Stable associations of syntrophic fermentative organisms and populations that consume fermentation products play key roles in the anaerobic biodegradation of chlorinated organic contaminants. The involvement of these syntrophic populations is essential for mineralization of chlorinated aromatic compounds under methanogenic conditions. The fermentative production of low levels of hydrogen (H₂) can also be used to selectively deliver a limiting electron donor to dehalogenating organisms and achieve complete dehalogenation of chlorinated aliphatic contaminants such as tetrachloroethene. Thus, tracking the abundance of syntrophically coupled populations should aid in the development and monitoring of sustainable bioremediation strategies. In this study, two complementary nucleic acid–based methods were used to identify and assess relative changes or differences in the abundance of potentially important populations in complex anaerobic microbial communities that mineralized chlorinated aromatic compounds. Population dynamics were related to the consumption and production of key metabolic substrates, intermediates, and products. Syntrophus–like populations were detected in 3-chlorobenzoate–degrading communities derived from sediment or sludge digesters. In the presence of H₂-consuming populations, characterized Syntrophus species ferment benzoate, a central intermediate in the anaerobic metabolism of 3-chlorobenzoate and 2-chlorophenol. A DNA probe that targeted characterized Syntrophus species was developed and used to quantify rRNA extracted from the 3-chlorobenzoate– and 2-chlorophenol–degrading communities. The level of rRNA targeted by the Syntrophus–specific probe tracked with the formation of benzoate during metabolism of the parent compounds. Hybridizations with an Archaea–specific probe and/or measurement of methane production demonstrated that methanogens directly benefited from the influx of benzoate–derived electron donors, and the activities of Syntrophus–like and methanogenic populations in the contaminant-degrading communities were closely linked. Key words: 2-chlorophenol, 3-chlorobenzoate, benzene, bioremediation, microbial communities, oligonucleotide probes, reductive dechlorination, ribosomal RNA, syntrophic associations, Syntrophus. Environ Health Perspect 113:310–316 (2005). doi:10.1289/ehp.6933 available via http://dx.doi.org/[Online 8 December 2004]
populations with PCE-degrading organisms for H₂.

One strategy for enhancing selective delivery of H₂ to dehalorespiring populations involves providing H₂ through the addition of certain fermentable substrates such as butyrate or propionate (Fennell et al. 1997). Fermentation of butyrate under typical culture conditions occurs slowly and is thermodynamically feasible only at H₂ partial pressures of 10⁻³ atm or less. Even lower H₂ levels are required to sustain fermentation of propionate. Dehalorespiring and other hydrogenotrophic populations in anaerobic environments maintain H₂ levels low enough to allow fermentation of a wide range of organic substrates to proceed. However, production of H₂ through the slow syntrophic fermentation of butyrate and similar compounds appears to favor dechlorination over other hydrogenotrophic processes. Thus, being able to track the abundance of syntrophic H₂-producing and H₂-consuming populations should be useful when developing and monitoring sustainable engineering strategies for remediation of chloroethene-contaminated sites.

The ability to track syntrophic populations that produce and consume H₂ (and/or acetate) in complex anaerobic microbial communities that degrade chlorinated aromatic compounds is also useful because of the key roles that these populations play in sustaining biodegradation of the parent compounds (Becker et al. 2001). Therefore, we used two model microbial communities to study the degradation of 3-chlorobenzoate (3-CB) and 2-chlorophenol (2-CP). The communities were maintained in batch laboratory systems derived from two different anaerobic habitats: lake sediment and municipal wastewater sludge. Two complementary nucleic acid-based methods were used to evaluate population changes relative to substrate production and use in these systems. The complementary analyses clearly resolved the role of syntrophic associations in the degradation of both chlorinated compounds.

**Materials and Methods**

**Source of inocula.** The 3-CB– and 2-CP–degrading communities were derived from environmental inocula obtained from anaerobic lake sediment or municipal wastewater sludge digesters. Sediment was obtained from the previously described (MacGregor et al. 1997) Fox Point sampling site in Lake Michigan during two sampling events. Anaerobic sediment was collected using a box corer and transferred to canning jars, which were filled to capacity and stored at 4°C until used (within ~3 months). Sediment collected during the first event provided the inoculum for a preliminary microcosm experiment conducted with 3-CB, whereas the inoculum used in mesocosm-scale experiments involving 2-CP or 3-CB was derived from sediment collected nearly 2 years later. A mixture of primary and secondary digester sludge was obtained from the North Shore Sanitary District’s Clavety Road Wastewater Treatment Plant in Highland Park, Illinois. After some material was flushed from a sampling line, canning jars were filled until they overflowed and stored at 4°C until used (within 24 hr). The digester contents were used as the inoculum in a second mesocosm-scale experiment involving 3-CB.

**Establishment of cultures.** The sediment and digester inocula were diluted in anaerobic mineral medium (1:9, vol/vol), and the cultures were maintained in microcosms and mesocosms comprising 160-mL and 2-L glass vessels, respectively, with serum bottle closures, using the methods described by Becker et al. (1999). Culture preparation, sampling, and amendments were performed using strict anaerobic techniques based on the methods described by Miller and Wolin (1974). The anaerobic mineral medium used in the sediment experiments has been described previously (Freedman and Gossett 1989). The medium used to dilute the digester inoculum had a slightly different formulation and contained (per liter): 0.5 g NH₄Cl, 0.4 g KH₂PO₄, 0.1 g MgCl₂·6H₂O, 0.001 g resazurin, 0.5 g Na₂S·5H₂O, 0.1 g CaCl₂·2H₂O, 4 g NaHCO₃, 0.05 g yeast extract, and 10 mL trace metal solution. The trace metal solution was modified from Tanner (1997) and contained (per liter): 1 g MnCl₂·4H₂O, 0.2 g CoCl₂·6H₂O, 0.2 g ZnSO₄·7H₂O, 0.019 g H₃BO₃, 0.02 g NiCl₂·6H₂O, 0.02 g Na₂MoO₄·2H₂O, 0.8 g Fe(NH₄)₂(SO₄)₂, 0.02 g CuCl₂·2H₂O, 0.02 g Na₃SeO₃, 0.02 g Na₂WO₄, and 2 g nitric acid. Cultures were maintained under a headspace of 30% CO₂:70% N₂ (vol/vol). The initial ratio of headspace volume to slurry phase volume in all culture vessels was 3:5 (vol/vol). All cultures and controls were incubated statically at 30°C.

**Microcosm-scale biodegradation experiment.** A preliminary experiment was conducted using 160-mL serum bottle microcosms inoculated with sediment and amended with 3-CB. Sediment microcosms were prepared in triplicate and amended with 3-CB (99%; Aldrich) at an initial concentration of 200 μM. Degrading and control reactors were sampled at approximately 1-week intervals. For each experiment, duplicate or triplicate microcosms (160-mL) were prepared in the same way as the viable 2-CP– and 3-CB–amended mesocosms and periodically sampled to evaluate the reproducibility of the nucleic acid and chemical data obtained with the mesocosms (Becker 1998). Aromatic substrates and metabolites were monitored in all reactors amended with 3-CB and/or 2-CP. rRNA was extracted from all viable reactors. All other analyses were performed only on the viable 2-L reactors. In the interest of brevity, only the results obtained with the mesocosms are reported here.

**Benzoate perturbation experiment.** At the conclusion of the mesocosm-scale biodegradation experiment involving the digester sludge inoculum, duplicate 50-mL aliquots of slurry were aseptically removed from the 3-CB–amended mesocosms and transferred to 160-mL serum bottles. Except for the difference in the ratio of headspace volume to slurry volume, the transferred 3-CB–degrading digestate cultures were maintained as described above. The cultures were amended with a sterile, deoxygenated stock solution of benzoate, resulting in an initial concentration of 13.8 mM, and regularly sampled for analysis of benzoate.

**Analytical methods.** Reverse-phase high-performance liquid chromatography with diode array detection was used to quantify chlorinated aromatic substrates and metabolites, as previously described (Becker et al. 1999). Headspace H₂ and methane (CH₄) concentrations were determined using gas chromatography and reduction gas and flame ionization detectors, respectively, according to the methods described by Becker et al. (2001).

**DNA extraction, amplification, separation, and sequencing.** DNA was isolated from sediment and digester slurry samples using a modification of the freeze-thaw and lysosome...
treatments, phenol–chloroform extraction, and ethanol precipitation described by Tsai and Olson (1991). Each slurry sample (1 mL) was transferred to a plastic centrifuge tube (14 mL; Sarstedt, Inc., Nümbrecht, Germany) and frozen (~20°C) until cell lysis and extraction and precipitation of DNA with ethanol was implemented. Extracted DNA was diluted (1:10) and amplified using the bacteria-specific primers GM5F-GC and S-^-Univ-0907-b-A-20 and the polymerase chain reaction (PCR) procedure described by Muyzer et al. (1993) with slight modification. The GM5F-GC primer contains a 40-bp GC clamp to introduce a high-melting-point domain into the amplified fragment. Together, primers GM5F-GC and S-^-Univ-0907-b-A-20 amplify bacterial small-subunit (SSU) rDNA in the region corresponding to positions 357–907 in Escherichia coli to produce a 550-bp fragment (not including the GC-clamp). The 50-µL reaction mixture consisted of 100–300 ng (2–5 µL) template DNA; 15 mM MgCl

RNA extraction, hybridization, and quantification of extracted RNA. The mechanical-disruption/phenol–chloroform extraction process of Stahl et al. (1988) was used with previously described modifications (Becker et al. 2001; MacGregor et al. 1997) to extract RNA from sediment and digester slurry samples. RNA slot blotting, probe labeling, prehybridization, hybridization, and washing were performed as previously described (Lin and Stahl 1995; Raskin et al. 1994; Stahl et al. 1988). Samples were transferred to nylon membranes in triplicate, prehybridized at 40°C, and washed at 56°C [S-D-Arch-0915-a-A-20; Amann et al. (1990) or 51°C [S-G-Syn-0424-a-A-18; Becker et al. (2001)]. It was assumed that archael rRNA, which was targeted by probe S-D-Arch-0915-a-A-20, was primarily derived from methanogenic species. This is reasonable because previous studies have shown that crenarchaeotal rRNA is a minor component of the Lake Michigan sediment (MacGregor et al. 1997). Probe S-G-Syn-0424-a-A-18 targets members of the genus Syntrophus and has been described previously (Becker et al. 2001) but was developed as part of this study. The relative abundance of rRNA that hybridized with the Archaea- and Syntrophus-specific probes in a given mesocosm is reported relative to the amount of archaenal and Syntrophus-like rRNA, respectively, in the reactor at time zero.

Results and Discussion

In the preliminary studies of biodegradation of 3-CB in sediment, biodegradation was not observed for approximately 78 days. Thereafter, 3-CB was rapidly degraded in the triplicate microcosms but not in the sterile controls (data not shown). 3-CB was repeatedly degraded after 29 replenishments (~ 200-µM amendments) over an approximately 2-year period. DNA samples were obtained from the microcosms on days 330, 500, and 600 and analyzed using DGGE (Figure 1). In each of the microcosms, the DGGE separation patterns varied over time, which suggests that the structures of the 3-CB–degrading communities were not static, even after degrading repetitive additions of 3-CB. Patterns also differed among microcosms, despite their similar treatment and behavior. However, several DNA fragments migrated to similar positions in the DGGE gel in all three microcosms, even after 500 or 600 days. A number of these bands were unique to the 3-CB–degrading microcosms and did not appear in the DGGE patterns obtained from microcosms that did not adapt to 3-CB (Becker 1998).

Figure 1. Ethidium bromide–stained DGGE profiles of rDNA PCR fragments derived from DNA extracted from triplicate 3-CB–degrading microcosms inoculated with Lake Michigan sediment (3CB1, 3CB2, and 3CB3) using a 20–70% denaturant gradient gel. The DNA extracts were obtained from the microcosms on the following days: lane a, day 330; lane b, day 500; lane c, day 600. Gel portions that were excised for additional analyses are indicated with arrows plus a letter and/or number. Throughout the text and in Figure 2, these bands are referred to using the microcosm name plus the label shown in Figure 1 (e.g., 3CB1-4). Sequences derived from reamplified DGGE bands were submitted to GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db_nucleotide) with the following accession numbers: 3CB1-4, AY646230; 3CB1-Nb, AY646231; 3CB1-N, AY646232; 3CB2-4, AY646233; 3CB2-6, AY646234; 3CB2-d15, AY646235; 3CB3-4, AY646236; and 3CB3-7, AY646237.
Selected bands were excised from the DGGE gel for reamplification and comparative sequencing (Figure 1). One band (position 4) was common to all lanes. The bands in position 4 actually consisted of two DNA fragments that comigrated in the 20–70% denaturant gradient gel. The comigrating fragments could be resolved using a 30–50% denaturant gradient gel (not shown). Each of the other amplified bands corresponded to a single sequence. Sequence lengths ranged from 338 to 467 nucleotides. The sequences contained a maximum of five ambiguities, and most of the sequences contained zero to two ambiguities. Ambiguous sequence regions were not included in similarity determinations.

Sequences 3CB2-4 and 3CB3-4 are closely related to uncultured members of anaerobic communities that dechlorinate 1,2-dichloropropane (SHA-207; Schlotelburg et al. 2000) and trichlorobenzene (SJA-162; von Wintzingerode et al. 1999). 3CB2-4 shares a sequence similarity of 99 and 98% with SHA-207 and SJA-16, respectively. 3CB3-4 shares 97% sequence similarity with SHA-207 and SJA-16. Another fragment from the 3-CB-degrading sediment microcosms (3CB1-Nb) originated from a close relative of Desulfomonile tiejedi, which is able to conserve energy via the reductive dehalogenation of 3-CB (DeWeerd et al. 1990). 3CB1-Nb and D. tiejedi share >99% sequence similarity over 429 bp.

The closest cultured relative of both 3CB1-N and 3CB2-d15 is Moorella sp. F21 (GenBank accession no. AB086398; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db_nucleotide). Strain F21 shares 87 and 91% sequence similarity with 3CB1-N and 3CB2-d15, respectively. 3CB3-7 shares 97% sequence similarity with its closest relative, a clone of an uncultured bacterium from a thermophilic terephthalate-degrading anaerobic sludge (GenBank accession no. AY297966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide).

Two sequences are closely related to benzoate-degrading syntrophs. Conversion of benzoate to H₂, acetate, and CO₂ is thermodynamically unfavorable at standard conditions. However, in the presence of hydrogen-consuming populations that maintain low H₂ partial pressures in anaerobic environments, the fermentation of benzoate to H₂, acetate, and CO₂ by syntrophic bacteria is thermodynamically feasible. The closest cultured relatives of 3CB1-4 are the characterized Syntrophus species S. buswellii (Mountfort et al. 1984), S. gentianae (Wallrabenstein et al. 1995), and S. aciditrophicus (Jackson et al. 1999) (Figure 2). For example, 3CB1-4 shares 99% sequence similarity with S. aciditrophicus. Although fermentation of benzoate to cyclohexane carboxylate and acetate by a pure culture of S. aciditrophicus has been observed (Elshahed and McInerney 2001), the characterized Syntrophus species appear to grow primarily via syntrophic fermentation of benzoate (Jackson et al. 1999; Mountfort et al. 1984; Wallrabenstein et al. 1995). 3CB2-6 shares 95% sequence similarity with the gram-positive benzoate-degrading syntroph Sporotomaculum syntrophicum (Qiu et al. 2003).

Benzoate is a central intermediate in the anaerobic biodegradation of a variety of aromatic compounds, including 3-CB and ortho-chlorinated phenols (Heider and Fuchs 1997) and is produced from the reductive dehalogenation of 3-CB. Consumption of H₂ by a dehalogenating organism (e.g., Desulfomonile tiejedi) and/or methanogenic populations creates an environment in which benzoate fermentation is feasible and biodegradation of 3-CB can be sustained without the addition of an electron donor (Dolfing and Tiedje 1986). During the mineralization of 2-CP, benzoate is formed from phenol, the primary dehalogenation product, via sequential carboxylation and dehydroxylation reactions (Becker et al. 1999; Sharak Gentnher et al. 1991; Zhang et al. 1990). A second pathway that sometimes occurs during the mineralization of 2-CP and involves benzoate has also been described (Becker et al. 1999). In this alternative pathway, 2-CP undergoes para-carboxylation and dehydroxylation to form 3-CB, which is reductively dehalogenated to form benzoate. Benzoate produced during the biodegradation of 2-CP via either pathway is subject to syntrophic conversion to H₂, acetate, and CO₂ in the presence of populations that consume the fermentation products (Zhang and Wiegel 1990).

We were interested in tracking the relative activity of populations that mediate syntrophic benzoate fermentation because of the importance of this process in sediment and other anaerobic environments. In particular, we focused on monitoring the activity of Syntrophus populations, partly because the DGGE results suggested that the activity of 3CB2-6 decreased with time, whereas 3CB1-4 appeared to increase in abundance (Figure 2).

A phylogenetic probe (S-G-Syn-0424-a-A-18), which specifically targets the SSU rRNAs of the three Syntrophus species characterized to date and the uncultured population from the sediment community, was designed. Probe S-G-Syn-0424-a-A-18 was used along with an existing probe that targets the SSU rRNA sequences of Archaea to quantify the relative activity of syntrophic benzoate-degrading and methanogenic populations in 2-L mesocosms inoculated with sediment and amended with 3-CB, 2-CP, or no substrate. As shown in Figure 3, transformation of 3-CB in the amended mesocosm followed the pattern observed in the microcosm study and was preceded by an adaptation period of approximately 77 days. During the adaptation period, no 3-CB removal occurred, and the
amounts of *Syntrophus*-like rRNA in the 3-CB–amended mesocosm and the no-substrate control fluctuated over time but did not increase significantly overall, compared with the levels initially present at the time of exposure to 3-CB. Once 3-CB biodegradation began, a significant increase in *Syntrophus*-like rRNA levels in the 3-CB–amended mesocosm was observed, and a second increase, which nearly tripled *Syntrophus*-like rRNA levels compared with the initial concentration in this culture, was observed after the addition of a second 3-CB dose. In contrast, during the period of 3-CB biodegradation in the amended mesocosm, *Syntrophus*-like rRNA levels in the no-substrate control decreased. Although benzoate was never detected in the 3-CB–amended mesocosm, it was presumably produced via the reductive dehalogenation of the parent substrate and rapidly consumed by *Syntrophus* species. Thus, the results of the hybridization conducted with mesocosm rRNA extracts support the idea that a *Syntrophus*-like population in the sediment culture was involved in 3-CB biodegradation, as suggested by the DGGE and sequencing analyses performed during the preliminary microcosm experiment. On the basis of previous characterizations of *Syntrophus* species (Jackson et al. 1999; Mountfort et al. 1984; Wallrabenstein et al. 1995), it is likely that this population played a role in fermenting benzoate.

Further, the results of the hybridization between rRNA extracted from the 3-CB–amended mesocosm and no-substrate control and the Archaea-specific probe suggested that the activity of the *Syntrophus*-like organisms in the 3-CB–amended mesocosm was sustained by methanogenic populations, which benefited from the production of benzoate-derived H₂ and/or acetate (Figure 4). In the 3-CB–amended sediment mesocosm, the level of archaean rRNA, which presumably was derived primarily from methanogens, remained fairly constant during the adaptation period, except for the peak at around day 50, which coincided with the onset of endogenous methane production (Becker 1998). A similar pattern in the level of archaean rRNA was observed in the no-substrate control during the adaptation period. However, after the onset of 3-CB biodegradation in the amended mesocosm, differences in the activities of methanogenic populations in the two sediment cultures began to emerge. Specifically, in the 3-CB–amended mesocosm, increases in *Syntrophus*-like rRNA were followed by increases in archaean rRNA. This trend is consistent with the idea that *Syntrophus* and methanogenic populations are members of a syntrophic association in which the *Syntrophus* population, because it increases in abundance and activity, releases greater amounts of electron donors (H₂ and acetate), which in turn stimulate growth of the methanogens. In contrast, an overall decrease in archaean rRNA was...
observed in the no-substrate control after day 77, in the absence of benzoate-derived H₂ and acetate.

Evidence of a syntrophic association between _Syntrophus_ and methanogenic populations in the 2-CP-degrading sediment community was also obtained. The mineralization of 2-CP and associated population changes in the sediment community have been reported previously (Becker et al. 2001). Briefly, 2-CP was rapidly removed in the amended mesocosm and was replenished 5 times during the remainder of the approximately 130-day experiment (Figure 5A). In contrast to the studies conducted with 3-CB, benzoate did transiently accumulate in 2-CP-transforming systems. The onset of benzoate fermentation to acetate (Becker et al. 2001) and H₂ and acetate.

The onset of benzoate fermentation to acetate (Becker et al. 2001) and H₂ and acetate occurred by day 49. No hydrogen and acetate.

(Figure 5B) occurred by day 49. No hydrogen and acetate.

The onset of benzoate fermentation to acetate (Becker et al. 2001) and H₂ and acetate. The onset of benzoate fermentation to acetate (Becker et al. 2001) and H₂ and acetate.
control until the conclusion of the experiment. In contrast, the onset of 3-CB biodegradation in the amended mesocosm was accompanied by a significant increase in *Syntrophus*-like rRNA levels. The detection of *Syntrophus*-like sequences in the DGGE profiles of the benzoate-perturbed digester community and the results of the hybridization conducted with the *Syntrophus*-specific probe strongly suggest that a *Syntrophus* population played a key role in the biodegradation of chlorinated aromatic compounds in the digester community, as well as in the sediment systems.

The production of CH$_4$ in the amended digester mesocosm increased significantly after the onset of 3-CB biodegradation, compared with methanogenesis in the no-substrate control (Becker 1998). The CH$_4$ data suggested that methanogens also played an important role in sustaining the biodegradation of the chlorinated substrates by the digester community and benefited from the activity of the *Syntrophus* population. However, the impact of syntrophic benzoate degradation on the activity of methanogenic populations could not be detected by hybridizing the Archaea-specific probe with rRNA extracted from the 3-CB-amended digester mesocosm and no-substrate control over time. Archaeal rRNA levels in the two cultures were of a comparable magnitude and underwent similar temporal changes. Apparently, the effect of metabolism of abundant substrates that were endogenous to the digester inocula significantly influenced archaeal SSU rRNA levels and masked the effect of syntrophic benzoate fermentation reflected in the methane levels.

**Conclusions**

The results of this study demonstrate that the general approach of using a DNA fingerprinting technique and comparative sequencing to identify potentially important populations in contaminant-degrading communities, followed by SSU rRNA-based hybridizations to quantify changes in the abundance of these populations, can be integrated with analysis of metabolic substrates and products and applied to different complex microbiological systems. Specifically, we demonstrated that it is possible to identify and track populations in syntrophic associations that are based on the fermentation of organic intermediates and are critical for sustaining mineralization of the chlorinated parent compounds in complex anaerobic microbial communities. We anticipate that the analytical approach and methods developed in this study will be useful in the development and monitoring of sustainable engineering strategies for remediation of sites contaminated with chlorinated organic compounds.

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