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# Characterization of methanogenic and methanotrophic assemblages in landfill samples

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**A greater understanding of the tightly linked trophic groups of anaerobic and aerobic bacteria residing in municipal solid waste landfills will increase our ability to control methane emissions and pollutant fate in these environments. To this end, we characterized the composition of methanogenic and methanotrophic bacteria in samples taken from two regions of a municipal solid waste landfill that varied in age. A method combining polymerase chain reaction amplification, restriction fragment length polymorphism analysis and phylogenetic analysis was used for this purpose. 16S rDNA sequence analysis revealed a rich assemblage of methanogens in both samples, including acetoclasts, $H_2/CO_2$-users and formate-users in the newer samples and $H_2/CO_2$-users and formate-users in the older samples, with closely related genera including *Methanoculleus*, *Methanofollis*, *Methanosaeta* and *Methanosarcina*. Fewer phylotypes of type 1 methanotrophs were observed relative to type 2 methanotrophs. Most type 1 sequences clustered within a clade related to *Methylobacter*, whereas type 2 sequences were broadly distributed among clades associated with *Methylocystis* and *Methylosinus* species. This genetic characterization tool promises rapid screening of landfill samples for genotypes and, therefore, degradation potentials.**

**Keywords:** landfill; methanogen; methanotroph; homoacetogen; phylogenetic analysis; 16S rDNA

## 1. INTRODUCTION

Out of the approximately 220 million metric tons of municipal solid waste (MSW) produced annually in the United States, nearly 61% is disposed of in landfills (USEPA 1999). As the population growth and MSW generation rates increase in many areas of the United States, there is an urgent need to develop methods to increase the rate of microbial decomposition of MSW while minimizing the release of methane, a potent greenhouse gas.

Most landfills are composed primarily of anaerobic zones, where microbial decomposition of MSW follows well-known decomposition processes, including microbial fermentations, sulphate reduction, acetogenesis and methanogenesis (Barlaz 1997). The last step in the conversion of complex polymers in MSW to methane requires the activity of methane-producing micro-organisms (methanogens), which use a limited range of growth substrates including acetate, $H_2/CO_2$, formate, methanol and methylated amines and dimethyl sulphide (Keltjens & Vogels 1993; Barlaz 1997). Despite estimates of global net emissions of methane from landfills ranging from 9 to 70 Tg $yr^{-1}$ (Nozhevnikova *et al.* 1993; Boeckx *et al.* 1996; Czepiel *et al.* 1996; Bogner *et al.* 1997; Visvanathan *et al.* 1999) (with a range of 40–70 Tg most often cited), methanotrophs have been reported to oxidize a significant amount of methane to $CO_2$, a far less potent greenhouse gas (Whalen *et al.* 1990; Boeckx *et al.* 1996; Czepiel *et al.* 1996; Bogner *et al.* 1997; USEPA 1999). Methanogens and methanotrophs have also been linked in their abilities to sequentially transform chlorinated solvents, such as perchloroethylene (PCE) and trichloroethylene (TCE), known to be present in landfills, yielding $CO_2$ as the final product (Wilson & Wilson 1985; Little *et al.* 1988). Thus, understanding the composition, metabolic potential and interrelationships of these individual groups of micro-organisms in landfills will facilitate the development of optimized landfill management strategies.

We have taken initial steps to a better understanding of MSW landfill micro-organisms by developing a tool using sequence analysis of genes characteristic of methanogens and methanotrophs to assess their presence and species richness in landfill samples. This approach to bacterial characterization shows potential for linking species richness and degradation potentials with landfill type, conditions and, ultimately, management practices.

## 2. MATERIAL AND METHODS

### (a) *Landfill site and sample characteristics*

The samples used in this study were obtained from the Alachua County Southwest Landfill (Archer, FL, USA), a lined MSW landfill containing mixed household and commercial/light industrial waste and covered with 30–45 cm of sandy soil. Samples, consisting of a mixture of MSW and soil, were collected during the installation of gas collection wells from two locations in sections of the landfill differing in age by *ca.* 4 years, the more recent being 2 years old. The average methane and oxygen concentrations and temperature of gas samples taken from each location during the sampling period were 60.8% v/v, 0.2% v/v and 41 °C, respectively, in the older location (referred to as GW60) and 56.5% v/v, 0% v/v, 33 °C, respectively, in the newer location (referred to as GW70).

A minimum of three 67 g samples were taken *ca.* 4 m below the surface from GW60 and *ca.* 3 and *ca.* 6 m below the surface in GW70 (referred to as GW60-13, GW70-10 and GW70-20, respectively). Because soil samples from *ca.* 4 m below the surface were not available in the newer section, the *ca.* 3 and *ca.* 6 m sampling depths were chosen for the best comparison to the GW60 sample. (These depths were chosen rather than more shallow cover soil depths, where an overabundance of methanotrophic populations was expected, to test the sensitivity of the genetic analysis method for the detection of methanotrophs.) Upon collection, all samples were immediately refrigerated. Before use, the three replicate samples taken from each location were manually homogenized by mixing with a sterile spatula in a laminar flow hood.

### (b) *Soil DNA isolation and PCR*

DNA from all samples was isolated by a soil DNA isolation kit (Mobio, Solana Beach, CA, USA). Primer names, sequences and target groups for amplification by PCR are presented in table 1. Reaction mixtures were subjected to 35 cycles in a Perkin–Elmer Model 2400 Thermal Cycler (Perkin–Elmer, Norwalk, CT, USA). PCR cycling was performed at 94 °C for 30 s for denaturation and at 72 °C for 30 s for chain extension. An initial activation step of 95 °C for 15 min was required for HotStarTaq master mix (Qiagen, Germany). Annealing was carried out at 58 °C for 30 s for methanogen and type


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Table 1. Primers used in this study.

| primer (sequence) | target gene | reference |
|---|---|---|
| 23F (5′-TCYGGTTGATCCTGCC-3′) | archaeal 16S rDNA gene | Burggraf *et al.* (1991) |
| 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) | archaeal 16S rDNA gene | Lane (1991) |
| MethT1dF (5′-CCTTCGGGMGCYGACGAGT-3′) | type 1 methanotrophic 16S rDNA gene | Wise *et al.* (1999) |
| MethT1bR (5′-GATTCYMTGSATGTCAAGG-3′) | type 1 methanotrophic 16S rDNA gene | Wise *et al.* (1999) |
| MethT2R (5′-CATCTCTGRCSAYCATACCGG-3′) | type 2 methanotrophic 16S rDNA gene | Wise *et al.* (1999) |

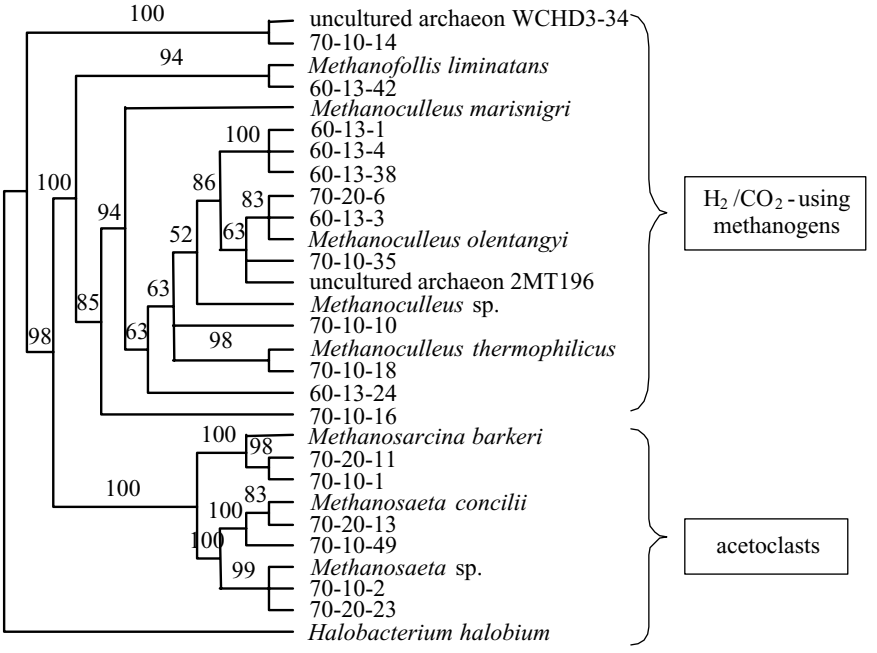


Figure 1. Phylogenetic tree of landfill soil methanogen 16S rRNA gene clone sequences. The tree was constructed with PAUP v. 4.0b8 using maximum parsimony. Bootstrap values are based on 100 replicates. *Halobacterium halobium* was used as the outgroup.

2 methanotroph primers and 55 °C for 30 s for type 1 methanotroph primers. An additional 7 min were added for chain extension.

PCR products were ligated to vector plasmid PCR@2.1 (Invitrogen, Carlsbad, CA, USA) and transformed to competent *Escherichia coli* cells (TOP10F′) according to the vendor's instructions. For archaeal rDNA, 50, 48 and 25 colonies were screened in samples GW60-13, GW70-10 and GW70-20, respectively. For methanotroph type 1 rDNA, 38, 50 and 41 colonies were screened in samples GW60-13, GW70-10 and GW70-20, respectively. For methanotroph type 2 rDNA, 55 and 61 colonies were only screened in samples GW60-13 and GW70-10, respectively. Sample GW70-20 was not screened for type 2 methanotrophs because of repeated difficulties in obtaining amplification products of suitable quality. The lack of a type 2 clone library from this sample is not considered to be significant because no comparable GW60 sample was available. Plasmid DNA from transformants was isolated with standard mini-prep plasmid isolation procedure (Sambrook *et al.* 1989).

### (c) *Restriction fragment length polymorphism analysis, sequencing and phylogenetic analysis*

Plasmid DNA-containing inserts of the appropriate size were digested with *Hha*I. Clones showing different digestion patterns were sequenced by the University of Florida's Interdisciplinary Center for Biotechnology Research core sequencing facility. Restriction fragment length polymorphism (RFLP) results were analysed by using analytic Rarefaction software (v. 1.2, S. M. Holland, University of Georgia, http://www.uga.edu/~strata) to determine the extent to which the number of clones sampled were sufficient to represent the diversity in the individual clone libraries.

Sequences were compared with previously identified sequences in the National Center Biotechnology Information (NCBI) database using BLAST (Altschul *et al.* 1990). The sequences obtained in this study were initially aligned with closely matched sequences from the NCBI database using the Pileup function of the Genetics Computer Group (Genetics Computer Group 1999) and adjusted manually with ClustalX v. 1.8 (Thompson *et al.* 1997). Phylogenetic trees were generated with PAUP v. 4.0b8 (Swofford 2001) and rDNA trees were constructed using maximum parsimony.

### (d) *Nucleic acid accession numbers*

The GenBank accession numbers obtained in this study for archaeal and types 1 and 2 methanotrophic 16S rDNA are AY062218–AY062235, AY063504–AY063510, AF450001–AF450 007, respectively.

## 3. RESULTS

### (a) *Characterization of methanogen assemblages*

16S rDNA gene sequence analysis revealed a rich assemblage of methanogens at both sampling locations and included acetoclastic and hydrogenotrophic methanogens (figure 1). All six archaeal 16S rDNA sequences obtained from GW60-13, and five of the sequences obtained from GW70-10 and GW70-20, clustered within the branch representing methanogens capable of using $H_2/CO_2$ and formate as substrates. All sequences in this branch are related to the *Methanoculleus* and *Methanofollis* genera of the *Methanomicrobiaceae* family. The other deep branches contain sequences obtained from the GW70 samples. Six of these sequences are closely related to *Methanosaeta concilii* (*Methanothrix soehngenii*) (which uses only acetate as an electron donor) and *Methanosarcina*

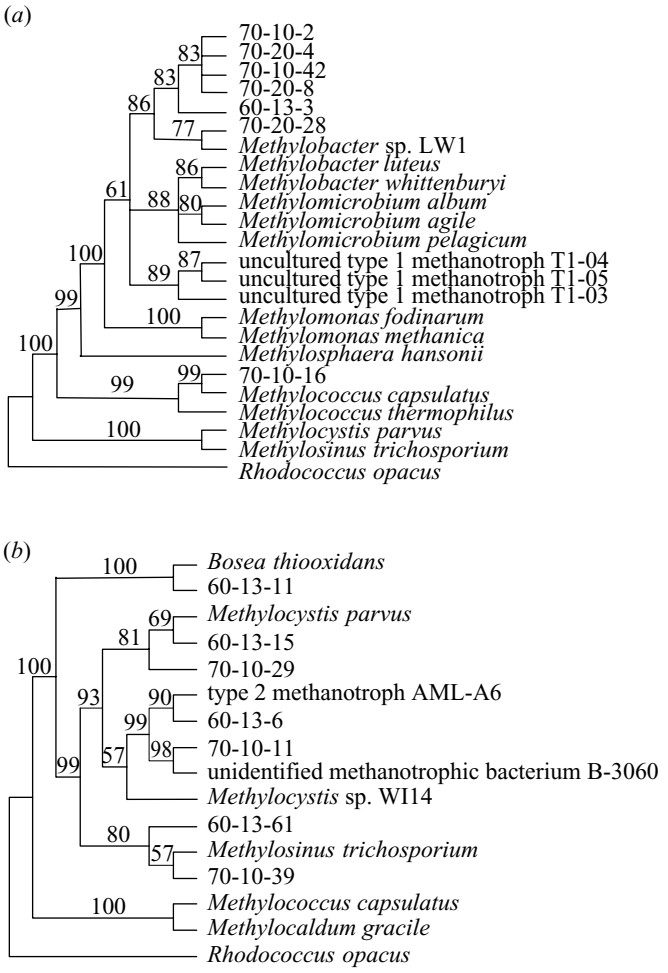


Figure 2. Phylogenetic trees of landfill soil methanotrophs. (*a*) Type 1 16S rRNA gene sequences. (*b*) Type 2 16S rRNA gene sequences. Trees were constructed with PAUP v. 4.0b8 using maximum parsimony. Bootstrap values are based on 100 replicates. *Rhodococcus opacus* was used as the outgroup.

*barkeri* (which can also use $H_2/CO_2$, methanol and methylamines as electron donors). Clone 70-10-14 is located in a deep branch, separate from either the hydrogenotrophic or the acetoclastic branches.

Rarefaction analysis indicated the likelihood that RFLP types were present in the GW60-13 library that were not represented among those clones sequenced, although most clones were accounted for in the other two samples (data not shown). No GW60-13 sequences clustered within the acetoclastic branch, although acetoclastic sequences in the GW60-13 clone library cannot be ruled out at this time.

### (**b**) *Methanotroph assemblage composition*

Type 1 methanotrophs were found in all samples (figure 2*a*), and most of these sequences clustered within a clade related to *Methylobacter* and not *Methylomicrobium*, as reported by a previous study on landfill methanotrophs (Wise *et al.* 1999). Rarefaction indicates that all probable unique RFLP types from both GW60-13 and GW70-10 clone libraries were analysed (data not shown).

Type 2 methanotroph phylotypes from GW70-10 and GW60-13 were broadly distributed among clades associated with *Methylocystis* and *Methylosinus* species (figure 2*b*), with no obvious bias of one sample for a particular clade. One sequence (60-13-11) did not cluster within known methanotrophs and probably arose from non-specific amplification. Four GW60-13 and three GW70-10 phylotypes were included in the analysis, and rarefaction predicted that these accounted for the richness of RFLP types in the individual libraries.

## 4. DISCUSSION

Efficient decomposition of MSW and detoxification of toxic organic compounds in landfills require the coordinated efforts of several trophic groups of anaerobic and aerobic bacteria. In an initial step to understand the individual processes that control the flow of carbon and energy in these systems, we have characterized assemblages of key microbial groups known to be involved in the production and consumption of the greenhouse gas methane and mineralization of other carbon compounds in landfill environments. Using samples taken from two locations of an MSW landfill, cloning and sequence analyses were coupled with rarefaction analysis to use as a guide to estimate the number of clones required to sequence all RFLP types present in our clone libraries and to provide an indication of the relative completeness of the analysis. In this study, this tool provided valuable information concerning the relative richness of methanogenic and methanotrophic populations in the landfill samples.

A rich assemblage of methanogens was found in the samples derived from three depths (figure 1), including representatives that cluster with either acetoclastic- or $H_2/CO_2$-using methanogens. The activities of methanotrophs may be a major factor in controlling landfill emissions not only of methane, a potent greenhouse gas, but also chlorinated solvents, commonly found in these environments. rDNA sequences characteristic of type 1 and type 2 methanotrophs were found in both samples (figure 2), despite the relatively deep sampling depths of 3 and 4 m. These positive results provided a strong indication of the effectiveness of using this method to detect methanotrophs throughout a landfill and not just in the cover soils where one expects a greater richness of these populations. According to the maximum-parsimony algorithm used in constructing figure 2, most type 1 sequences clustered within a clade that was different from those previously reported (Wise *et al.* 1999). When a neighbour-joining algorithm was used with these sequences, the two groups clustered together (data not shown). The significance of this difference, if any, is not understood at this time.

A diversity of methanogens and methanotrophs was observed in these landfill samples using this method of genetic analysis. These results represent the first basis for assessing the variation in communities throughout a landfill environment. While the links between the microbial groups and their capacity to transform hazardous chemicals with landfill geochemical conditions are not fully understood, the results of this study emphasize the need for further work to enable better prediction and control of carbon cycling in these environments.

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Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. 1990 Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Barlaz, M. A. 1997 Microbial studies of landfill and anaerobic refuse decomposition. In *Manual of environmental microbiology* (ed. C. J. Hurst, G. R. Knudsen, M. J. McInerney & L. D. Stetzenbach), pp. 541–557. Washington, DC: American Society for Microbiology.

Boeckx, P., Van Cleemput, O. & Villaralvo, I. 1996 Methane emission from a landfill and the methane-oxidizing capacity of its cover soil. *Soil Biol. Biochem.* **28**, 1397–1405.

Bogner, J., Meadows, M. & Czepiel, P. 1997 Fluxes of methane between landfills and the atmosphere: natural and engineered controls. *Soil Use Mngmt* **13**, 268–277.

Burggraf, S., Stetter, K. O., Rouviere, P. & Woese, C. R. 1991 *Methanopyrus kandleri*: an archaeal methanogen unrelated to all other known methanogens. *Syst. Appl. Microbiol.* **14**, 346–351.

Czepiel, P. M., Mosher, B., Crill, P. M. & Harriss, R. C. 1996 Quantifying the effect of oxidation on landfill methane emissions. *J. Geophys. Res.* D **101**, 16 721–16 729.

Genetics Computer Group (GCG) 1999 Wisconsin package v. 10.2. Madison, WI: GCG.

Keltjens, J. T. & Vogels, G. D. 1993 Conversion of methanol and methylamines to carbon dioxide and methane. In *Methanogenesis: ecology, physiology, biochemistry, and genetics* (ed. J. G. Ferry), pp. 253–303. New York: Chapman & Hall.

Lane, D. J. 1991 16S/23S rRNA sequencing. In *Nucleic acid techniques in bacterial systematics* (ed. E. Stackebrandt & M. Goodfellow), pp. 115–175. New York: Wiley.

Little, C. K., Palumbo, A. V., Herbes, S. E., Lidstrom, M. E., Tyndall, R. L. & Gilmer, P. J. 1988 Trichloroethylene biodegradation by a methane-oxidizing bacterium. *Appl. Environ. Microbiol.* **45**, 951–956.

Nozhevnikova, A. N., Nekrasova, V. K., Lebedec, V. S. & Lifshits, A. B. 1993 Microbiological processes in landfills. *Water Sci. Tech.* **27**, 243–252.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989 *Molecular cloning: a laboratory manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Swofford, D. L. 2001 *Phylogenetic analysis using parsimony (PAUP)*, v. 4.08b. Sunderland, MA: Sinauer.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. 1997 The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876–4882.

US Environmental Protection Agency (USEPA) 1999 *US methane emissions 1990–2020: inventories, projections and opportunities for reductions*. EPA 430-99-013.

Visvanathan, C., Pokhrel, D., Cheimchaisri, W., Hettiaratchi, J. P. A. & Wu, J. S. 1999 Methanotrophic activities in tropical landfill cover soils: effects of temperature, moisture content and methane concentration. *Waste Mngmt Res.* **17**, 313–323.

Whalen, S. C., Reeburgh, W. S. & Sandbeck, K. A. 1990 Rapid methane oxidation in a landfill cover soil. *Appl. Environ. Microbiol.* **56**, 3405–3411.

Wilson, J. T. & Wilson, B. H. 1985 Biotransformation of trichloroethylene in soil. *Appl. Environ. Microbiol.* **49**, 242–243.

Wise, M. G., McArthur, J. V. & Shimkets, L. J. 1999 Methanotroph diversity in landfill soil: isolation of novel type I and type II methanotrophs whose presence was suggested by culture-independent 16S ribosomal DNA analysis. *Appl. Environ. Microbiol.* **65**, 4887–4897.