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Influence of Substituents on Reductive Dehalogenation of 3-Chlorobenzoate Analogs

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The biochemical effects of aryl substituents on the reductive dechlorination of 3-chlorobenzoate analogs were quantified with (i) a stable 3-chlorobenzoate-grown methanogenic sludge enrichment, (ii) *Desulfomonile tiedjei* DCB-1, isolated from this enrichment and able to catalyze the reductive dechlorination of 3-chlorobenzoate, and (iii) a defined 3-chlorobenzoate-degrading methanogenic consortium with *D. tiedjei* as the key dechlorinating organism. The addition of hydrogen stimulated the dechlorination rate in the consortium. The extent of this stimulation depended on the substituent. The data were evaluated with various sets of substituent constants compiled for the Hammett equation. None of the sets yielded a satisfactory correlation between experimental values and theoretical constants. This suggests that the microbially catalyzed reductive dechlorination of 3-chlorobenzoate cannot be described simply as either a nucleophilic or an electrophilic substitution reaction. Nevertheless, observations that the presence of a *para*-amino or -hydroxy group inhibited the rate of dechlorination suggest that the rate-limiting step in the reductive dechlorination of 3-chlorobenzoate is a nucleophilic attack on the negatively charged π electron cloud around the benzene nucleus.

The presence of a carbon-halogen bond in organic compounds strongly affects their biodegradability (17, 25). Although naturally occurring organohalides have been identified, mainly in marine environments (7, 16), halogenated organic compounds are frequently considered to be xenobiotics. This concept is reinforced by the observation that many anthropogenic halogenated compounds tend to persist once they have entered the biosphere (29).

Some halogenated aromatic compounds are dehalogenated under aerobic conditions by enzymatic hydrolysis (13, 14, 19, 22) or by specific monooxygenases (12, 23), but many of them may eventually end up in anaerobic environments. Fortunately, however, the anaerobic microflora has the ability to remove halogens from at least some halogenated organics. Reductive dehalogenation has been observed for a variety of both aromatic and nonaromatic organohalides (35, 36). The mechanism of the aryl dehalogenation reaction, in which halosubstituents on the aromatic ring are replaced by hydrogen, is not known. Electrophilic and nucleophilic attacks on the aromatic ring can both be envisaged (1).

The first observations of the reductive dechlorination of chlorinated aromatic compounds were made with a 3-chlorobenzoate-mineralizing methanogenic sludge enrichment (32). This enrichment culture was also able to dechlorinate a number of substituted 3-chlorobenzoate analogs (3, 32). The organism responsible for the reductive dechlorination, *Desulfomonile tiedjei* (2), formerly known as strain DCB-1 (31), has been isolated in pure culture and is also capable of dechlorinating a variety of substituted 3-chlorobenzoate analogs (18). This capacity allows the establishment of structure-activity relationships which may give insight into the chemical mechanism of biologically mediated reductive dechlorination. Electron-withdrawing substituents such as halogens lower the electron density of the aromatic nucleus and render the ring less susceptible to electrophilic attack. Electron-contributing substituents such as amino, methyl, and hydroxy groups, on the other hand, increase the electron density on the aromatic nucleus, thus making the ring more susceptible to electrophilic attack and less susceptible to nucleophilic attack. Besides this influence of the electronegativity of a substituent, the possibility of mesomeric structures for intermediates will also affect the reaction pathway. Efforts to quantify such effects have met considerable success, especially for purely chemical, nonenzymatic reactions (15), but successful application of these ideas to biological systems has also been reported (6, 24, 26, 27). Structure-activity relationships could also be of value in providing rate constants for models designed to predict the environmental fate of chemicals (24).

Structure-activity relationships frequently follow (modifications of) the Hammett equation, written as $\log (k_x/k_h) = \rho \cdot \sigma$, where k_x is the reaction rate of the substituted analog, k_h is the reaction rate of the nonsubstituted analog, σ is a constant which reflects the chemical properties of the substituent, and ρ is a constant which reflects the effect of the substituent on the reaction rate. This equation provides a tool to evaluate quantitatively the effects of a series of substituents on the rate of a chemical reaction, which in turn gives information on the mechanism of this reaction. This approach is only applicable for substituents *meta* or *para* relative to the reaction center, since *ortho* substituents introduce steric effects in addition to the desired electronic effects (15).

In this paper we present results on the rate of dechlorination of a series of 3-chlorobenzoate analogs, as exhibited by the original 3-chlorobenzoate-mineralizing enrichment, the dechlorinating bacterium *D. tiedjei*, and a defined 3-chlorobenzoate degrading methanogenic consortium in which *D. tiedjei* is the dechlorinating organism. The objectives were (i) to establish the effects of various substituents on the rate of dechlorination, (ii) to evaluate the obtained structure-activity relationship for various sets of Hammett constants, and (iii) to use this information for a preliminary discussion of

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TABLE 1. Characteristics of substituted 3-chlorobenzoate analogs used in the present study (9, 15, 30)

Test compound	Substituent	Position[a]	σ	$pK_{substrate}$	$pK_{product}$[b]	σ'[c]
2-Amino-5-chlorobenzoate	Amino	*para*	−0.66	1.69	2.17	−0.48
2,5-Dichlorobenzoate	Chloro	*para*	0.23	2.47	2.91	0.44
3,5-Dichlorobenzoate	Chloro	*meta*	0.37	3.54	3.82	−0.28
2-Hydroxy-5-chlorobenzoate	Hydroxyl	*para*	−0.37	2.65	2.97	−0.32
2-Methyl-5-chlorobenzoate	Methyl	*para*	−0.17	3.63	3.98	−0.35

[a] Position relative to the 3-chloro position.
[b] Product after dechlorination at the 3-chloro position.
[c] σ', $pK_{substrate} - pK_{product}$; σ'', $pK_{substrate}$ − pK3-chlorobenzoate, i.e., −2.13, −1.35, −0.28, −1.17, −0.19 (pK3-chlorobenzoate = 3.82); σ''', $pK_{substrate} - pK_{benzoate}$; σ'''', $pK_{product} - pK_{benzoate}$.

possible reaction mechanisms for the reductive dechlorination of aromatic compounds.

MATERIALS AND METHODS

Organisms. The 3-chlorobenzoate-degrading methanogenic sludge enrichment, as described elsewhere (32, 33), was a gift of T. G. Linkfield in our laboratory. This enrichment had been stably maintained for over 4 years by monthly transfer into an inorganic salts medium with 3-chlorobenzoate as the sole energy source. The dechlorinating organism, *D. tiedjei* (2), had been isolated in our laboratory (31) from the above-mentioned sludge enrichment. The defined 3-chlorobenzoate-degrading methanogenic consortium consisted of *D. tiedjei*, a syntrophic benzoate-oxidizing hydrogen producer (strain BZ-2), a hydrogenotrophic methanogen (*Methanospirillum hungatei* strain PM-1), and low numbers of a *Desulfovibrio* sp. (strain PS-1). All the organisms present in the consortium had been isolated from the 3-chlorobenzoate-degrading enrichment (31). The construction and characteristics of the defined consortium have been described previously (4, 5).

Media and culture conditions. Preparation of the media and all manipulations were carried out under rigorous exclusion of O_2 by using strict anaerobic techniques (11, 20). Both the 3-chlorobenzoate-degrading enrichment and the 3-chlorobenzoate-degrading defined consortium were cultivated in a bicarbonate-buffered inorganic salts medium (pH = 7), which after autoclaving was supplemented with filter-sterilized 3-chlorobenzoate (final concentration, 3.2 mM) under an atmosphere of N_2-CO_2 (80:20). For cultivation of *D. tiedjei*, this medium was supplemented with clarified rumen fluid (clarified by centrifuging twice at 10,000 × *g* for 20 min) (20%) and sodium pyruvate (2 g/liter), while the 3-chlorobenzoate concentration was lowered to 800 μM. Cultures were grown in volumes of 50 ml in serum bottles with a volume of 158 ml at 35°C in the dark.

Test compounds. The substituted 3-chlorobenzoate analogs used in this study are listed in Table 1, together with the characteristics of the substituents that are pertinent to this study. The test compounds were individually dissolved in double-distilled water plus sodium hydroxide to final concentrations of 100 mM. These stock solutions were made anaerobic by flushing with O_2-free nitrogen.

Kinetic experiments. After 3-chlorobenzoate had been depleted from the growth medium, the cultures were individually supplemented with filter-sterilized test compounds to final concentrations of 0.8 to 1.0 mM and incubated on a shaker at 35°C in the dark; the disappearance of the 3-chlorobenzoate analogs and the appearance of products were monitored from samples (3 ml) taken at regular intervals. The samples were filtered through syringe-mounted 0.22-μm-pore-size Millipore filter units and frozen until analysis. All progress curves were run in triplicate.

The rates of dechlorination did not increase when the initial substrate concentrations were reduced from 1 to 0.5 mM, indicating that substrate inhibition due to toxicity did not occur; in some cases, however, a slight decrease in the rate of dechlorination was observed at 0.5 mM, probably because the substrate concentration was not always saturating.

Chemical analysis. Substituted 3-chlorobenzoate analogs and other benzenoids were quantified by high-pressure liquid chromatography as described previously (4), except for the aminobenzenoids, which were analyzed at 320 rather than at 284 nm. Hydrogen was measured by gas chromatography as described by Robinson and Tiedje (28) with a 3-ml sampling loop.

RESULTS

The effects of aryl substituents on the rates of reductive dechlorination of chlorinated benzoates were tested with the 3-chlorobenzoate-grown enrichment, the defined consortium, and *D. tiedjei* in pure culture. 3-Chlorobenzoate was readily dechlorinated in all three test systems. Its dechlorination product, benzoate, was not further metabolized in the pure culture system. In the mixed culture systems, however, benzoate was only detectable as an intermediate and at low concentrations unless hydrogen was added to the headspace. Under the latter conditions, benzoate accumulated in stoichiometric amounts. In the mixed culture systems, hydrogen was routinely detected at partial pressures of 1 to 10 Pa, indicating that this compound was present as a potential source of reducing equivalents needed for the reductive dechlorination. In the pure culture system, in which hydrogen was not detectable as a free intermediate (detection limit, 0.5 Pa), this function was probably taken by the products of the oxidation of pyruvate (the compound on which *D. tiedjei* had been cultivated), which were still present in the assay system. An indication of the availability of internal reducing equivalents from pyruvate in this strain is its reduction of CO_2 to form additional acetate (21).

The substituted 3-chlorobenzoate analogs listed in Table 1 were all dechlorinated in the three different assay systems. In all cases the chlorine in the *meta* position relative to the carboxyl group was stoichiometrically replaced by hydrogen. When the *para* position relative to the 3-chloro position was occupied by an amino, chloro, hydroxy, or methyl substituent, this was the only transformation that occurred. When the chlorine substituent was *meta* relative to the 3-chloroposition, however, 3-chlorobenzoate accumulated and was subsequently more rapidly dechlorinated once 3,5-dichlorobenzoate was depleted from the medium, in

TABLE 2. Influence of various substituents on the rate of dechlorination of substituted 3-chlorobenzoate analogs in various test systems

Test compound	Mean (SD) rate of dechlorination (μM/h) with the following assay system[a]			
	Enrichment	Cons $-H_2$	Cons $+H_2$	*D. tiedjei*
3-Chlorobenzoate	21.5 (2.1)	16.9 (1.6)	32.2 (4.7)	2.9 (0.1)
2,5-Dichlorobenzoate	9.1 (0.2)	7.6 (0.3)	34.1 (4.2)	2.3 (0.3)
3,5-Dichlorobenzoate	7.5 (0.6)	7.0 (1.4)	34.7 (4.0)	2.4 (0.4)
2-Methyl-5-chlorobenzoate	7.6 (0.6)	8.1 (0.3)	29.8 (4.7)	1.6 (0.6)
2-Hydroxy-5-chlorobenzoate	1.2 (0.1)	3.4 (1.2)	26.1 (1.9)	2.2 (0.6)
2-Amino-5-chlorobenzoate	0.9 (0.1)	0.2 (0.1)	ND	0.7 (0.4)

[a] Cons $-H_2$, consortium without H_2; Cons $+H_2$, consortium with H_2. $n = 3$ for all experiments. ND, Not determined.

agreement with a previous report (33). Further degradation of the dechlorinated product is, however, necessary for the generation of H_2, a source of reducing equivalents necessary for dechlorination. Thus, hydrogen was not produced in the presence of substituents *para* relative to the 3-chloroposition and was produced only at low rates during the dechlorination of 3,5-dichlorobenzoate. The availability of hydrogen is important for reductive dechlorination, since it provides the reducing power necessary for reductive dechlorination when the consortium grows on 3-chlorobenzoate (4). Therefore, a series of experiments was included in which the consortium plus hydrogen (partial pressure, 100 kPa) was used as the assay system. For a proper understanding of the results, it should be mentioned here that in the absence of hydrogen, acetate can serve as a source of reducing equivalents for reductive dechlorination in the consortium (unpublished data).

Table 2 shows the rates of dechlorination of the substituted 3-chlorobenzoate analogs for the four test systems. In the experiments with H_2 added to the consortium, the dechlorination rates for the chlorinated or methylated 3-chlorobenzoate analogs were roughly equal to the rate for 3-chlorobenzoate, while in the absence of H_2, the rates for these substituted 3-chlorobenzoate analogs were about half the rate for 3-chlorobenzoate. This indicates that the generation of reducing equivalents needed for reductive dechlorination was probably rate limiting in the absence of added H_2. For a proper evaluation of the effects of the various substituents it is thus necessary not only to normalize the rates of dechlorination to the rates of dechlorination of 3-chlorobenzoate but also to be critical towards the data generated in the absence of extra H_2.

The normalized values (Table 3) for the defined consortium were similar to the normalized values for the 3-chlorobenzoate-degrading enrichment. This indicates that the test conditions in the consortium were similar to those in the enrichment and supports the idea that the defined consortium is a good model system for the study of methanogenic 3-chlorobenzoate degradation under environmentally relevant conditions. The normalized values for the defined consortium in the presence of extra hydrogen, on the other hand, were different from those of the aforementioned two systems but agreed fairly well with the normalized values obtained with *D. tiedjei* in pure culture (Table 3). This result is in agreement with the idea that *D. tiedjei* is indeed the only dechlorinating organism present in the defined consortium and with the hypothesis that the presence of reduced fermentation products of pyruvate allows for a more rapid mobilization of reducing power needed in the dechlorination reaction than acetate allowed for.

The application of the Hammett approach with traditional sigma values taken from the literature does not result in a good correlation between chemical characteristics of the substituents and their effect on the rate of dechlorination (Fig. 1). The chemical literature gives various sets of σ constants for use in the Hammett equation to be used with specific types of intermediates, appropriate for charge and possible interactions with a substituent (9, 15). Plotting the

TABLE 3. Influence of various substituents on the rate of dechlorination of substituted 3-chlorobenzoate analogs in various test systems

Test compound	Log (k_x/k_h) for the following assay systems[a]:			
	Enrichment	Cons $-H_2$	Cons $+H_2$	*D. tiedjei*
3-Chlorobenzoate	0.00	0.00	0.00	0.00
2,5-Dichlorobenzoate	−0.37	−0.35	0.02	−0.10
3,5-Dichlorobenzoate	−0.46	−0.38	0.03	−0.08
2-Methyl-5-chlorobenzoate	−0.45	−0.32	−0.03	−0.26
2-Hydroxy-5-chlorobenzoate	−1.25	−0.70	−0.09	−0.12
2-Amino-5-chlorobenzoate	−1.38	−1.93	ND	−0.62

[a] Cons $-H_2$, consortium without H_2; Cons $+H_2$, consortium with H_2; ND, not determined.

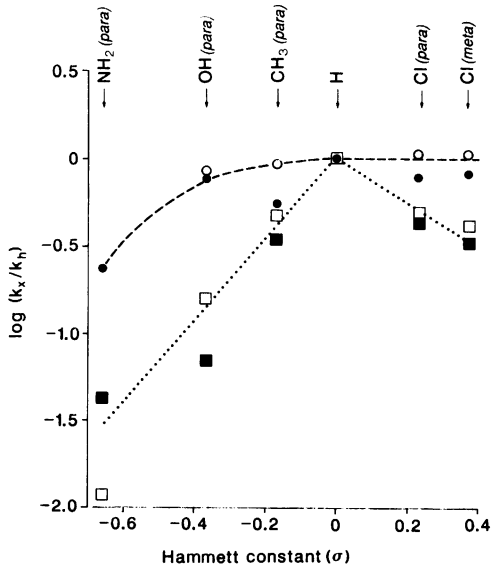

FIG. 1. Hammett plot of the normalized rate of reductive dechlorination of substituted 3-chlorobenzoate analogs. Symbols: ■, enrichment culture; □, consortium; ○, consortium plus H_2; ●, *D. tiedjei*.

relative reaction rates obtained with the consortium in the presence of hydrogen against these sets of values did not yield a meaningful correlation either (data not shown). In the original demonstration of his approach, Hammett defined his sigma values as the difference between the pK values of substrates and products (8). The σ values that are currently listed in the literature deviate from these values. Thus, we also calculated our own set of σ values for the substrates and products of interest in the present study, by comparing the pK values of substituted 3-chlorobenzoate analogs with their dechlorinated products (σ′ in Table 1). A possible interfering effect may have been introduced by this approach because the position *para* to the 3-chloro position is *ortho* to the carboxyl group and thus may result in interactions between the *para* substituent and the carboxyl group. Another approach would be to define a σ value by comparing the pK value of the substituted 3-chlorobenzoate analog with the pK value for 3-chlorobenzoate (Table 1, σ″) or benzoate (Table 1, σ‴) or by comparing the pK value of the dechlorinated analog with the value for benzoate (Table 1, σ⁗). None of these approaches, however, yielded a good correlation between the numeric value of the substituent and the relative rate of dechlorination.

DISCUSSION

This study demonstrates that the presence of substituents on the aromatic ring of 3-chlorobenzoate influences the rate of dechlorination and that this influence depends on the chemical properties of the substituent and may vary with the assay system used. This is not surprising, since substituents can be expected to have an influence on both the chemistry and the biology of the dechlorination reaction. Ideally, the Hammett approach should have been able to give some insight into the chemistry of the dechlorination. The problem in the present study, however, was that the substituents probably also influenced the biology of the system. Not only was the study performed with whole cells and did the substrates have to be taken up by the cells, but there probably was also an effect of the presence of the substituents on the fit of the substrate in the enzyme active site. The steric and electronic effects of the presence of the substituent may alter the fit of the substrate on the enzyme and/or the conformation of the enzyme and thus may alter its ability to catalyze the dechlorination. Another possible effect of the presence of a substituent is that it may affect the high-energy-state complex that is thought to be formed during the reductive dechlorination reaction. If the presence of a substituent affects the energy of the transition state differently from the energy of the ground state of the substrates, the energy of activation and consequently the rate of the reaction will be affected.

In the absence of hydrogen, the reducing equivalents in the defined consortium are derived from acetate (unpublished data). The observations that the addition of hydrogen stimulated the rate of the reductive dechlorination in the consortium and that the extent of this stimulus depended on the substituent indicate that the generation of reducing power is affected by the presence of aryl substituents.

Dechlorination of 2-amino- and 2-hydroxy-5-chlorobenzoate (Table 2) had not been observed before: screening for dechlorination of a wide range of compounds in which digestor sludge or sediment served as the inoculum had previously yielded negative results for both these compounds (10). A major difference between the systems used in the present investigation and the sludge and sediment used previously is that our systems had been enriched for 3-chlorobenzoate dechlorinating activity before they were tested for their potential to dechlorinate hydroxy- and aminochlorobenzoate. Thus, analog enrichment may be useful when designing strategies to obtain dechlorinating activity for compounds that score negative in dechlorination assays with sludges and sediments per se.

In summary, we conclude that the presence of substituents on the aromatic ring of 3-chlorobenzoate affects the rate of dechlorination at the 3 position and that the inhibiting effects of both the hydroxy and the amino groups suggest that a nucleophilic attack on the aromatic ring may be one of the reaction steps in an intricate series of biologically catalyzed steps which are too complicated to be resolved with this straightforward approach. The recent finding that reductive dechlorination of chlorinated benzoates (and phenols) can be obtained chemically under mild conditions by using a Raney-type catalyst (34) makes it tempting to speculate that more meaningful structure-activity relationships for the reductive dechlorination of halogenated aromatic compounds are now within reach and will shed more light on the reaction mechanism.

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REFERENCES

1. **Carey, F. A., and R. J. Sundberg.** 1977. Advanced organic chemistry. Plenum Press, New York.
2. **DeWeerd, K. A., L. Mandelco, R. S. Tanner, C. R. Woese, and J. M. Suflita.** 1990. *Desulfomonile tiedjei* gen. nov. and sp. nov., a novel anaerobic, dehalogenating, sulfate-reducing bacterium. Arch. Microbiol. **154:**23–30.
3. **DeWeerd, K. A., J. M. Suflita, T. G. Linkfield, J. M. Tiedje, and P. H. Pritchard.** 1986. The relationship between reductive dehalogenation and other aryl substituent removal reactions catalyzed by anaerobes. FEMS Microbiol. Ecol. **38:**331–339.
4. **Dolfing, J., and J. M. Tiedje.** 1986. Hydrogen cycling in a three-tiered food web growing on the methanogenic conversion of 3-chlorobenzoate. FEMS Microbiol. Ecol. **38:**293–298.
5. **Dolfing, J., and J. M. Tiedje.** 1987. Growth yield increase linked to reductive dechlorination in a defined 3-chlorobenzoate degrading methanogenic coculture. Arch. Microbiol. **149:**102–105.
6. **Dorn, E., and H.-J. Knackmuss.** 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of catechol. Biochem. J. **174:** 85–94.
7. **Gschwend, P. M., J. K. MacFarlane, and K. A. Newman.** 1985. Volatile halogenated organic compounds released to seawater from temperate marine macroalgae. Science **227:**1033–1035.
8. **Hammett, L. P.** 1935. Some relations between reaction rates and equilibrium constants. Chem. Rev. **17:**125–136.
9. **Hansch, C., and A. Leo.** 1979. Substituent constants for correlation analysis in chemistry and biology. John Wiley & Sons, Inc., New York.
10. **Horowitz, A., D. R. Shelton, C. P. Cornell, and J. M. Tiedje.** 1982. Anaerobic degradation of aromatic compounds in sediments and digested sludge. Dev. Ind. Microbiol. **23:**435–444.
11. **Hungate, R. E.** 1968. A roll tube method for the cultivation of strict anaerobes, p. 117–132. *In* J. R. Norris and D. W. Ribbons (ed.), Advances in microbiology, vol. 3B. Academic Press, Inc., New York.
12. **Janssen, D. B., G. Grobben, R. Hoekstra, R. Oldenhuis, and B. Witholt.** 1988. Degradation of *trans*-1,2-dichloroethene by mixed and pure cultures of methanotrophic bacteria. Appl. Microbiol. Biotechnol. **29:**392–399.
13. **Janssen, D. B., R. Oldenhuis, and A. J. van den Wijngaard.** 1990. Hydrolytic and oxidative degradation of chlorinated aliphatic compounds by aerobic microorganisms, p. 105–125. *In* D.

Kamely, A. Chakrabarty, and G. S. Omenn (ed.), Biotechnology and biodegradation. Gulf Publishing Company, Houston.
14. **Janssen, D. B., A. Scheper, L. Dijkhuizen, and B. Witholt.** 1985. Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. Appl. Environ. Microbiol. **163:**635–639.
15. **Johnson, C. D.** 1973. The Hammett equation. Cambridge University Press, Cambridge.
16. **King, G. M.** 1986. Inhibition of microbial activity in marine sediments by a bromophenol from a hemichordate. Nature (London) **323:**257–259.
17. **Knackmuss, H.-J.** 1981. Degradation of halogenated and sulfonated hydrocarbons, p. 189–212. *In* T. Leisinger, A. M. Cook, R. Hütter, and J. Nusch (ed.), Microbial degradation of xenobiotics and recalcitrant compounds. Academic Press, Inc. (London), Ltd., London.
18. **Linkfield, T. G., and J. M. Tiedje.** 1990. Characterization of the requirements and substrates for reductive dehalogenation by strain DCB-1. J. Ind. Microbiol. **5:**9–16.
19. **Marks, T. S., R. Wait, A. R. W. Smith, and A. V. Quirk.** 1984. The origin of the oxygen incorporated during the dehalogenation/hydroxylation of 4-chlorobenzoate by an *Arthrobacter* sp. Biochem. Biophys. Res. Commun. **124:**669–674.
20. **Miller, T. L., and M. J. Wolin.** 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl. Microbiol. **27:**985–987.
21. **Mohn, W. W., and J. M. Tiedje.** 1990. Catabolic thiosulfate disproportionation and carbon dioxide reduction in strain DCB-1, a reductively dechlorinating anaerobe. J. Bacteriol. **172:**2065–2070.
22. **Müller, R., J. Thiele, U. Klages, and F. Lingens.** 1984. Incorporation of [^{18}O]water into 4-hydroxybenzoic acid in the reaction of 4-chlorobenzoate dehalogenase from *Pseudomonas* spec. CBS3. Biochem. Biophys. Res. Commun. **124:**178–182.
23. **Oldenhuis, R., R. L. J. M. Vink, D. B. Janssen, and B. Witholt.** 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble monooxygenase. Appl. Environ. Microbiol. **55:**2819–2826.
24. **Paris, D. F., N. L. Wolfe, and W. C. Steen.** 1982. Structure-activity relationships in microbial transformation of phenols. Appl. Environ. Microbiol. **44:**153–158.
25. **Reineke, W.** 1984. Microbial degradation of halogenated aromatic compounds, p. 319–360. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.
26. **Reineke, W., and H.-J. Knackmuss.** 1978. Chemical structure and biodegradability of halogenated aromatic comounds. Substituent effects on dehydrogenation of 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid. Biochim. Biophys. Acta **542:**424–429. 429.
27. **Reineke, W., and H.-J. Knackmuss.** 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of benzoic acid. Biochim. Biophys. Acta **542:**412–423.
28. **Robinson, J. A., and J. M. Tiedje.** 1982. Kinetics of hydrogen consumption by rumen fluid, anaerobic digestor sludge, and sediment. Appl. Environ. Microbiol. **44:**1374–1384.
29. **Rochkind-Dubinski, M. L., G. S. Sayler, and J. W. Blackburn.** 1987. Microbial decomposition of chlorinated aromatic compounds. Marcel Dekker, Inc., New York.
30. **Serjeant, E. P., and P. Dempsey.** 1979. Ionisation constants of organic acids in aqueous solution. IUPAC chemical data series, no 23. Pergamon Press, Oxford.
31. **Shelton, D. R., and J. M. Tiedje.** 1984. Isolation and characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. Appl. Environ. Microbiol. **48:**840–848.
32. **Suflita, J. M., A. Horowitz, D. R. Shelton, and J. M. Tiedje.** 1982. Dehalogenation: a novel pathway for the anaerobic biodegradation of haloaromatic compounds. Science **218:**1115–1117.
33. **Suflita, J. M., J. A. Robinson, and J. M. Tiedje.** 1983. Kinetics of microbial dehalogenation of haloaromatic substrates in methanogenic environments. Appl. Environ. Microbiol. **45:**1466–1473.
34. **Tashiro, M., H. Tsuzuki, J. Matsumoto, S. Mataka, K. Nakayama, Y. Tsuruta, and T. Yonemitsu.** 1989. Reductive dechlorination of chloro-phenols and -benzoic acids by Raney Co-Al alloy in an alkaline deuterium oxide solution and preparation of deuterated salicylic acids. J. Chem. Res. **M:**2826–2851.
35. **Tiedje, J. M., S. A. Boyd, and B. Z. Fathepure.** 1987. Anaerobic degradation of chlorinated aromatic hydrocarbons. Dev. Ind. Microbiol. **27:**117–127.
36. **Vogel, T. M., C. S. Criddle, and P. L. McCarty.** 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. **21:**722–736.