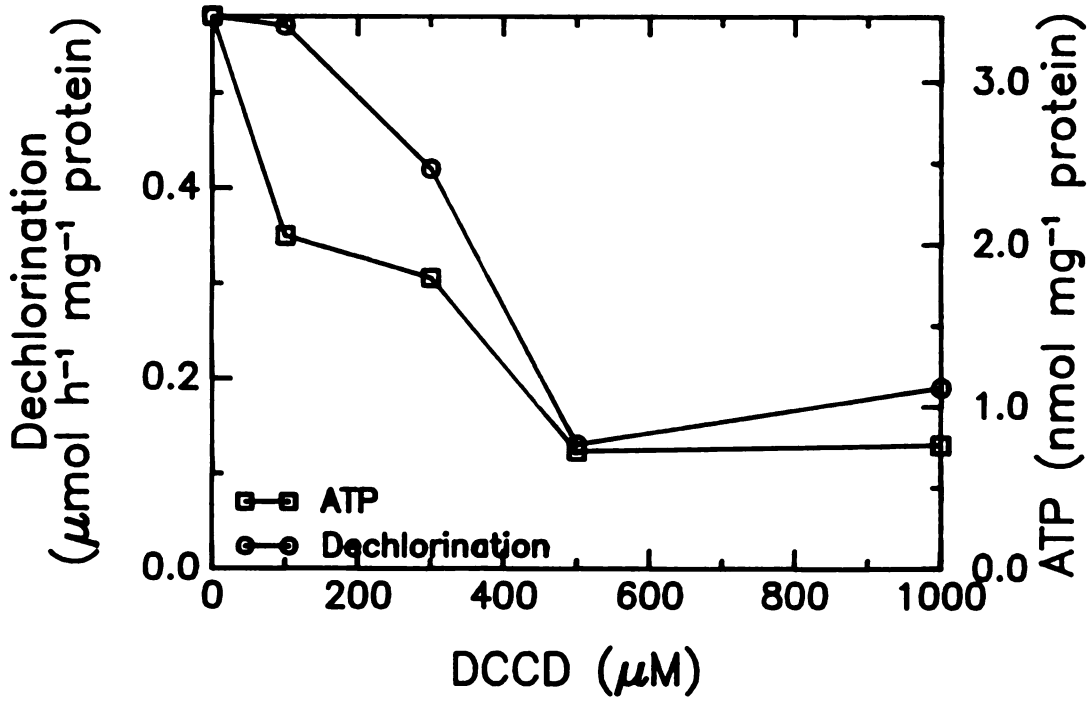

FIGURE 3. Effect of DCCD on dechlorination rate and ATP concentration. Cells were resuspended in Tris buffer.

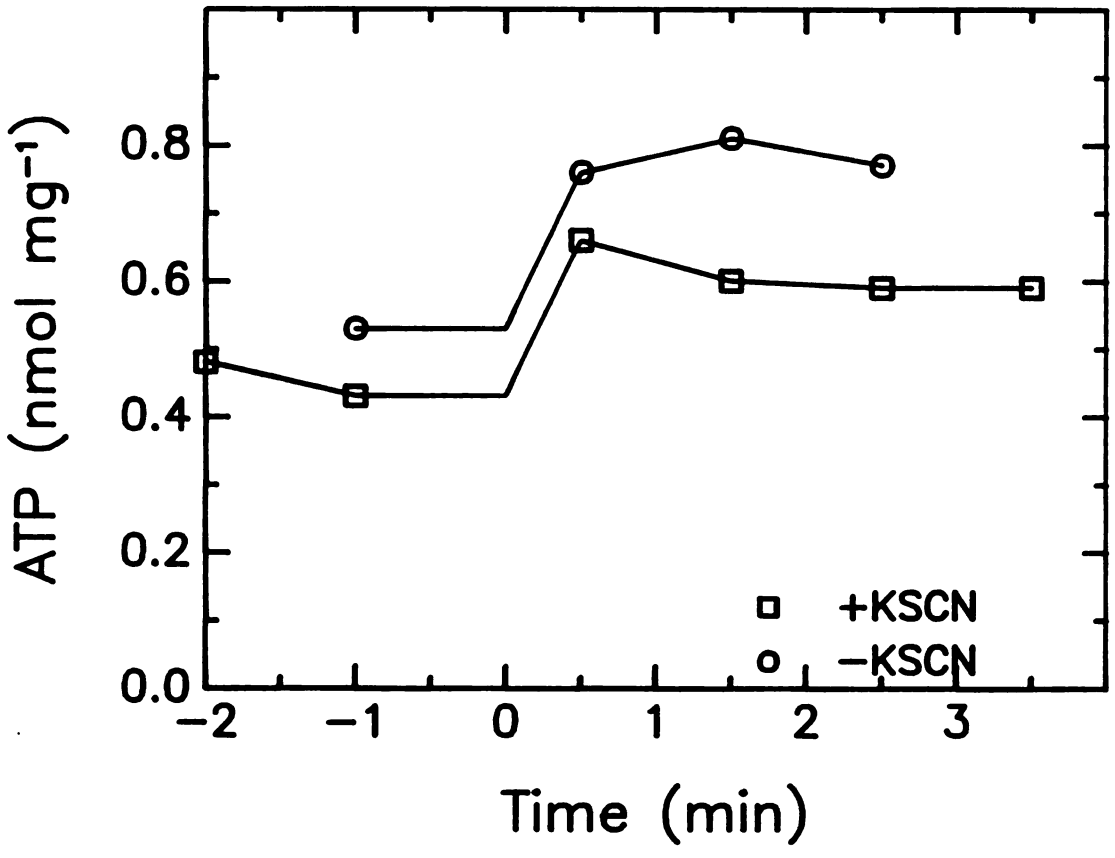

FIGURE 4. ATP pool increase in *Desulfomonile tiedjei* resulting from an imposed pH gradient. Cells were resuspended and starved in HEPS buffer without formate. At 0 min the pH was reduced from 7.5 to 2.9 by HCl addition.

catabolic electron acceptor. Despite some acetate production in the cultures of this study, *D. tiedjei* could not grow acetogenically on formate plus CO_2 alone. This inability is in agreement with previous findings (DeWeerd et al., 1990; Mohn and Tiedje, 1990b). The acetate production might account for the formate consumption observed which was in excess of that required for dechlorination; however, the energetic consequences of such a process are not clear. The coupling of formate oxidation to dechlorination was previously shown in cultures and cell suspensions (Mohn and Tiedje, 1990b), and, as in this study, formate consumption was in excess of that required for dechlorination.

The ATP increase in resuspended cells upon addition of 3CB (Fig. 2) agrees with previous findings of Dolfing (1990) using stationary phase cultures and indicates that dechlorination and ATP synthesis are somehow coupled. Uncouplers and ionophores are both able to dissipate a proton-motive force (ΔP). In cell suspensions of *D. tiedjei*, these agents had the common effect of reducing the ATP increase relative to the dechlorination rate (Table 1), thus, apparently reducing the efficiency of dechlorination-dependent ATP synthesis. Such an effect suggests chemiosmotic coupling of dechlorination and ATP synthesis in which dechlorination supports formation of ΔP which then supports ATP synthesis. An alternative hypothesis is that dechlorination supports ATP synthesis which then supports formation of ΔP; however, in the latter case, uncouplers and ionophores would not be expected to affect the efficiency of dechlorination-dependent ATP synthesis. The similar effect of such a

variety of agents lessens the possibility that the observations are artifacts of unexpected activities of these agents. However, the possibility exists that the dechlorinating enzyme, which is believed to be membrane associated (K.A. DeWeerd, 1989, personal communication), could be directly inhibited by these agents. While these results suggest the involvement of H^+ as a coupling ion, they do not rule out the additional significance of other ions.

The finding that low concentrations of DCCD do not inhibit dechlorination while they do inhibit the ATP increase (Fig. 3; Table 1) suggests that the action of DCCD is specific to a proton-driven ATPase which is involved in converting ΔP to ATP. The existence of such an ATPase in *D. tiedjei* is further supported by the finding that an imposed pH gradient (inside alkaline) also causes an increase in ATP (Fig. 4). It would be very desirable to test whether this pH driven ATP increase is also sensitive to DCCD.

The inability of uncouplers, ionophores and DCCD to completely inhibit ATP synthesis while allowing dechlorination may indicate that either ATP or ΔP are required for dechlorination. Possible explanations would be that dechlorination requires active transport or an activation step. These two situations have been proposed for dissimilatory sulfate reduction (Cypionka, 1987) and methanogenesis (Mountfort, 1978), respectively.

The above results provide a consistent body of evidence supporting chemiosmotic coupling of reductive dechlorination and ATP synthesis in *D. tiedjei*. The general agreement of the above results is critical, as the use of respiratory inhibitors provides only indirect

evidence which is subject to unexpected activities of the inhibitors. Lancaster (1986) has even argued that results with methanogens similar to those reported here with *D. tiedjei* are not inconsistent with ATP synthesis via substrate-level phosphorylation. It would be informative to obtain more direct evidence such as measurement of dechlorination-dependent proton extrusion, isolation of ATPase activity or isolation of dechlorinating membrane vesicles. The latter might be especially useful in better understanding the dechlorination reaction. However, such evidence may be difficult to obtain owing to the slow growth rate and low yields of *D. tiedjei* in laboratory culture.

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Chapter 5:

Attempts to identify or isolate dehalogenating anaerobes

Introduction

Recent advances in our understanding of the physiology of the dehalogenating anaerobe, *Desulfomonile tiedjei* (formerly strain DCB-1) (DeWeerd et al., 1990; Dolfing, 1990; Mohn and Tiedje, 1990a; Mohn and Tiedje, 1990b; Stevens and Tiedje, 1988; Stevens et al., 1988) suggested new strategies for finding other dehalogenating anaerobes. First, the identification of DCB-1 as a sulfate-reducing bacterium suggested screening existing isolates of this physiological group for dehalogenation activity. A very limited attempt had previously been made by Linkfield (1985) using three *Desulfovibrio* spp. grown under sulfate-reducing conditions with 3-chlorobenzoate. Emphasis in the present study was given to genera with physiological similarities to DCB-1 and to substrates known to be readily dehalogenated in enrichment cultures. The conditions selected for testing dehalogenation activity were those found to be favorable for this activity in DCB-1.

The second strategy was to attempt to isolate from existing enrichment cultures dehalogenating anaerobes which have physiological similarities to DCB-1. These efforts yielded a new strain from the same source as DCB-1 which is morphologically and physiologically similar to DCB-1. Dehalogenating anaerobes were not obtained from other enrichment cultures.

Materials and Methods

ENRICHMENT CULTURES. Strains were isolated from four enrichment cultures (Table 1). Sewage sludge was collected prior to 1983 from

TABLE 1. Enrichment cultures used as sources for isolates.

Name	Source	Substrate	Medium
Sed 3BB	Lake sediment	3-bromobenzoate	RAMM
Slg 3BB	Sewage sludge	3-bromobenzoate	RAMM
Mang 3CB	Estuary sediment	3-chlorobenzoate	SS-RAMM
Ditch 2CP	Flooded ditch	2-chlorophenol	W+P

an anaerobic digester (Shelton and Tiedje, 1984). Lake sediment was collected from the highly eutrophic Wintergreen Lake, Kalamazoo Co., Mich. prior to 1985 by T.G. Linkfield. Estuary sediment was collected from a mangrove swamp in the Florida Keys in 1987 by T.O. Stevens. Sediment was collected from a flooded ditch near Lake Lansing, Ingham Co., Mich. in 1989. Enrichments from these sources have been maintained in our laboratory since those times. The Reduced Anaerobic Mineral Medium (RAMM) medium is from Shelton and Tiedje (1984). The SS-RAMM medium is the above mineral medium with added sea salts (220 mM NaCl, 26 mM $MgCl_2$, 10 mM KCl and 5 mM $CaCl_2$). The W+P mineral medium is one formulated for *Desulfobulbus* spp. (Widdel and Pfennig, 1984). The W+P medium was buffered (pH 7.0) with 30 mM $NaHCO_3$, and had a headspace of N_2-CO_2 (95:5). Media were reduced with 1.0 mM cysteine added before autoclaving and 0.1 mM titanium (III) citrate added after autoclaving, unless otherwise stated (Table 2). A vitamin mixture was added to all media after autoclaving; this included vitamins generally required by anaerobic bacteria (Wolin et al., 1963). Halobenzoates were added at 1 mM and

TABLE 2. Media used for isolations and numbers of isolates.

Abbreviation	Components (mM)[1]	No. of isolates from each enrichment			
		Sed 3BB	Slg 3BB	Man 3CB	Dit 2CP
P	pyruvate (10)	11	-	11	11
PR	pyruvate (10), rumen fluid (10%),	7	5	4	4
Prif	pyruvate (10), rifampicin	-	-	-	1
RT	rumen fluid (10%), titanium (III) citrate (1) as reductant	-	-	-	-
ThA	thiosulfate (10), acetate (1), dithionite (0.5) as reductant	5	-	4	2
FATh	formate (10), acetate (1), thiosulfate (5), dithionite (0.5) as reductant	2	-	-	-
MbTh	3-methoxybenzoate (2.5), thiosulfate (5), dithionite (0.5) as reductant	7	-	-	-
FA	formate (10), acetate (1)	10	-	12	11
$PThN_2$	pyruvate (10), thiosulfate (5), dithionite (0.5) as reductant, NH_4^+ omitted	5	2	-	-

[1]Media also contained the haloaromatic compound of the enrichment culture which served as the inoculum.

replenished after disappearance. 2-Chlorophenol was added at 0.25 mM and replenished after disappearance. Haloaromatic compounds were analyzed by high-pressure liquid chromatography as described by Stevens et al. (1988) except that the eluent was water-acetonitrile-phosphoric acid (66:33:0.1) and the UV detector was set at 230 nm for halobenzoates and 218 nm for 2-chlorophenol. Enrichment cultures were subcultured by 10% transfers into homologous media.

ISOLATIONS. Strains were isolated from the above enrichments by serial dilution into agar shakes. Mineral media homologous to the corresponding enrichments were used with 1.5% agar and various substrates (Table 2). In addition to the above vitamins, others required by *Desulfomonile tiedjei* for dehalogenation (Apajalahati et al., 1989; DeWeerd et al., 1990) were also added. The latter vitamins were nicotinamide (500 μg/l), 2,4-naphthoquinone (200 μg/l) and thiamine (50 μg/l) dissolved in NaOH solution. Colonies were picked with a syringe and transferred to homologous liquid media containing the haloaromatic compound of the original enrichment. Dehalogenation activity of the isolates was assayed by incubating the liquid cultures for at least two weeks after stationary phase was reached, assaying the haloaromatic substrates and comparing the values to those of uninoculated control cultures. When CO_2 was omitted from the mineral medium, the phosphate concentration was increased to 10 mM.

CULTURES OF SULFATE-REDUCING BACTERIA. The sulfate reducers used in this study (Table 3) were cultured on W+P medium described above with various substrates (Table 4). Stock cultures had the higher sulfate

TABLE 3. Sulfate-reducing bacteria tested for dehalogenation activity.

Name	Strain no.	Source	Media
Desulfovibrio desulfuricans	DG2	lab collection	P, FS[1], LS[1]
Desulfovibrio desulfuricans	DDS	lab collection	P, FS[1], LS[1]
Desulfovibrio sp.	G11	lab collection	FS
Desulfovibrio sp.	PS1	lab collection	FS[1]
Desulfovibrio sp.	P3	lab collection	P
Desulfovibrio sulfodismutans	ThAc01	F. Bak	P, ThA
none	ThA-2	this study	ThA, LS
none	ThA-3	this study	P, LS
Desulfobacterium autotrophicum	DSM 3382	R. Devereaux	HS[1], FS
Desulfobacterium niacini	DSM 2650	R. Devereaux	HS[1], FS, AS[1]
Desulfococcus multivorans	ATCC 33890	R. Devereaux	FS, BS
Desulfobacter hydrogenophilus	DSM 3380	R. Devereaux	HS[1], AS[1]

[1]Only tested for dehalogenation of 3-chlorobenzoate on this medium.

TABLE 4. Media used for growth of sulfate-reducing bacteria.

Abbreviation	Components (mM)
P	pyruvate (20)
HS	H_2-CO_2 (80:20, 2 atm), sulfate (1 or 5)
FS	formate (20), sulfate (1 or 5)
AS	acetate (20), sulfate (1 or 5)
LS	lactate (20), sulfate (1 or 5)
BS	benzoate (3), sulfate (1 or 5)
ThA	thiosulfate (5), acetate (1)

concentrations, and dehalogenation test cultures had the lower. All sulfate-containing media also had 0.1 mM $Na_2S_2O_4$ as an extra reductant and potential nutrient. Media for strains of *Desulfobacterium*, *Desulfobacter* and *Desulfococcus* had additional NaCl (6 g/l) and $MgCl_2.6H_2O$ (1.3 g/l). Dehalogenation activity was assayed as described above.

POTENTIAL ELECTRON ACCEPTORS. An important consideration in attempts to identify or isolate dehalogenating anaerobes is the possible effect on dehalogenation of any available electron acceptors (see Chapter 1). Specific concerns in this study were sulfoxy anions commonly used as substrates (i.e., $SO_4^=$, $S_2O_3^=$, $SO_3^=$) and media reductants (i.e., $S_2O_4^=$, Na_2S). The latter reductant was considered to be of concern because upon chemical oxidation it can yield $S_2O_3^=$. The preceding compounds were avoided or minimized unless specifically desired as electron acceptors. Cysteine plus titanium (III) citrate were therefore usually used as medium reductants.

Results and Discussion

SCREENING OF SULFATE-REDUCING BACTERIA. Sulfate reducers were screened under conditions thought most likely to allow dehalogenation activity. For the reasons stated above, sulfate reducers were tested with excess electron donor and limiting sulfate, and those capable of fermentative growth were additionally tested in the absence of sulfoxy anions. The former condition presumably allowed any inhibitory effect of sulfate to terminate after its exhaustion. Three haloaromatic compounds known to be readily dehalogenated in enrichment cultures were separately tested with each sulfate reducer, 3-chlorobenzoate (0.5 mM), 2-chlorophenol (0.25 mM) and 4-chlororesorcinol (0.25 mM). A total of 52 tests were performed (Table 3), but no dehalogenation activity was detected. Of course it is possible that the organisms tested might have activity under different conditions, or that other sulfate reducers available in pure culture, but not tested here, might have activity. It is perhaps more likely that other sulfate reducers not available in pure culture, and more closely related to *D. tiedjei* than those available, account for dehalogenation activity in certain enrichment cultures. This possibility was addressed by the following attempts to isolate from various enrichment cultures organisms with physiological similarities to *D. tiedjei*.

ENRICHMENTS. The Sed 3BB and Slg 3BB enrichment cultures were modified for this study by the addition of 20 mM formate, 1 mM acetate and 1 mM bromoethane sulfonate. The rationale was to select for organisms like *D. tiedjei* which can grow with formate as an

electron donor and halobenzoates as an electron acceptor. Acetate was an additional carbon source and bromoethane sulfonate was used to prevent methanogens from growing on the formate. The enrichments were serially transferred on this medium twice, retaining dehalogenation activity, and the resulting enrichments were used as inocula for isolations.

ISOLATIONS. Over 114 isolates were obtained and tested for dehalogenation activity. Media used (Table 2) selected for various physiological groups and were all previously found to support *D. tiedjei*. Several general observations were made on the various media.

P and PR media selected pyruvate fermenters. These isolates were usually large (5 μm diameter) cocci in clumps which were somewhat translucent under phase-contrast optics. The Man 3CB enrichment yielded very long (>30 μm) filaments on this medium. These morphotypes appeared to be only minor components of the enrichment cultures. These isolates did not show dehalogenation activity.

Prif medium was employed in an attempt to isolate spirochetes observed in the Ditch 2CP enrichment. However, this medium yielded only motile rods which showed no dehalogenation activity.

RT medium was used to select for organisms adapted to mixotrophic growth on substrates commonly at low concentrations in anaerobic habitats. Small colonies were obtained on this medium, but these did not grow when transferred to homologous liquid medium.

ThA medium selected thiosulfate disproportionaters. Most of these isolates were motile vibrios resembling *Desulfovibrio sulfodismutans* which could ferment pyruvate. Such isolates were tested for dehalogenation activity on P medium, but none showed activity. Two of these isolates from the Sed 3BB enrichment (ThA-2 and ThA-3) were further tested with the sulfate reducer cultures (Table 3). A large rod resembling *D. tiedjei* was obtained from the Sed 3BB enrichment on ThA medium, but this culture was contaminated with cocci. The rod could not be purified in further agar shakes or by passage on various selective media, suggesting that perhaps it was dependent on the cocci. On P medium the cocci outgrew the rod and no dehalogenation activity was observed.

MbTh medium was intended to select thiosulfate reducers which could O-demethylate and possibly further degrade methoxybenzoate. Again, motile vibrios which could ferment pyruvate were obtained, and again, no dehalogenation activity was detected on P medium.

FA medium was used in an attempt to select organisms capable of using formate as an electron donor and haloaromatic compounds as an electron acceptor (acetate was an additional carbon source). None of the isolates from this medium showed dehalogenation activity. Presumably the isolates (mostly rods) were homoacetogens growing on formate plus CO_2.

$PThN_2$ medium selected diazotrophic organisms. The Sed 3BB enrichment yielded motile vibrios which could ferment pyruvate but which had no dehalogenation activity on P medium. The Slg 3BB enrichment yielded large rods. These rods had visible collars and

were indistinguishable from *Desulfomonile tiedjei* by phase-contrast microscopy. These isolates fermented pyruvate and dehalogenated 3BB on P medium. One isolate (DCB-2) was selected for further study.

Strain DCB-2 also dehalogenated 3CB on P medium (Fig. 1). In contrast to *D. tiedjei*, the DCB-2 culture also consumed the benzoate product after transient accumulation. On this medium *D. tiedjei* produced 18 mM acetate plus 1.2 mM lactate; while, DCB-2 produced 22 mM acetate plus 1.9 mM lactate. These cultures did not produce methane. Also in contrast to *D. tiedjei* (see Chapter 4), DCB-2 did not grow on FA medium with 3CB. DCB-2 does appear able to use the same electron acceptors as *D. tiedjei* (see Chapter 2), since sulfate, thiosulfate and CO_2, but not nitrate, stimulate growth on pyruvate (CO_2 was omitted when testing other electron acceptors). The DCB-2 culture appeared pure, but these results remain to be confirmed after repurification of DCB-2. The possibility exists that a benzoate-degrading contaminant caused the observed benzoate consumption. The new isolate, DCB-2, thus is very similar to *D. tiedjei* morphologically and physiologically, but may have significant differences. DCB-2 probably warrants further physiological characterization. Molecular methods (e.g., comparing restriction fragment length polymorphisms or testing 16S rRNA homology) would also be very helpful in comparing DCB-2 to *D. tiedjei*.

Conclusions. With the caveat that not all culture conditions or substrates could be tested, it appears that reductive dehalogenation is not a general property of sulfate-reducing bacteria and is not a specific property of members of that group tested here. It should be

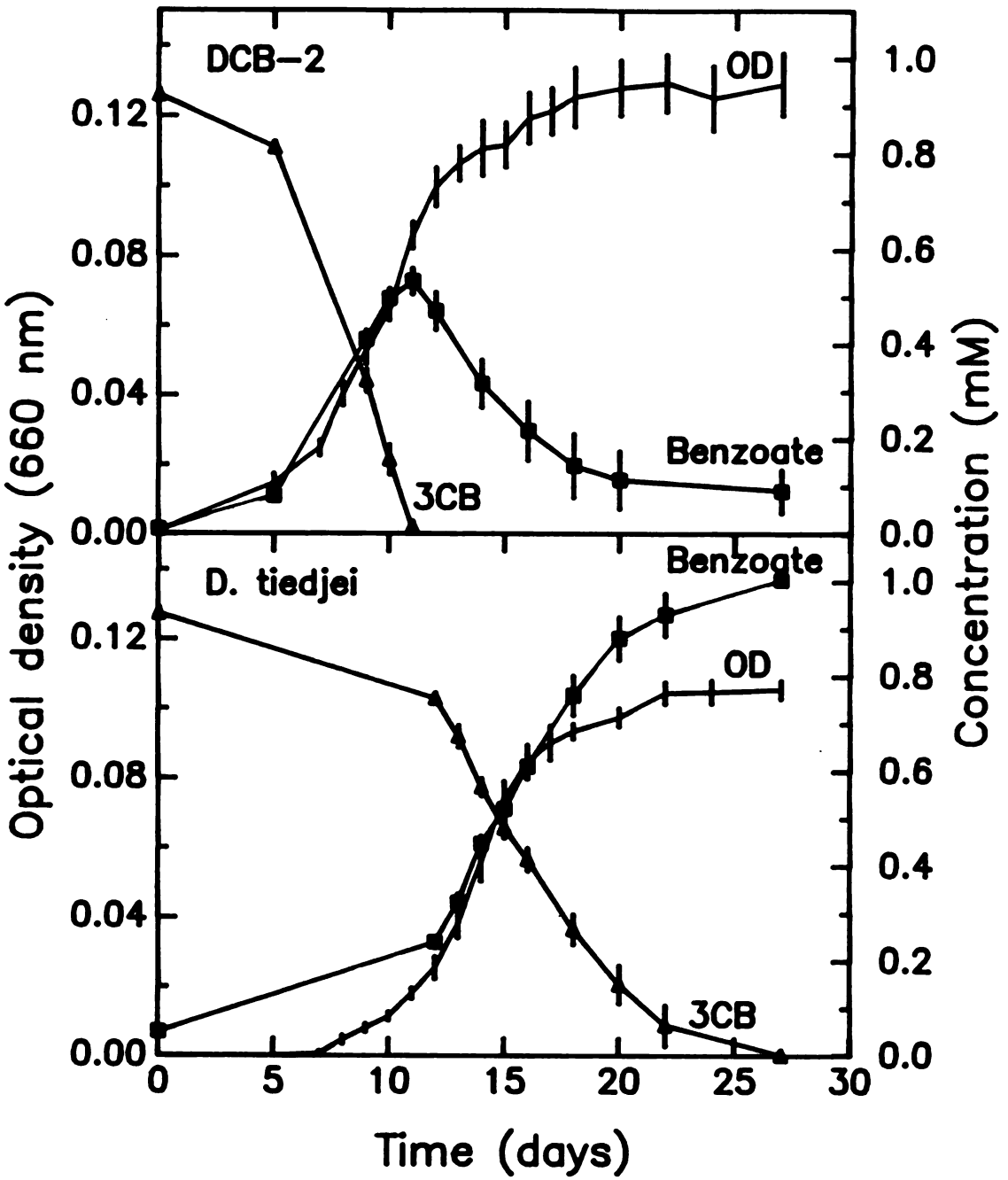

FIGURE 1. Growth on pyruvate and metabolism of 3-chlorobenzoate by strain DCB-2 and *Desulfomonile tiedjei*. Data are means of triplicates with standard error bars.

noted that a major subgroup, the gram-positive sulfate reducers, were not tested.

Enrichment cultures with halobenzoates as sole substrates maintain diversity even after long periods and multiple transfers, as demonstrated by the variety of physiological groups isolated. Isolates with a variety of physiological characteristics common to *D. tiedjei* were selected from such enrichment cultures, but in only one case was this selection specific for a dehalogenating organism. In that case selection was for diazotrophy, and the enrichment culture was the source of *D. tiedjei*. This isolate appears to be very closely related to *D. tiedjei*. It seems likely that dehalogenating organisms in the other enrichment cultures are physiologically different than *D. tiedjei* and cannot grow on the media used. The possibility exists, however, that the dehalogenating organisms are similar to *D. tiedjei* but are outnumbered by other organisms which grew on the media used. Future attempts to isolate reductively dehalogenating anaerobes will probably require new means of selection, optimally selection for dehalogenation activity itself.

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APPENDIX

Involvement of a collar structure in polar growth and cell division of strain DCB-1

The following was collaborative work. My contribution was to provide the scanning electron microscopy samples, to perform and analyze the cell measurements, to make the figures (excluding the prints), and to write the manuscript.

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Involvement of a Collar Structure in Polar Growth and Cell Division of Strain DCB-1

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Microscopic methods were used to investigate the unique collar structure of the gram-negative sulfate-reducing bacterium, strain DCB-1. Polar cell growth apparently occurred from the collar. When the daughter cell was approximately equal in length to the mother cell and the collar was thus centrally located, cell division occurred within the collar region. Division was by a novel mechanism which conserved the collar of the mother cell and gave rise to a new collar of the daughter cell. Cells of DCB-1 were also found to contain stacked internal membranes and glycogen bodies.

Strain DCB-1 was isolated from an anaerobic enrichment culture which mineralized 3-chlorobenzoate (8). Apparently, this isolate is a unique type of sulfate-reducing bacterium (6b, 9, 10). The isolate is capable of reductive dehalogenation of 3-chlorobenzoate (8), other halogenated benzoates (3), and tetrachloroethylene (4) and is of special interest since it is the only pure culture capable of anaerobic reductive dehalogenation of aromatic compounds. Dechlorination has recently been shown to provide energy to DCB-1 as a terminal electron-accepting process (3a, 6a).

Strain DCB-1 is a large, gram-negative rod with a unique morphological feature: a collar which girdles each cell (8). To our knowledge, no similar structure has been observed in other bacteria, with the possible exception of an unidentified organism from sediment of a hypereutrophic lake (2). The purpose of this study was to better characterize this collar and its origin. The collar was found to be involved in a unique mechanism of cell division.

MATERIALS AND METHODS

Cultures. Strain DCB-1 was obtained from our laboratory culture collection. Cultures were grown to late log phase on a previously described reduced anaerobic mineral medium (8) with 20 mM pyruvate and 10 mM thiosulfate as substrates.

Transmission electron micrographs. Agar-embedded cells were fixed overnight in cold 2.5% glutaraldehyde in 0.1 M Na_2HPO_4-KH_2PO_4 (pH 7.2), washed in the phosphate buffer, and postfixed for 1 h at room temperature in 1% OsO_4 in the phosphate buffer. The cells were dehydrated through a graded ethanol series and propylene oxide and were embedded in Poly/Bed 812 (Polysciences Inc., Warrington, Penn.). Thin sections were cut with a diamond knife mounted on an Ultotome III (LKB Instruments, Inc., Rockville, Md.). Sections were stained with uranyl acetate and lead citrate and were examined with a Philips EM 300 microscope.

Scanning electron micrographs. Cells were fixed in glutaraldehyde, mounted on a glass cover slip by using poly-L-lysine, and dehydrated by the procedure of Klomparens et

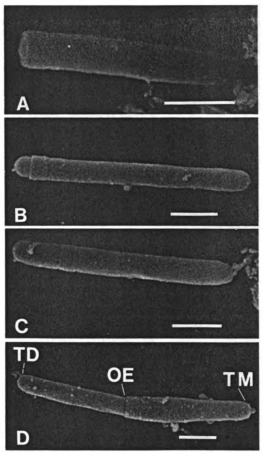

FIG. 1. Scanning electron micrographs of strain DCB-1 showing the collar in various locations. OE, Open end of collar; TM, terminus of mother cell; TD, terminus of daughter cell. Bar = 1 μm.

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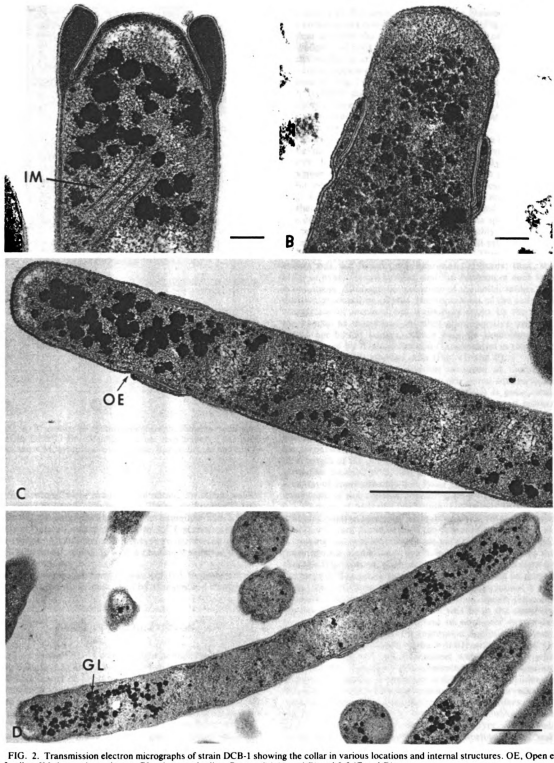

FIG. 2. Transmission electron micrographs of strain DCB-1 showing the collar in various locations and internal structures. OE, Open end of collar; IM, internal membrane; GL, glycogen bodies. Bars = 0.1 (A and B) and 0.5 (C and D) μm.

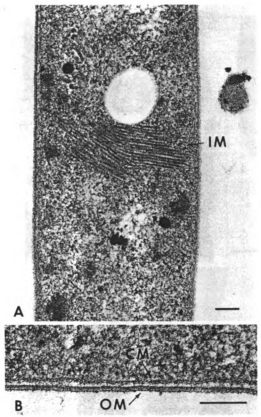

FIG. 3. Transmission electron micrographs showing membranes of strain DCB-1. IM, Stacked internal membranes; OM, outer membrane; CM, cytoplasmic membrane. Bars = 0.1 (A) and 0.5 (B) μm.

al. (6). Samples were mounted on aluminum stubs, coated with gold (20-nm thickness) in an Emscope Sputter Coater model SC 500 purged with argon, and examined with a Japan Electron Optics Limited model JSM-35CF scanning electron microscope. All cell measurements were performed on scanning electron micrographs with a randomly selected population of 28 cells.

Assay of glycogen. Glycogen was isolated, hydrolyzed, and assayed as glucose as described by Hanson and Phillips (5).

RESULTS AND DISCUSSION

Cells of strain DCB-1 were straight or slightly curved rods 3.2 to 8.7 μm long and 0.5 to 0.7 μm wide, in agreement with the initial report of Shelton and Tiedje (8). Each cell had a collar which varied in its location (Fig. 1). The width of the collar (measured parallel to the length of the cells) was 0.3 to 0.5 μm. The collar had a definite orientation, with an open end apparent on both scanning and transmission electron micrographs (Fig. 1 and 2). In all cells observed, the open end of the collar was the end closest to a cell terminus. The collar was faintly visible by phase-contrast microscopy. The cell wall of DCB-1 appeared to have the bimembrane structure common to gram-negative procaryotes (Fig. 3B). It was unclear whether the cytoplasmic membrane extended into the collar of all cells as was suggested in an earlier study (8). Occasionally, in cells with terminal collars, the interior of the collar appeared to be more electron dense than the cytoplasm (Fig. 2A). In the majority of cells, including those with terminal collars, the interior of the collar and the cytoplasm were of equal electron density, and the cytoplasmic membrane was not clearly discernible throughout the region of the collar.

The collar is shown by transmission electron micrographs to be the site of cell division (Fig. 4). Division was not observed at other locations. Division apparently involved (i) invagination of the membrane, which formed a small second collar beneath the first oriented in the opposite direction (Fig. 4A and B); (ii) further invagination, separating the mother and daughter cells (Fig. 4C and D); and (iii) completion of the new cell termini, leaving an open space between the cells (Fig. 4E and F). The cells presumably separated as the new collar slid out of the old. Each cell produced from this mode of division had a terminal collar. This sequence of events was not based on synchronous cultures; thus, the order of events is not certain, and the duration of each step is unknown. Although the presence of the collar makes this division process remarkable, the separation of the cells and completion of terminal cell walls may occur by the same mechanism as that found in other gram-negative bacteria. Membrane-bound bodies, which may be homologous to blebs formed by *Escherichia coli* (1), appeared in the space between the newly divided cells (Fig. 4E and F).

The observations that division occurred at the collar region and that all cells presumably originated with terminal collars suggest that growth of strain DCB-1 is polar, with daughter cells elongating from within the collars of mother cells. This mode of growth was further substantiated by analysis of cell measurements. The relative collar location was defined as the distance from the open end of the collar to the terminus of the presumed daughter cell divided by the total length of the cell (Fig. 1D). Thus, a terminal collar has a relative location of 0, and a central collar has a relative location of approximately 0.5. Polar growth should result in movement of this relative position from 0 to approximately 0.5. Consistent with this prediction, cell collars appeared at different relative locations with about equal frequency, and collars were not found beyond the center of the cell (Fig. 5). Assuming that mother cells do not vary too greatly in length, polar growth should also result in terminal collars on the shortest (most recently divided) cells and central collars on the longest. The relative collar location (Y) would be related to the total cell length (X) by the following equation: $Y = 1 - (b/X)$, where b is the initial total cell length (i.e., the mother cell length). This equation was fit to the observed data using the Marquadt method of nonlinear regression (Fig. 6). All assumptions for regression analysis were met, and the fit was significant (alpha = 0.01) with an r^2 of 0.81. Thus, the observed cell dimensions were consistent with polar growth. Variability not accounted for by the equation was most likely due to variation in the initial total cell length (b). The only alternative explanation for our observations is migration of the collar from the termini to the centers of cells during intercalary growth, but that seems unlikely if the collar includes the rigid peptidoglycan layer. The direct observation of growing cells and the labeling of cell wall components to determine where growth of the wall occurs were not possible because of the strict requirement for

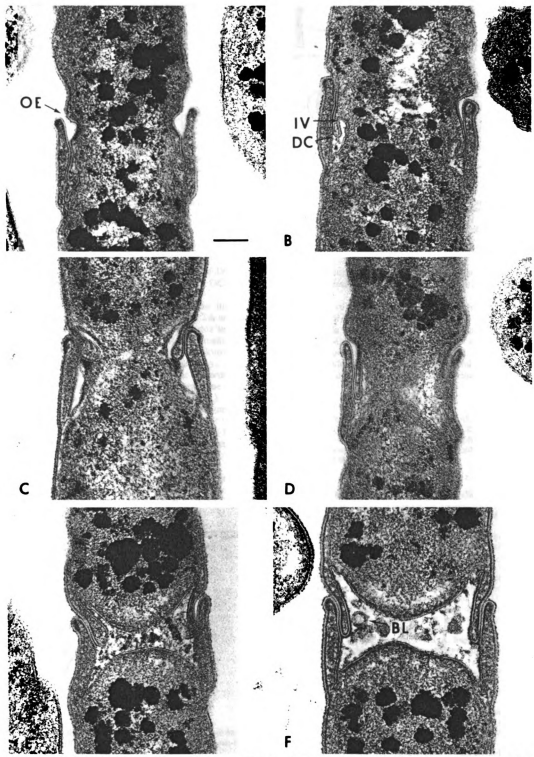

FIG. 4. Transmission electron micrographs showing the collar region of dividing cells of strain DCB-1. OE, Open end of collar; IV, invagination; DC, daughter cell collar; BL, blebs. Bar = 0.1 μm.

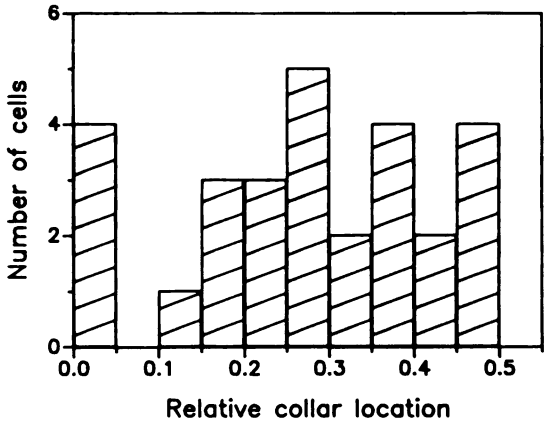

FIG. 5. Histogram showing distribution of collar locations in a randomly selected population of 28 cells.

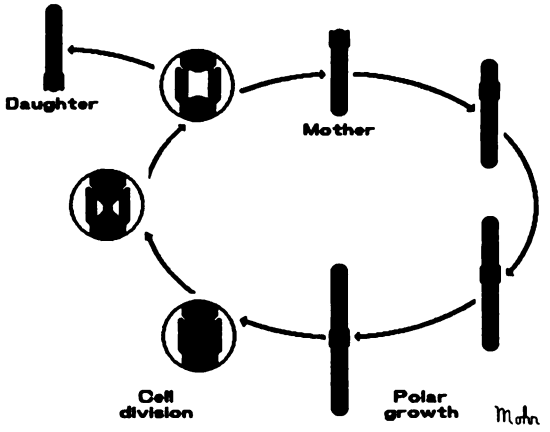

FIG. 7. Proposed mode of growth and division of strain DCB-1.

anaerobiosis and the low growth rate (t_d = 4d) of DCB-1. Our interpretation of growth and division of strain DCB-1 is summarized in Fig. 7.

Another feature observed in DCB-1 cells was internal membranes having a stacked configuration (Fig. 2A and 3). Other bacteria known to have internal membranes include sulfate reducers (7), anoxic phototrophs, methylotrophs, and nitrifiers. Many members of these groups are known to exhibit polar growth (e.g., *Rhodopseudomonas* spp., *Methylosinus* spp., and *Nitrobacter* spp.). Polar growth also occurs in other groups that have complex membrane structures such as prosthecae (e.g., *Caulobacter* spp.). Polar growth can be understood as adaptive for such organisms, since intercalary growth would disrupt membrane structures. Electron-transparent spheres such as that in Fig. 3 were observed only rarely and did not normally accompany internal membranes. The sphere was not distinctly membrane bound, and its composition is unknown.

An additional feature visible in the transmission electron micrographs is electron-dense, spherical bodies which occurred preferentially near the poles of DCB-1 cells (Fig. 2).

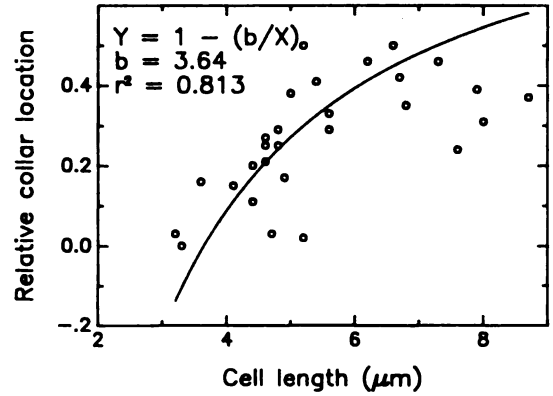

FIG. 6. Relative collar location as a function of cell length for the same population of cells as in Fig. 5 with nonlinear regression fit of polar-growth model.

These bodies have the appearance of glycogen. The extractable polysaccharide of DCB-1 was determined to be 80% glycogen.

The collar of strain DCB-1 appeared to be intimately involved in cell division. No other function for the collar was apparent. This unique morphological feature distinguishes DCB-1 from other procaryotes, as does its unique combination of metabolic characteristics (6b, 9, 10). It remains to be determined whether the morphology and physiology of DCB-1 define a novel group of sulfate-reducing bacteria.

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