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Isolation and Partial Characterization of Bacteria in an Anaerobic Consortium That Mineralizes 3-Chlorobenzoic Acid†

DANIEL R. SHELTON[1]‡ AND JAMES M. TIEDJE[1,2]*

Departments of Crop and Soils Sciences[1] and Microbiology and Public Health,[2] Michigan State University, East Lansing, Michigan 48824

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A methanogenic consortium able to use 3-chlorobenzoic acid as its sole energy and carbon source was enriched from anaerobic sewage sludge. Seven bacteria were isolated from the consortium in mono- or coculture. They included: one dechlorinating bacterium (strain DCB-1), one benzoate-oxidizing bacterium (strain BZ-2), two butyrate-oxidizing bacteria (strains SF-1 and NSF-2), two H_2-consuming methanogens (*Methanospirillum hungatei* PM-1 and *Methanobacterium* sp. strain PM-2), and a sulfate-reducing bacterium (*Desulfovibrio* sp. strain PS-1). The dechlorinating bacterium (DCB-1) was a gram-negative, obligate anaerobe with a unique "collar" surrounding the cell. A medium containing rumen fluid supported minimal growth; pyruvate was the only substrate found to increase growth. The bacterium had a generation time of 4 to 5 days. 3-Chlorobenzoate was dechlorinated stoichiometrically to benzoate, which accumulated in the medium; the rate of dechlorination was ca. 0.1 pmol bacterium^{-1} day^{-1}. The benzoate-oxidizing bacterium (BZ-2) was a gram-negative, obligate anaerobe and could only be grown as a syntroph. Benzoate was the only substrate observed to support growth, and, when grown in coculture with *M. hungatei*, it was fermented to acetate and CH_4. One butyrate-oxidizing bacterium (NSF-2) was a gram-negative, non-sporeforming, obligate anaerobe; the other (SF-1) was a gram-positive, sporeforming, obligate anaerobe. Both could only be grown as syntrophs. The substrates observed to support growth of both bacteria were butyrate, 2-DL-methylbutyrate, valerate, and caproate; isobutyrate supported growth of only the sporeforming bacterium (SF-1). Fermentation products were acetate and CH_4 (from butyrate, isobutyrate, or caproate) or acetate, propionate, and CH_4 (from 2-DL-methylbutyrate or valerate) when grown in coculture with *M. hungatei*. A mutualism among at least the dechlorinating, benzoate-oxidizing, and methane-forming members was apparently required for utilization of the 3-chlorobenzoate substrate.

Many chlorinated organic compounds are not biodegraded under aerobic conditions, often because the chlorine substitutions prevent ring cleavage and thus subsequent dechlorination. Recently, however, certain chlorinated aromatic compounds have been shown to be dechlorinated in anaerobic habitats such as sediment, flooded soil, and digested sludge. These chemicals include chlorinated benzoates (5, 15, 16), chlorinated phenols (2, 3, 6, 12), and the pesticides diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] (1), Techlofthalam [*N*-(2,3-dichlorophenyl)-3,4,5,6-tetrachlorophthalmic acid] (7), chloronitrofen (4-nitrophenyl-2,4,6-trichlorophenyl ether) (19), and 2,4,5-trichlorophenoxyacetic acid (17). In all of these cases, chlorine is removed from the aromatic ring before ring cleavage, which is in contrast to aerobic metabolism of chloroaromatic compounds. Thus, this reaction—a reductive dechlorination—is of particular interest because it has the potential for making some of the highly chlorinated, serious pollutants less persistent and less toxic.

To further study this reaction and the responsible organisms, we enriched a bacterial community from anaerobic sludge that grows on 3-chlorobenzoate and produces CH_4 and CO_2 (15, 16). Microscopic examination of this consortium revealed a stable community with a large number of different morphological types. Our objectives were to isolate and characterize the bacterium or bacteria responsible for the dechlorination as well as other bacteria involved in this food chain. Since the free-energy change of this reaction is positive under standard conditions, it was assumed that some members of this consortium would be syntrophic with H_2 consumers.

MATERIALS AND METHODS

Enrichment. A stable methanogenic consortium growing on 3-chlorobenzoate as the sole carbon and energy source was selected from municipal digestor sludge from Adrian, Mich., over a 2-year period. The enrichment was maintained by making a 10% inoculum transfer into fresh mineral salts medium (14) approximately every 2 months. 3-Chlorobenzoate (Na^+ salt) at 2 to 3 mM was added to the enrichment from a 10% stock solution initially and whenever the substrate was depleted.

Isolation. Methanogens and a sulfidogen used in coculture for the isolation of syntrophs were also isolated from the enrichment. Samples of the 3-chlorobenzoate enrichment were incubated with either 80% H_2–20% CO_2 or 80% H_2–20% CO_2 plus 20 mM SO_4^{2-}. Serial dilutions of the methanogen or sulfate reducer enriched samples were inoculated into anaerobic culture tubes (Bellco Glass, Inc., Vineland, N.J.) containing 8 ml of the basal medium, 5% clarified rumen fluid, 0.5% sodium acetate for cell carbon, and 2% agar rolled onto the tube walls. The gas phase was either 80% H_2–20% CO_2 (2 atm [202 kPa]) or 80% H_2–20% CO_2 (2 atm [202 kPa]) plus 20 mM SO_4^{2-} for the methanogens and sulfidogen, respectively.

The techniques used for isolating the benzoate and bu-

* Corresponding author.
† Journal article no. 11121 of the Michigan Agricultural Experiment Station.
‡ Present address: Department of Soil and Environmental Sciences, University of California, Riverside, CA 92521.

tyrate oxidizers were essentially those described by McInerney et al. for their isolation of volatile fatty acid (VFA)-degrading bacteria (9). Serial dilutions of the 3-chlorobenzoate enrichment (10^{-5} and 10^{-6}), or subsamples of the enrichment that had been fed once on 0.1% butyrate, were inoculated into anaerobic culture tubes containing 8 ml of the basal medium, 5% clarified rumen field, 0.1% of either benzoate or butyrate, and 2% molten agar which was then rolled onto the tube wall. A methanogen and a sulfidogen were added (1 ml of a turbid culture) in separate tubes to form a lawn of H_2 consumers.

The basal medium consisted of (per liter): 0.27 g of KH_2PO_4 and 0.35 g of K_2HPO_4 (adjusted to pH 7.0), 0.53 g of NH_4Cl, 15 mg of $CaCl_2 \cdot 2H_2O$, 20 mg of $MgCl_2 \cdot 6H_2O$, and 4 mg of $FeCl_2 \cdot 4H_2O$; trace metals modified from Zehnder and Wuhrman (21), 0.5 mg of $MnCl_2 \cdot 4H_2O$, 0.05 mg of H_3BO_3, 0.05 mg of $ZnCl_2$, 0.05 mg of $CaCl_2 \cdot 6H_2O$, 0.05 mg of $NiCl_2 \cdot 6H_2O$, 0.03 mg of $CuCl_2$, 0.01 mg of $NaMoO_4 \cdot 2H_2O$; the vitamin solution of Wolin et al. (18); 2.4 g of $NaHCO_3$; and 1 mg of resazurin. The headspace gas was 80% N_2–20% CO_2. Tubes were reduced before use with 0.5 mM cysteine-hydrochloride and 0.5 mM $Na_2S \cdot 9H_2O$.

Colonies were picked from the roll tubes by the use of a sterile Pasteur pipette with a bent tip and transferred to 10 ml of liquid medium (basal medium, 5% rumen fluid and 0.1% either benzoate or butyrate). A methanogen or sulfidogen was also added to the liquid medium (3 ml of a turbid culture) to ensure a low partial pressure of H_2. Strict anaerobic techniques were employed throughout. Incubations were at 37°C and in the dark.

Characterization. Experiments to assess substrate range were performed by transferring 10% of a turbid culture to anaerobic culture tubes containing the basal medium, 5% clarified rumen fluid, and 0.2% of the substrate of interest. Growth was assessed visually by comparison with the uninoculated medium. All substrates were tested in triplicate. Nitrate reduction was tested by adding 5 mM NO_3^- to the basal medium and assaying for both nitrate and nitrite, using a Technicon Autoanalyzer (Technicon Instruments Corp., Inc.) at the beginning and end of incubation. Sulfate was added at 20 mM where indicated.

The fermentation balance for the benzoate-oxidizing bacterium was performed with benzoate; fermentation balances for the butyrate-oxidizing bacteria were performed with butyrate, isobutyrate, 2-DL-methylbutyrate, valerate, and caproate. Five milliliters of turbid culture was transferred to 50 ml of basal medium plus 5% clarified rumen fluid with 0.15% substrate in 160-ml serum bottles. Subsamples of medium (3 ml) were collected at the beginning and end of incubation and frozen until analysis. Bottles were incubated statically in the dark for 3 or 4 weeks at 37°C.

Generation time was determined from optical density measured in anaerobic culture tubes (18 mm diameter), using a Turner spectrophotometer set at 660 nm. Cell numbers were determined with a Petroff-Hauser chamber and a high-dry phase microscope objective.

Microscopy. Common methods for electron microscopy of bacterial cells were used. For thin-section electron micrographs, cells were fixed overnight (4°C) in 2% glutaraldehyde and 0.1 M phosphate buffer (pH 7.2). Cells were centrifuged and washed in 0.1 M phosphate buffer. Cells were then embedded in 1% Noble agar and postfixed for 2 h (ambient temperature) in 1% osmium tetroxide and Ryter-Kellenberger fixative (pH 6.1). Samples were dehydrated through an ethanol series followed by propylene oxide, then were embedded in DER (332/732) epoxy resin. Thin sections were poststained with uranyl acetate and lead citrate. Whole-cell electron micrographs were obtained by floating Parlodion-coated copper grids (330 mesh) on a drop of bacterial suspension for 1 min. Grids were blotted and then were negatively stained for 30 s with 2% uranyl acetate. Electron micrographs were taken at 80 kV on a Philips 300 electron microscope.

Phase photomicrographs were obtained by adding one drop of 1% agarose to one drop of bacterial suspension on a clean slide. A cover slip was pressed firmly onto the slide surface, and the agar-bacteria solution was allowed to solidify for ca. 10 min. Photomicrographs were taken on a Zeiss photomicroscope.

Chemical analysis. 3-Chlorobenzoate and benzoate were quantified by using a Varian-3700 high-pressure liquid chromatograph coupled to a Hitachi variable wavelength detector set at 284 nm (15). The analytical column was a C-18 reverse-phase column (250 by 4.6 cm). The mobil phase consisted of methanol-water-acetic acid (60:40:5). The flow rate was 1.5 ml/min.

Acetate, propionate, butyrate, isobutyrate, 2-DL-methylbutyrate, valerate, and caproate were quantified by using a PE-900 gas chromatograph equipped with a flame ionization detector and a 2-m glass column (2 mm inside diameter) packed with Carbopack C–0.3% Carbowax 20 M–0.1% H_3PO_4. The oven temperature was 80°C for 2 min and then increased 16°C per min up to 120°C. The N_2 carrier gas flow was 30 to 40 ml/min. Samples were acidified with H_3PO_4 (15% by volume) before injection.

Methane was quantified by injecting 0.2 ml of headspace gas into a Carle 8500 gas chromatograph equipped with microthermister detector and a 2-m column packed with Porapak QS.

RESULTS

Isolation of methanogens and sulfate reducer. After several days, colonies appeared in culture tubes pressurized with 80% H_2–20% CO_2, containing bacteria consistent with the morphologies of known methanogens and sulfate reducers and consistent with organisms observed in the enrichment. Isolates were subcultured two additional times to ensure culture purity. After subculturing, only the individual dis-

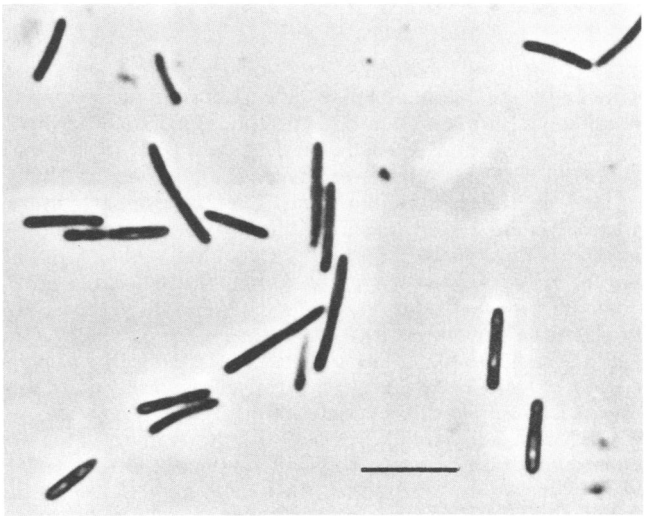

FIG. 1. Phase photomicrograph of the dechlorinating bacterium, DCB-1. Bar, 5.0 μm.

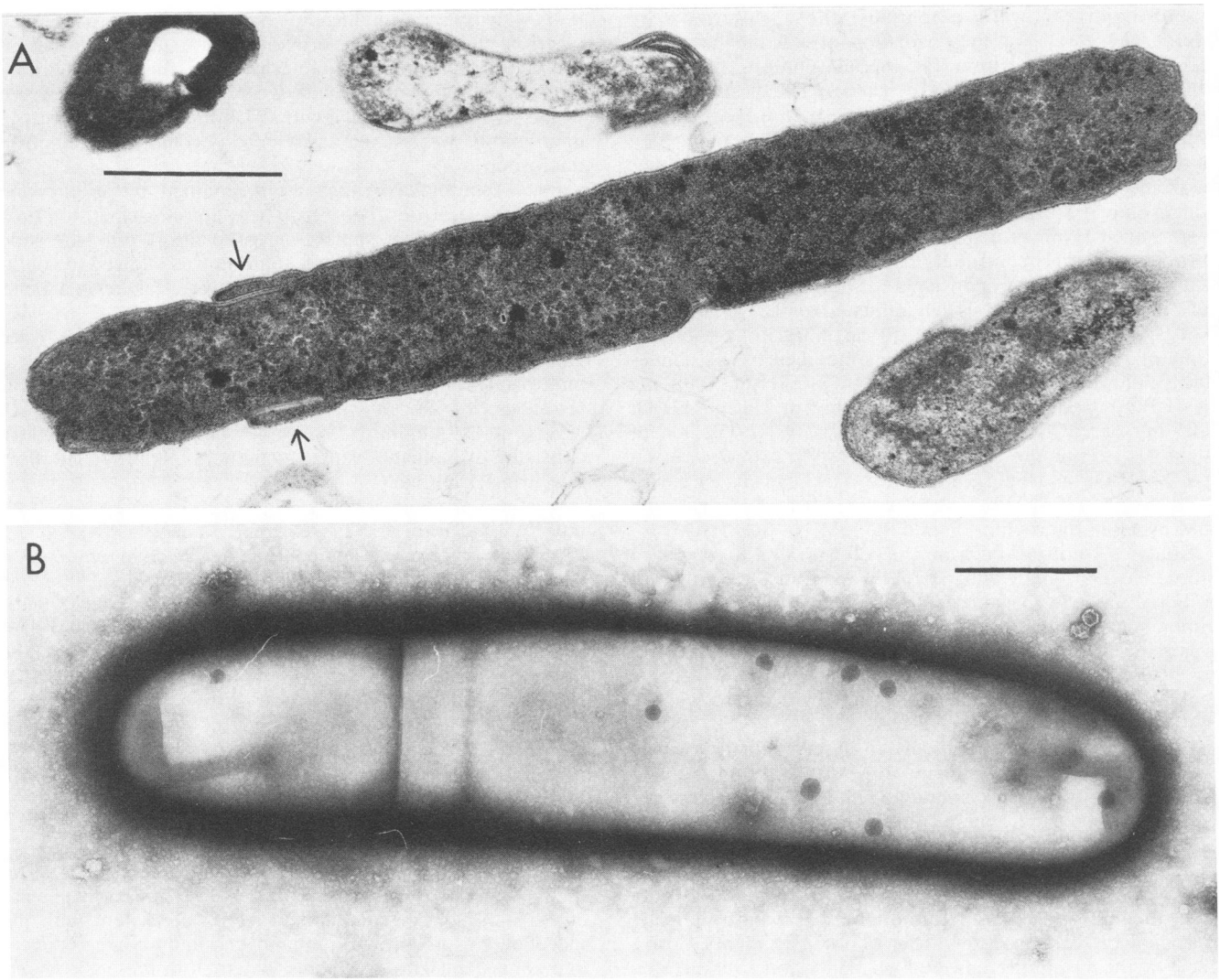

FIG. 2. (A) Electron micrograph of the dechlorinating bacterium DCB-1, showing collar (arrows) in relation to the rest of the cell. Bar, 0.5 μm. (B) Electron micrograph of the dechlorinating bacterium DCB-1 positively stained with uranyl acetate, showing the outer appearance of the collar. Bar, 0.5 μm.

tinctive cell morphologies were observed and no turbid growth of the methanogen isolate occurred in a complex medium (0.1% each of glucose, Trypticase [BBL Microbiology Systems], and yeast extract). A low level of turbidity occurred with the sulfate reducer in the complex medium.

Two methane-producing and UV-fluorescing cultures were isolated. Based on morphology, these were identified as *Methanospirillum hungatei* (strain PM-1) and *Methanobacterium* sp. (strain PM-2). *M. hungatei* was used as an H_2 consumer for the isolation of benzoate- and butyrate-oxidizing bacteria because of its low K_m for H_2 (2.0 μM) (13). One sulfidogen was isolated and identified as a *Desulfovibrio* sp. (strain PS-1). This strain was also used as an H_2 consumer for the isolation of the benzoate-oxidizing bacterium because of its low K_m for H_2 (0.7 μM) (13). Although an extensive characterization was not performed, the sulfidogen (PS-1) was observed to metabolize pyruvate and lactate in the presence of *M. hungatei* (no SO_4^{2-}).

Isolation of dechlorinating bacterium. Our initial isolation strategy was based on the assumption that the dechlorinating bacterium or bacteria must use benzoate or a possible intermediate of benzoate metabolism, such as butyrate, as a source of carbon and energy for growth. 3-Chlorobenzoate was not tried as a growth substrate since preliminary experiments had indicated that concentrations of 0.1% (6.4 mM) were inhibitory to the enrichment. After 8 to 10 weeks, small white colonies of up to 0.5 mm diameter appeared in culture tubes containing either benzoate or butyrate. Most of the colonies consisted of large rods typical of one of the dominant morphological types observed in the enrichment. Colonies were transferred to liquid culture (basal medium, 5% rumen fluid and 0.1% benzoate or butyrate), and by week 8 a very slight turbidity was observed. Only the large rod was seen by phase-contrast microscopy. Analysis of benzoate or butyrate in liquid culture indicated that these substrates were not being consumed. The bacterium did not appear to be a syntroph since viable cells of the H_2-consuming bacteria were not observed in the colonies or in liquid culture. Therefore, we presumed that the bacterium was able to obtain sufficient carbon and other nutrients from the 5%

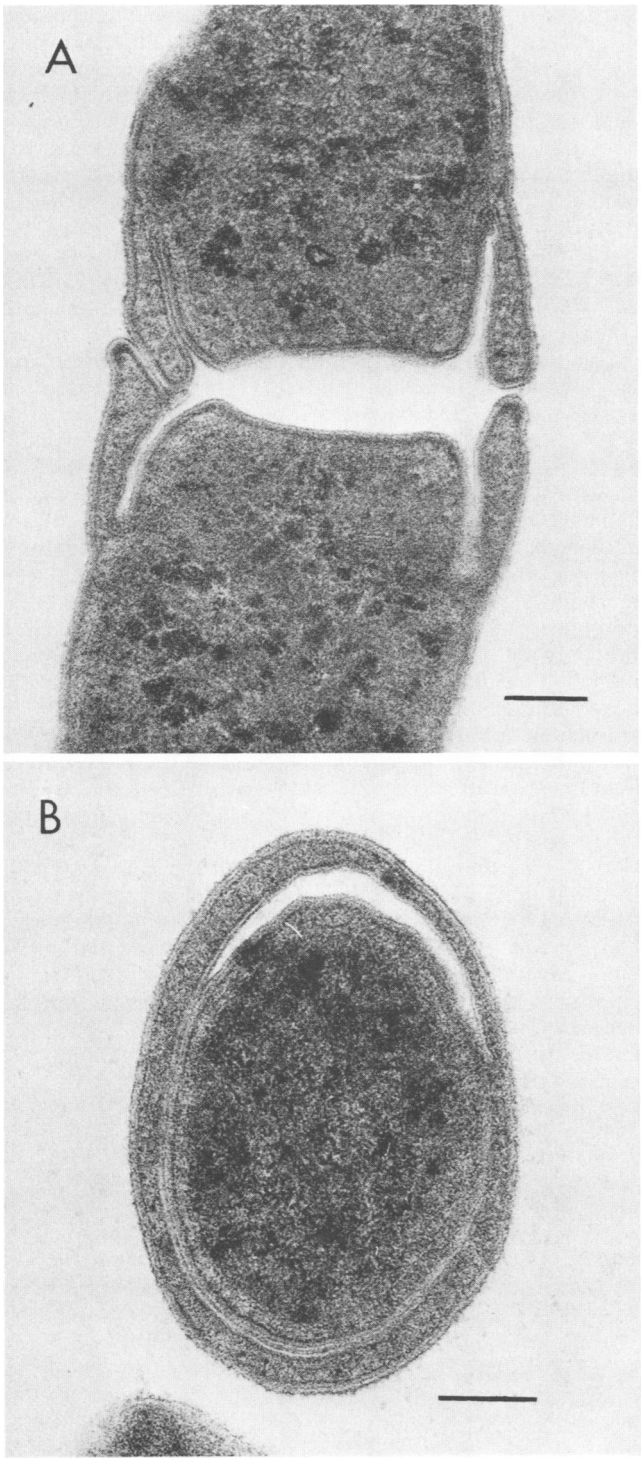

FIG. 3. (A) Electron micrograph of the dechlorinating bacterium DCB-1, showing the collar at high magnification. Bar, 0.1 μm. (B) Electron micrograph of the dechlorinating bacterium DCB-1, showing collar in cross-section. Note the concentric rings. Bar, 0.1 μm.

rumen fluid to support growth in the roll tubes and in liquid culture. When 3-chlorobenzoate was added to liquid cultures, dechlorination was observed.

To ensure culture purity, the bacterium was subcultured two additional times; benzoate, butyrate, and the H_2 consumers were omitted from the medium and 0.05% yeast extract was added as a nutritional supplement. After subculturing, only the one distinctive cell morphology was observed, and no turbid growth occurred in a complex medium (0.1% each of glucose, Trypticase, and yeast extract). Growth was still meager on 5% rumen fluid plus yeast extract medium, but was improved by the use of 20% rumen fluid. However, cell yields were never high enough to quantify in a spectrophotometer. Initially, the culture was maintained on the basal medium plus 20% clarified rumen fluid. The culture obtained after the third subculture was designated strain DCB-1 (for dechlorinating bacterium) and was used for all subsequent experiments.

The bacterium was a straight rod, 3 to 6.5 μm in length and 0.5 to 0.8 μm in width (Fig. 1). The cells stained gram negative and appeared to have a gram-negative cell wall as observed in the thin sections (Fig. 2 and 3). A unique feature was the "collar" that surrounded the cell (Fig. 2 and 3). The transverse thin sections show that cytoplasm extended into this collar (Fig. 2 and 3). Cross sections show the concentric ring expected for sections cut through the collar (Fig. 3B). Almost all of the cells in the culture possessed the collar. Cells were nonmotile.

Twenty-eight chemicals were examined as substrates, but only pyruvate plus clarified rumen fluid supported increased growth. Other chemicals tested included: alanine, arginine, aspartate, benzoate (± *M. hungatei*), butyrate (± *M. hungatei*), Casamino Acids (plus SO_4^{2-}), formate, fructose, fumarate, galactose, glucose (plus SO_4^{2-}), glutamate, glycerol (plus SO_4^{2-}), glycine, histidine, lactate (plus SO_4^{2-}), lysine, proline, Difco Proteose Peptone (plus SO_4^{2-}), ribose (plus SO_4^{2-}), serine, threonine, Trypticase (plus SO_4^{2-}), tryptic soy broth (Difco), valine, xylose, and yeast extract (plus SO_4^{2-}). Growth with these substrates was not noticeably greater than with 20% clarified rumen fluid alone. The rumen fluid could be replaced with a complex protein source (0.1% of Trypticase, Casamino Acids, Proteose Peptone, or tryptic soy broth) or 0.1% yeast extract; pyruvate alone did not support growth of the bacterium. The growth rate with pyruvate was not enhanced by the presence of sulfate. Combinations of amino acids known to support growth of *Clostridium* species that carry out a Stickland reaction did not support growth. Growth did not occur in the presence of oxygen. Nitrate was reduced stoichiometrically to nitrite. The bacterium was routinely grown at 37°C and grew poorly or not at all at 25 or 45°C.

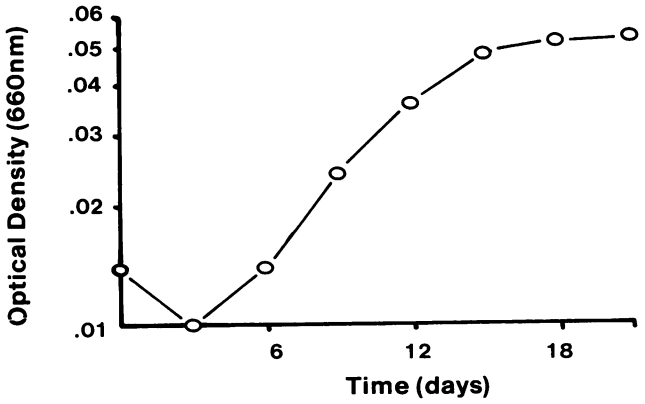

FIG. 4. Growth of the dechlorinating bacterium on 0.2% pyruvate plus 20% rumen fluid. Coefficients of variation ranged from 4 to 8% for individual points.

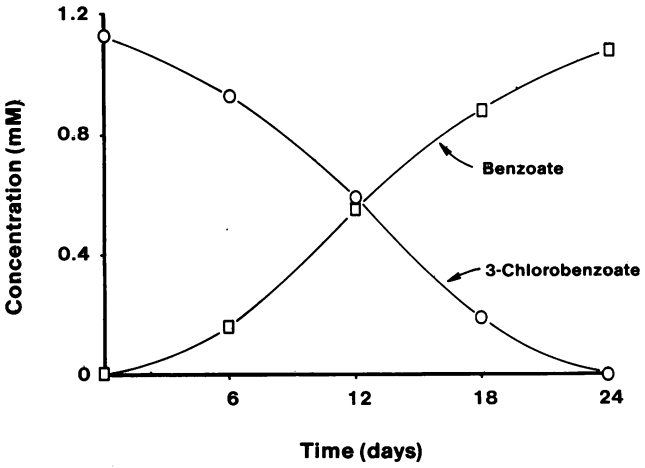

FIG. 5. Dechlorination of 3-chlorobenzoate by the dechlorinating bacterium after inoculation into fresh medium.

The growth curve of DCB-1 on pyruvate plus 20% clarified rumen fluid in the basal medium is shown in Fig. 4. We estimate the generation time was 4 to 5 days. The low maximum yield of 0.05 to 0.06 optical density may indicate a nutrient limitation (Fig. 4), although increased concentrations of pyruvate and Trypticase in the medium did not result in increased cell yields.

When 3-chlorobenzoate was added to the pyruvate medium, it was dechlorinated stoichiometrically to benzoate (Fig. 5). The rate of dechlorination was ca. 0.1 pmol bacterium^{-1} day $^{-1}$. 3-Chlorobenzoate (1 mM) was inhibitory to growth. If cultures were grown in the absence of 3-chlorobenzoate for several doublings, dechlorination occurred only after a 3- to 6-day lag, which suggests the activity was inducible.

Isolation of benzoate-oxidizing bacterium. The enrichment metabolized benzoate without a lag and at a faster rate than it metabolized 3-chlorobenzoate. After 8 to 9 weeks, pinpoint colonies of ca. 0.1 mm diameter appeared in culture tubes containing benzoate in coculture with both the methanogen and the sulfidogen; colonies did not appear in tubes to which an H_2 consumer had not been added. When colonies were transferred to liquid culture, however, only those incubated with *Desulfovibrio* sp. strain PS-1 grew. Turbidity was evident visually after 8 to 9 weeks of incubation. The bacterium was subcultured once to assure purity. After subculturing, purity was assessed by inoculation into complex medium (0.1% each glucose, Trypticase, and yeast extract); a low level of turbidity was observed visually. However, microscopic examination revealed the presence of only *Desulfovibrio* sp. strain PS-1. We have had no difficulty in shifting the benzoate-oxidizing bacterium from coculture with *Desulfovibrio* sp. to *M. hungatei* as the H_2 consumer. However, even after several transfers of 10% inoculum, low numbers of *Desulfovibrio* sp. cells persisted in sulfate-free coculture with *M. hungatei*.

Both straight and curved rod-shaped cells were seen. The cells were 1 to 2 μm in length and 0.2 to 0.4 μm in width (Fig. 6). The cells stained gram negative and appeared to have a gram-negative envelope, as observed in the thin sections (Fig. 7). The wavy outer membrane of both cell types (Fig. 7) appeared to be a distinctive feature of these organisms. We occasionally observed what appeared to be a twitching type of motility; however, it was difficult to distinguish from Brownian motion. Whether the curved and straight bacteria are distinct strains or simply represent variable morphology is unclear. We were able to isolate two subcultures in which the curved or the straight rod morphologies were predominant (but not purely one type); however, we were unable to demonstrate any physiological differences between these two cultures. Thus, we cannot conclude that there are two different strains. The culture subsequently used contained both cell types and was designated strain BZ-2 (for benzoate degrading).

The only substrate that supported growth of the organism was benzoate. Chemicals that did not support growth included: adipate, butyrate, Casamino Acids, *trans*-cinnamate, ethanol, fructose, fumarate, galactose, glucose, glycerol, *o*-, *m*-, and *p*-hydroxybenzoate, phenylacetate, phenylpropionate, phthalate, pimelate, ribose, Trypticase, xylose, and yeast extract. Benzoate (0.27 mmol) was fermented to acetate (0.82 mmol), CH_4 (0.20 mmol), and presumably either formate or CO_2 when grown in coculture with *M. hungatei*. Based on 0.75 mol of CH_4 produced per mol of benzoate metabolized, recoveries of C and H were 101 and 99%, respectively. In time course experiments, neither butyrate nor other VFAs were observed as intermediates during benzoate metabolism. Nitrate was not reduced. Growth did not occur in the presence of oxygen. The organism is probably not a sulfate reducer since a coculture rather than a monoculture fluorished when sulfate was added to the benzoate medium.

Isolation of butyrate-oxidizing bacteria. Subsamples of the 3-chlorobenzoate enrichment metabolized 0.1% butyrate in liquid culture after a lag of 4 to 5 days. After 2 to 3 weeks, small white colonies up to 1 mm in diameter appeared in roll tubes containing butyrate in coculture with *M. hungatei* (PM-1). The morphology of bacteria in the colonies examined was that of a sporeforming bacterium in coculture with *M. hungatei*. Colonies were transferred to a liquid culture, and by week 2 visible turbidity was observed. After the initial isolation, two distinct bacteria were observed in liquid culture: a sporeforming bacterium and a non-sporeforming bacterium.

To ensure purity of the non-sporeforming bacterium, cells

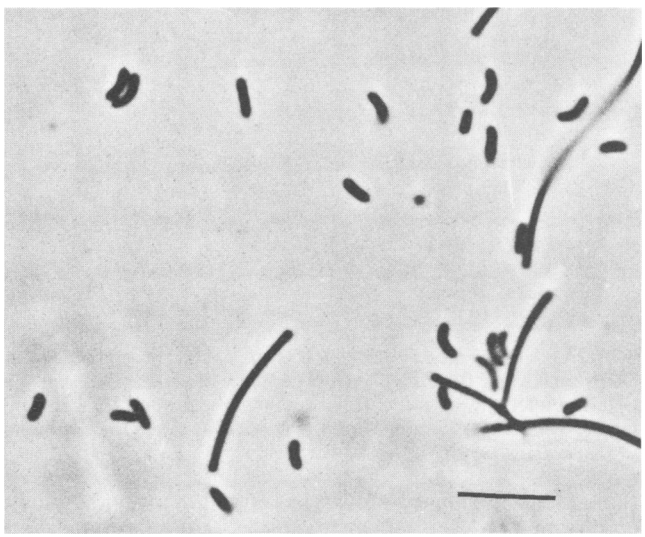

FIG. 6. Photomicrograph of the benzoate-oxidizing bacterium BZ-2 (short rods) in coculture with *M. hungatei* PM-1, showing curved and straight cells. Bar, 5 μm.

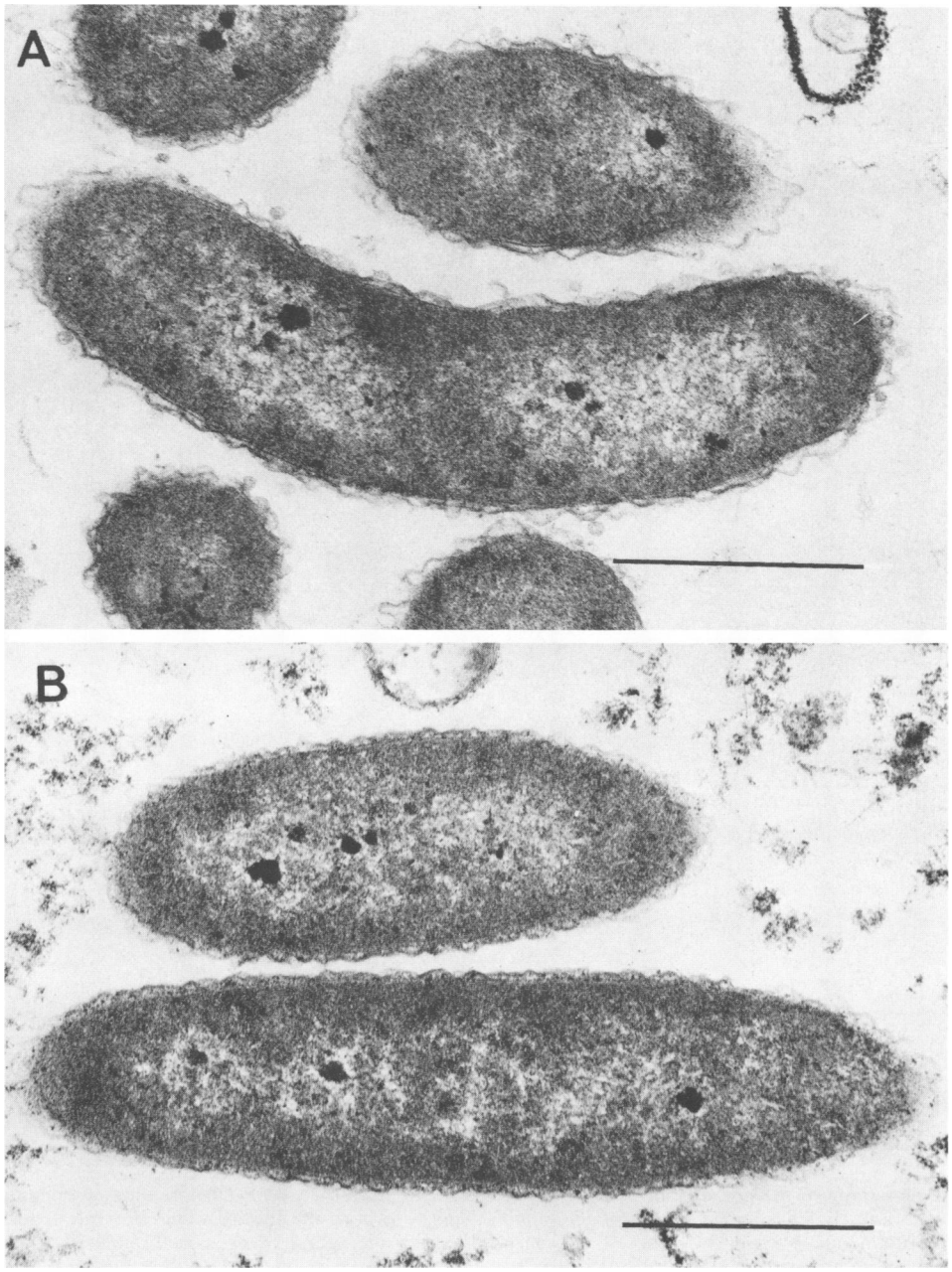

FIG. 7. (A) Electron micrograph of the benzoate-oxidizing bacterium BZ-2, showing curved cells. Bar, 0.5 μm. (B) Electron micrograph of the benzoate-oxidizing bacterium BZ-2, showing straight cells. Bar, 0.5 μm.

were subcultured two additional times. After subculturing, only the one distinctive cell morphology was observed (in addition to *M. hungatei*) and no turbid growth occurred in a complex medium (glucose, Trypticase, and yeast extract at 0.1% each). This isolate was designated NSF-2 (for non-sporeforming).

To purify the sporeforming bacterium, the culture was pasteurized three times at 80°C for 30 min. Before the final heat treatment, the culture was sparged with air until the resazurin in the medium had turned pink. After each pasteurization, 5 ml of the treated culture and 5 ml of a turbid culture of *Desulfovibrio* sp. or *M. hungatei* was added to 50 ml of liquid medium containing 0.15% butyrate. After spore germination, only the one distinctive cell morphology was observed (in addition to the H_2 consumer) and no turbid growth occurred in a complex medium (glucose, Trypticase, and yeast extract at 0.1% each). After four or five transfers, however (10% inoculum transfer), we observed the appearance of a non-sporeforming bacterium that would eventually displace the sporeformer as evidenced by the disappearance of spores from the culture. Repeated heat treatments always reselected the sporeforming culture, with nonsporing forms not appearing until after four to five transfers. The sporeforming organism was designated SF-1 (for sporeforming).

The non-sporeforming bacterium (strain NSF-2) was a slightly curved rod 2 to 4 μm in length and 0.2 to 0.3 μm in width (Fig. 8A). The cells stained gram negative. We occasionally observed what appeared to be a twitching type of

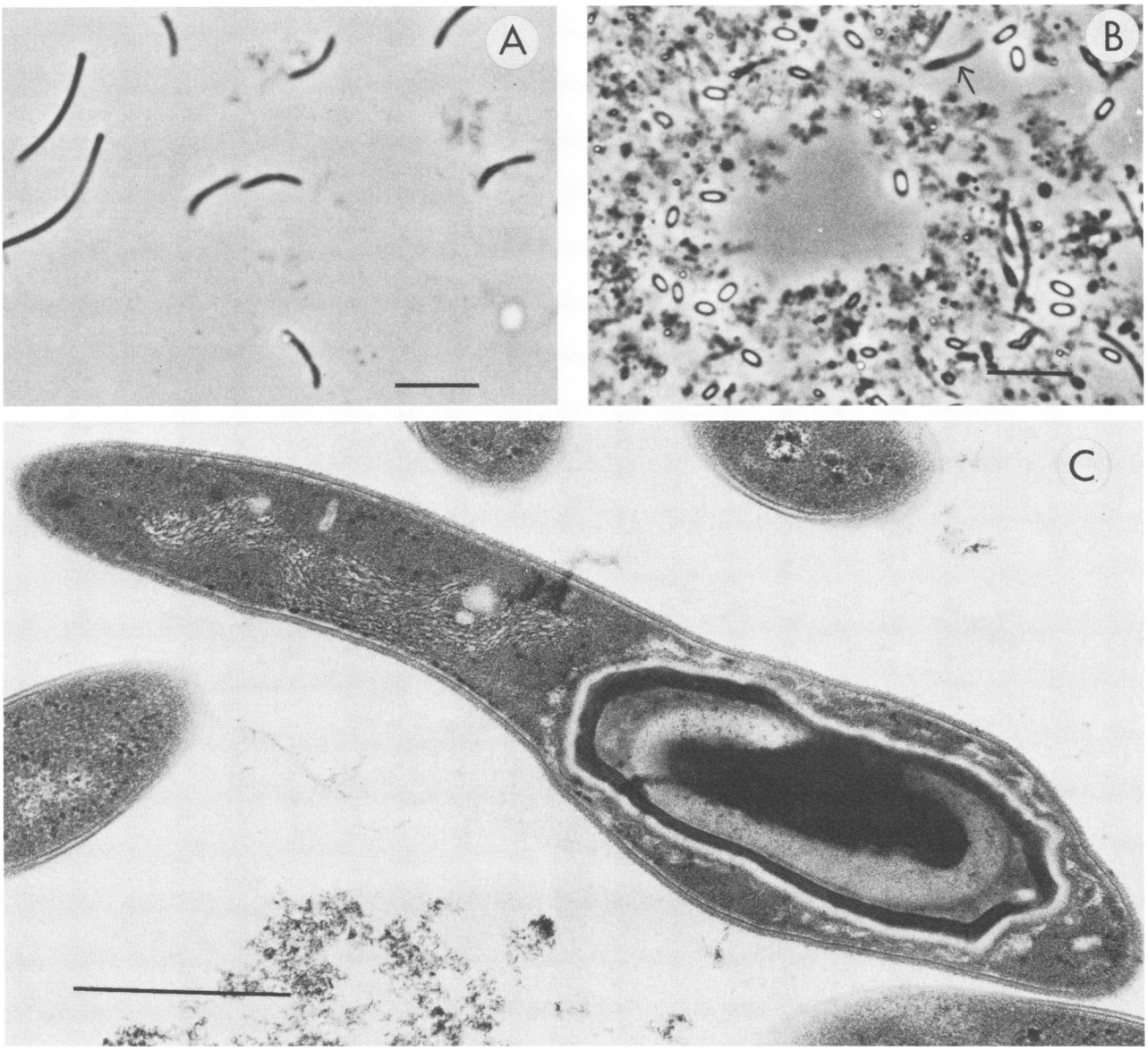

FIG. 8. (A) Photomicrograph of the non-sporeforming, butyrate-oxidizing bacterium NSF-2 (short rods) in coculture with *M. hungatei* PM-1 (long rods). Bar, 5 μm. (B) Phase-contrast photomicrograph of spores forming the sporeforming, butyrate-oxidizing bacterium. Note occasional intact cells with incomplete spores (arrow). Bar, 5 μm. (C) Electron micrograph of the sporeforming, butyrate-oxidizing bacterium SF-1, showing incipient spore and gram-positive cell wall. Bar, 0.5 μm.

motility; however, it was difficult to distinguish from Brownian motion. The sporeforming bacterium was a slightly curved rod 3 to 5 μm in length and 0.4 to 0.6 μm in width. The cells stained gram positive and appeared to have a gram-positive cell wall as observed in thin section (Fig. 8C). The culture produced spores resistant to heat and oxygen when grown in coculture with *M. hungatei* (Fig. 8B); spores were not produced when grown in coculture with *Desulfovibrio* sp.

Substrates observed to support growth of both bacteria were butyrate, 2-DL-methylbutyrate, valerate, and caproate (higher VFAs were not tested); isobutyrate supported growth of only the sporeforming bacterium; chemicals that did not support growth of either isolate included adipate, alanine, benzoate, butanol, Casamino Acids, ethanol, fructose, fumarate, glucose, glycerol, glycine, histidine, isoleucine, isovalerate, lactate, leucine, pimelate, proline, propionate, Proteose Peptone, pyruvate, ribose, Trypticase, valine, xylose, and yeast extract. Combinations of amino acids known to support growth of *Clostridium* species that carry out a Strickland reaction did not support growth. Fermentation products were acetate and CH_4 (from butyrate, isobutyrate, or caproate) or acetate, propionate, and CH_4 (from 2-DL-methylbutyrate or valerate) (Tables 1 and 2). Neither isolate reduced nitrate. When sulfate was added to bottles of NSF-2 and SF-1 with *M. hungatei* as the H_2 consumer, CH_4 production was not affected. Neither isolate grew in the presence of oxygen.

The sporeformer does not appear to have been made up of only two strains (SF-1 + methanogen) because of the displacement by a non-sporeformer resembling NSF-1 after several transfers. However, we believe that the sporeformer

is a separate VFA-oxidizing syntroph since (i) immediately after heat treatment, the culture oxidized VFAs and most cells showed evidence of spores, (ii) the colonies from roll tubes showed only spores plus methanogens, and (iii) isobutyrate was oxidized by the culture, but it was not metabolized after displacement by the non-sporeformer.

DISCUSSION

Based on the organisms isolated from the 3-chlorobenzoate-degrading consortium, there appears to be a three-tiered food chain: (i) 3-chlorobenzoate → benzoate, (ii) benzoate → acetate + H_2 + (CO_2), and (iii) acetate → CH_4 + CO_2 and H_2 + CO_2 → CH_4. The dechlorinating bacterium (DCB-1) converted 3-chlorobenzoate to benzoate and did not consume benzoate. We cannot conclude that other dechlorinating bacteria were absent; however, DCB-1 was present at greater than 10^6 bacteria ml^{-1} and was one of the dominant morphological types observed in the enrichment. A benzoate-oxidizing bacterium (BZ-2) was also present at greater than 10^6 bacteria ml^{-1}, and a similar morphological type was dominant in the enrichment. BZ-2 fermented benzoate to acetate, H_2 (represented by CH_4 in coculture with a methanogen), and presumably either formate or CO_2. We suspect the presence of other benzoate-oxidizing bacteria in the enrichment since we have observed colonies with cells of different morphologies growing on benzoate in coculture with *M. hungatei* or *Desulfovibrio* sp. that are consistent with morphologies observed in the enrichment. However, we have not succeeded in isolating them. Acetate was undoubtedly cleaved by the acetate-utilizing methanogen *Methanothrix soehgenii* (20), which was also a prevalent morphlogical type in the enrichment. H_2 was consumed by *M. hungatei* PM-1 and *Methanobacterium* sp. strain PM-2, both of which were isolated from the enrichment.

The presence of butyrate-oxidizing bacteria and the sulfidogen in the enrichment is unexplained since they do not appear to be in the main path of carbon flow. Morphologies consistent with either butyrate-oxidizing bacterium were not observed in the enrichment, although samples of enrichment readily metabolized butyrate after a lag of only 4 to 5 days; propionate was not metabolized by the enrichment. Butyrate or other VFAs were not detected in the enrichment supernatant and butyrate, and other VFAs were not observed as metabolites during benzoate catabolism by BZ-2. We periodically observed a highly motile bacterium with a vibrio morphology in the enrichment, which was consistent with the morphology of *Desulfovibrio* sp. strain PS-1. Since sulfate was never added to the enrichment, it seems unlikely that the bacterium was growing at the expense of H_2 and SO_4^{-2}. We do not feel that the presence of these bacteria can be attributed to contamination or carry-over because (i) these isolates are strict anaerobes, (ii) the only carbon and energy source added for more than 2 years was 3-chlorobenzoate, and (iii) the high dilution of the original sludge (ca. 10^{12}).

TABLE 1. Fermentation products formed from VFAs by the non-sporeforming isolate NSF-2 in coculture with *M. hungatei* PM-1

Substrate	Amt of substrate consumed (mmol)	Amt of product (mmol)			% Recovered	
		Acetate	Propionate	CH_4	C	H
Butyrate	0.78	1.53	0	0.39	98	100[a]
2-Methyl-butyrate	0.71	0.65	0.58	0.31	86	87[a]
Valerate	0.63	0.65	0.58	0.35	97	111[a]
Caproate	0.53	1.42	0	0.55	89	104[b]

[a] Based on 0.5 mol of CH_4 produced per mol of substrate consumed.
[b] Based on 1.0 mol of CH_4 produced per mol of substrate consumed.

TABLE 2. Fermentation products formed from VFAs by the sporeforming isolate SF-1 in coculture with *M. hungatei* PM-1

Substrate	Amt of substrate consumed (mmol)	Amt of product (mmol)				% Recovered	
		Acetate	Propionate	Butyrate	CH_4	C	H
Butyrate	0.72	1.59	0	0	0.35	110	97[a]
Isobutyrate	0.28	0.51	0	0	0.16	91	114[a]
2-Methyl-butyrate	0.30	0.28	0.30	0	0.14	97	93[a]
Valerate	0.49	0.46	0.53	0	0.23	102	94[a]
Caproate	0.54	1.20	0	0.17	0.45	95	99[b]

[a] Based on 0.5 mol of CH_4 produced per mol of substrate consumed.
[b] Based on 1.0 mol of CH_4 produced per mol of caproate consumed minus 0.5 mol of CH_4 per mol of butyrate produced.

The growth substrate(s) of the dechlorinating bacterium in the enrichment is not clear. Presumably, one or more of the organisms in the enrichment were cross-feeding the dechlorinating bacterium. This may offer an explanation for the presence of the butyrate-oxidizing and sulfate-reducing bacteria, which played no apparent role in the mineralization of 3-chlorobenzoate.

The dechlorinating bacterium (DCB-1) appears to be unique among procaryotes. The presence of a collar, the extremely restricted substrate range, the slow growth rate, and the ability to reductively dechlorinate aromatic compounds all are uncommon traits. The function of the collar is unknown; perhaps it is associated with cell division, as suggested by some electron micrographs. We are aware of only one previous report in which a structure resembling the collar was seen in a procaryote (4). In the previous case, the similar structure was shown in a thin section of a bacterium found in a community collected from anaerobic waters near the sediment surface of a eutrophic lake.

The only substrate that we observed to support significant growth of DCB-1 was pyruvate in conjunction with clarified rumen fluid or a complex protein source. The medium used for culturing of the organisms may not have been optimized if judged by the low cell yields obtained. We have had no difficulty, however, in growing other anaerobes to high density using this medium.

The dechlorination reaction appeared to be enzymatic since it occurred after induction and because of the previously reported evidence obtained with the enriched consortium, e.g., a low substrate K_m of 67 μM, loss of activity at temperatures >39°C, a high degree of substrate specificity, and the absence of any dechlorination in sterilized or inhibited controls (5, 16).

The benzoate-oxidizing bacterium (BZ-2) is similar to the benzoate-oxidizing bacterium isolated by Mountfort and Bryant (11) and recently named *Syntrophus buswellii* (10). The only distinction is that we observed both curved and straight cells in our cultures.

The non-sporeforming butyrate-oxidizing bacterium (NSF-2) is morphologically and physiologically consistent with *Syntrophomonas wolfei* (8), with the exception that our isolate catabolized 2-DL-methylbutyrate. The isolation of a gram-positive, sporeforming, butyrate-oxidizing bacterium has not been previously reported. According to current taxonomy, this would be a *Clostridium* species since it did not appear to reduce sulfate, formed spores, and was an obligate anaerobe. However, it is an atypical clostridium

since the bacterium was neither proteolytic or saccharolytic. Why this bacterium did not produce spores when grown in coculture with *Desulfovibrio* sp. is not known. In the vegetative state, the two butyrate-oxidizing bacteria are morphologically and physiologically similar, with the exception that the sporeformer was slightly larger and could catabolize isobutyrate.

This rather diverse consortium seems to have not only an obligate requirement for H_2 consumers but also a requirement for a dechlorinating organism to release the carbon and energy that supports all consortium members. Thus, this is a mutualism among at least three members. The particularly striking feature is that the dechlorinating organism seems to exist only by scavenging products from subsequent members in the food chain. Nonetheless, the consortium grows reasonably well on 3-chlorobenzoate and uses that substrate at a rate of ca. 20 $\mu M\ h^{-1}$ (16).

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