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Isolation and characterization of refuse methanogens

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J.A. LADAPO AND M.A. BARLAZ. 1997. Four mesophilic, irregular, rod-shaped methanogenic bacteria were isolated from decomposing refuse recovered from laboratory-scale reactors and a municipal solid waste landfill. H_2/CO_2 was the only substrate on which the isolates could grow in a complex medium. Isolates grew between either 25° or 30° and 45°C and between pH 6 and 8. One isolate exhibited growth at pH 5. Growth of each isolate was enhanced by yeast extract and inhibited by anaerobic sewage sludge supernatant fluid. No isolate showed greater than 25% lysis on exposure to 1% sodium dodecyl sulphate (SDS) for 24 h, as is typical of methanogens with a proteinaceous cell wall. The physiological traits of the methanogens isolated here are similar to many previously characterized isolates.

INTRODUCTION

Approximately 188 million metric tons of municipal solid waste were generated in the US in 1993 and 63% of this material was disposed of by burial in landfills (Anon. 1994a). In addition to the 6600 existing landfills, numerous facilities were closed prior to 1986 (Anon. 1988). Methane is the terminal product of the decomposition of municipal solid waste in landfills. Its production from landfills is well documented (Barlaz *et al.* 1990), and it is recovered in commercial quantities from 119 sites in the US and Canada (Thorneloe and Pacey 1994). In addition to its potential as an energy source, methane is a greenhouse gas with a heat trapping potential about 20 times that of carbon dioxide (Khalil and Rasmussen 1989). Approximately 8% of global anthropogenic methane emissions are attributed to landfills (Anon. 1994b).

A complex series of biological and chemical reactions is initiated with the burial of refuse in a landfill. The process has been described in a sequence of four phases (Barlaz *et al.* 1989). Initially, oxygen entrained in the refuse at burial is depleted, and the system becomes anaerobic. Due to an imbalance among the fermentative, acetogenic and methanogenic activities, carboxylic acids typically accumulate once a landfill becomes anaerobic, resulting in an acidic pH. Over time, acetogenic and methanogenic activities increase, leading to vigorous methane production. Initially, methane production results from consumption of the accumulated soluble fermentation products. Later, methane production is dependent upon hydrolysis of cellulose and hemicellulose, the principal biodegradable components of refuse (Barlaz *et al.* 1990). In the presence of nitrate or sulphate, electrons will be diverted to these electron acceptors prior to and concurrent with methanogenesis, respectively (Barlaz *et al.* 1989).

Despite the presence of thousands of landfills in the US, their microbiology is poorly characterized relative to that of anaerobic sludge digesters (Mah and Sussman 1967; Chartrain and Zeikus 1986), the rumen (Bryant 1959; Miller *et al.* 1986) and rice paddies (Murase and Kimura 1994). Previous studies on micro-organisms which either participate in refuse decomposition or are present in landfills have been summarized (Pahren 1987; Barlaz *et al.* 1990). Recent work has established the presence of anaerobic protozoa in landfills which carry viable methanogens in their cysts (Finlay and Fenchel 1991). There have also been recent reports on both hydrolytic enzyme activity (Palmisano *et al.* 1993a) and the enumeration of micro-organisms (Palmisano *et al.* 1993b) in excavated refuse samples. Research on parameters that influence methane production has also been summarized (Barlaz *et al.* 1990). Higher moisture contents and pHs around neutral corresponded to samples with higher methane production rates in both laboratory studies and samples of refuse excavated from landfills (Suflita *et al.* 1992; Ham *et al.* 1993).

In contrast to the rumen and sludge digesters, retention times in landfills are essentially infinite. However, the ecosystem changes over time with respect to pH and the nature of the available carbon. Initially, soluble fermentation inter-

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mediates are readily available, while later, methane production is dependent upon hydrolysis of complex carbohydrates. In previous work, a four-order of magnitude increase in the most probable number (MPN) of hydrogenophilic and acetoclastic methanogens was observed, while the pH remained below 6·2 (Barlaz *et al.* 1989). This, plus the documentation of methane production from full-scale landfills, suggested that either acidophilic or acid-tolerant methanogens may dominate, or at least be present in decomposing refuse. In previous reports of methanogens isolated from landfills, seven methanogens were isolated and identified as *Methanobacterium* sp., *Methanosarcina barkeri* and an unidentified *Methanococcus* sp. (Fielding *et al.* 1988). In addition, five species have been identified in landfill leachate by the use of species-specific probes: *Methanoculleus bourgense*, *Methanobacterium formicicum*, *Methanosarcina barkeri*, *Methanosphaera stadtmanae* and *Methanobrevibacter ruminantium* (Maule *et al.* 1994). The objective of this research was to isolate methanogens active during refuse decomposition and to evaluate the extent to which acidophilic or acid-tolerant methanogens are present in decomposing refuse. In this paper, an initial characterization of four methanogens isolated from both laboratory-scale and full-scale landfills is presented.

MATERIALS AND METHODS

Inoculum formation

Inocula for methanogen enrichments for the isolation work were obtained from decomposing refuse excavated from a landfill and from two laboratory-scale reactors. The landfill sample, designated LF, was excavated from a section of the Wilder's Grove landfill (Raleigh, NC) containing refuse buried prior to 1980 and known to be actively producing methane. The refuse was collected with a hand auger after removing about 2·1 m of overlying soil. The sample was placed in a refuse bag and flushed with N_2 for about 30 min before transport to the laboratory for immediate processing. Inocula were also produced from two laboratory-scale reactors initially filled with solid waste. The decomposition history of these reactors has been presented elsewhere (Rhew and Barlaz 1995). Reactor 6 (R6) produced methane at pH 7, while reactor 9 (R9) produced methane at pHs between 5·5 and 6. Thus, it was thought that R9 would be an excellent inoculum for enrichment of acidophilic or acid-tolerant methanogens.

The inoculum was prepared by blending 100 g of each sample in 23 mmol l^{-1} phosphate buffer (pH 7·0) in an autoclaved, N_2-sparged Waring blender equipped with a 3·8 l stainless steel jar as described previously (Barlaz *et al.* 1989). The resulting slurry was then hand-squeezed (hands covered with gloves and cleaned in methanol) to produce a liquid inoculum. This liquid served as the inoculum for methanogenic enrichments that began in February 1993 for the laboratory-scale refuse samples and August 1993 for the landfill sample.

Media

The composition of the basal medium was modified from that used previously (Barlaz *et al.* 1989) and is presented in Table 1. With reference to Table 1, the pH was adjusted to 5·5 or 7·0 with concentrated HCl or NaOH after addition of the deionized water to the medium. The medium was then boiled under oxygen-free N_2 and reduced with L-cysteine hydrochloride. The reduced medium was then dispensed into 26-ml anaerobic culture tubes in 9-ml aliquots and sealed with butyl-rubber stoppers and aluminium crimps (Belco Biotechnology Inc., Vineland, NJ) in an anaerobic glove box maintained in an atmosphere of 95% N_2/5% H_2. The tubes were then autoclaved for 30 min. After cooling, various growth substrates were added to the individual tubes as described below. Sodium sulphide, at a final concentration of 0·5 g l^{-1}, was added to the medium before inoculation. All enrichment experiments were conducted by using basal medium supplemented with yeast extract and trypticase peptone,

Table 1 Medium composition

Component	per l
PO_4 solution*	100 ml
M3 solution†	100 ml
Mineral solution‡	10 ml
Vitamin solution§	10 ml
Resazurin (0·1%)	1 ml
$NaHCO_3$	1 g
Deionized water	768 ml
Cysteine hydrochloride (5%)‖	10 ml

* The phosphate solution contained 16·1 g of KH_2PO_4 and 31·89 g of $Na_2HPO_4 . 7H_2O$ l^{-1}. It was prepared in carbonate-free water and stored under N_2 at 4°C.

† The M3 solution contained (l^{-1}): NH_4Cl, 10 g; NaCl, 9 g; $MgCl_2 . 6H_2O$, 2 g; $CaCl_2 . 2H_2O$, 1 g.

‡ As given by Kenealy and Zeikus (1981) (l^{-1}): nitrilotriacetic acid, 1·5 g; $FeSO_4 . 7H_2O$, 0·1 g; $MnCl_2 . 4H_2O$, 0·1 g; $CoCl_2 . 6H_2O$, 0·17 g; $CaCl_2 . 2H_2O$, 0·1 g; $ZnCl_2$, 0·1 g; $CuCl_2$, 0·02 g; H_3BO_3, 0·01 g; $NaMoO_4.2H_2O$, 0·01 g; NaCl, 1·0 g; Na_2SeO_3, 0·016 g; $NiSO_4 . 6H_2O$, 0·026 g, with the addition of 0·033 g $Na_2WO_4 . 2H_2O$ l^{-1}.

§ As given by Wolin *et al.* (1963) (l^{-1}): biotin, 2 mg; folic acid, 2 mg; pyridoxine hydrochloride, 10 mg; riboflavin (B_2), 2 mg; pantothenic acid, 5 mg; vitamin B_{12}, 0·1 mg; *p*-aminobenzoic acid, 5 mg; thioctic (lipoic) acid, 5 mg.

‖ Added after adjustment of the media to pH 7·2 and boiling under N_2.

each at a final concentration of 0·5 g l^{-1}. The basal medium plus these supplements will be referred to as enrichment medium. The enrichment medium pH was adjusted to the desired final pH, as appropriate.

Media were supplemented with either 202·6 kPa of an 80:20 H_2/CO_2 mixture or 20 mmol l^{-1} sodium acetate for the enrichment and isolation procedures. Supplements were added after autoclaving the medium. Isolates were tested for growth on a number of additional substrates. In each case, substrates were filter-sterilized (0·2 μm) and kept in the anaerobic chamber for 48 h before they were added to sterile basal medium at a final concentration of 20 mmol l^{-1}.

A contaminant medium was used to verify that the pure methanogen cultures could not grow in a medium that supports the growth of a wide range of fermentative microorganisms. This medium contained (l^{-1}): trypticase soy broth, 30 g; yeast extract, 1 g; glucose, 5 g; and resazurin and cysteine hydrochloride at the same concentrations as given in Table 1.

Enrichment and isolation techniques

Enrichments were conducted with either H_2/CO_2 or sodium acetate as the growth substrate. Tubes containing H_2/CO_2 were repressurized weekly. Enrichments with both substrates were conducted in media adjusted to both pH 5·5 and 7·0. Selected tubes of media were checked before inoculation and after 30 d of incubation to verify that the media pH was not altered during autoclaving or after growth. The media pH did not vary by more than $\pm$0·2 pH units throughout the experimental period. All enrichment studies were conducted at 37°C.

Liquid inocula were serially diluted in phosphate buffer (pH 7, 23 mmol l^{-1}) and inoculated into three tubes at each dilution to measure the MPN of the initial inoculum. Tubes were checked for methane synthesis after 30 d by gas chromatography (Gow-Mac, Bridgewater, NJ, and Shimadzu, Columbia, MD). Positive results were recorded if tubes contained greater than 1% methane. Methanogenic MPN populations were calculated by using published tables (de Man 1975).

Cultures that were positive for methane were used to further enrich for methanogens. The highest positive dilution for each enrichment condition was serially diluted and transferred to fresh medium. After seven enrichment sequences over 9 months, final enrichments were made in enrichment medium supplemented with a mixture of filter-sterilized (0·2 μm) benzyl penicillin and vancomycin at 20 and 40 μl ml^{-1} (Florencio *et al.* 1993).

Enrichment cultures from the tubes containing antibiotics were grown on plates by diluting the highest positive dilution for each enrichment condition in phosphate buffer in the anaerobic glovebox. A 0·1 ml aliquot was plated onto the enrichment medium supplemented with 2% noble agar (Difco Laboratories, Detroit, MI). Plates were incubated in a pressurizable canister at 37°C for 30 d. The H_2/CO_2 canister was pressurized with H_2/CO_2 (202·6 kPa), while the acetate canister was pressurized with N_2. The H_2/CO_2 canister was repressurized as necessary. Broth-grown cultures were inoculated each time an enrichment culture was inoculated onto plates. This broth was checked for methane before canisters were checked for colonies. Once methane was present in the broth tubes, usually after about 14 d, plates were checked for growth. Single colonies were picked with sterile needles into acetate or H_2/CO_2 broth and again plated onto noble agar. The plating procedures in agar and broth were repeated several times until isolates were considered pure. Purity of strains was established by (1) light microscopy, (2) the absence of growth in contaminant medium, and (3) streaking isolates on plates and observing homogeneous colonial morphology. Initial attempts to isolate methanogens in Difco nutrient agar were unsuccessful as no isolates picked from such agar synthesized methane in acetate or H_2/CO_2 broth.

All characterization of isolates was conducted at pH 7 and 37°C with H_2/CO_2, as no acetoclastic isolates were confirmed. Of course, pH and temperature were varied in experiments to identify the response of isolates to these parameters. With the exception of the nutrient amendment study, enrichment medium was used for all characterization work.

Growth rate experiments

Growth rate experiments were conducted to determine the pH and temperature range of each isolate, to determine the range of substrates that each isolate could use for growth, and to evaluate the effect of media supplements. Growth was measured by an increase in absorbance at 660 nm (A_{660}) and verified by measurement of methane formation at the termination of each growth rate experiment. Maximum growth rates were determined from the linear portion of a plot of A_{660} *vs* time. All growth measurements were conducted in triplicate. To initiate a growth experiment, 0·1 ml of logarithmic-phase culture was added to 9 ml of media in the presence of H_2/CO_2.

Growth substrates tested included sodium formate, methanol, sodium acetate and trimethylamine. In addition to growth substrates, complex carbon sources were added separately to the basal medium to determine whether they stimulated growth of each isolate on H_2/CO_2. Acetate, formate and trimethylamine were added at 20 mmol l^{-1}. Yeast extract and trypticase peptones were added at a final concentration of 0·5 g l^{-1}, and anaerobically digested sewage sludge was added at 10% (v/v). Sludge was obtained from the Orange County (NC, USA) wastewater treatment plant. Sludge was centrifuged at 1600 *g* for 30 min in a refrigerated centrifuge. The supernatant fluid was subsequently filtered through What-

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man no. 1 filter paper and a 0·2 μm membrane filter. Aliquots were then degassed with N_2 and stored at −20°C until use.

Sensitivity to sodium dodecyl sulphate

Sensitivity to sodium dodecyl sulphate (SDS) was tested by the addition of 0·1 ml of a 1% SDS solution to 5 ml of early stationary growth cultures. Lysis was measured by a decline in A_{660}. Measurements were taken every 15 min for the first 2 h and hourly for the next 22 h.

Microscopy

A Nikon Optiphot-2 epifluorescence microscope, equipped with a BV-2B filter set (excitation at 400–440 nm, emission at 475 nm), was used to examine the morphology and fluorescence of each isolate.

RESULTS

Methanogen populations in initial enrichments

The MPN of methanogens from each inoculum is presented in Table 2. With the exception of the R6 inoculum enriched on acetate, MPNs were two to seven orders of magnitude greater at pH 7 relative to pH 5·5. MPNs for R9 at pH 5·5 were well below the corresponding MPNs at pH 7 for both acetate and H_2/CO_2. While inhibition of methanogenesis at pH 5·5 relative to pH 7 is well known (Zinder 1993), the occurrence of this inhibition in the R9 inoculum but not in the R6 inoculum is surprising. This is because the reactor from which the R9 inoculum was derived produced substantial methane at pHs of 5·5–6·0 (Rhew and Barlaz 1995). In contrast, the inoculum from which the R6 inoculum was derived only produced methane at pHs about 7. In all cases the primary inocula contained substantial methanogenic populations from which to begin enrichments. The pH 7 MPNs for R9 on both H_2/CO_2 and acetate were surprisingly high.

Table 2 MPN of methanogens in primary enrichments (cells ml^{-1} of inoculum)

Inoculum	Acetate pH 5·5	Acetate pH 7	H_2/CO_2 pH 5·5	H_2/CO_2 pH 7
R6	$4{\cdot}3\times10^{9}$	$2{\cdot}4\times10^{8}$	$2{\cdot}1\times10^{8}$	$5{\cdot}5\times10^{10}$
R9	$2{\cdot}1\times10^{5}$	2×10^{12}	$5{\cdot}7\times10^{6}$	$2{\cdot}3\times10^{12}$
LF	$2{\cdot}9\times10^{5}$	$5{\cdot}9\times10^{8}$	$3{\cdot}5\times10^{6}$	$6{\cdot}3\times10^{10}$

Isolates

Four isolates were obtained in pure culture and will be referred to as follows: R6H7, R9H7 and LFH7 isolated from pH 7 enrichments from reactors R6 and R9, and the landfill sample, respectively, and R9H5, isolated from reactor R9 in a pH 5·5 enrichment. R9H5 was unable to grow on either Difco or noble agar at pH 5·5. However, it grew in broth at pH 5·5 and on noble agar at pH 7·0. Its purity was verified after growth on agar at pH 7·0. The growth of methanogenic isolates in broth but not on plates under acidic conditions has also been reported for peat bog enrichments (Williams and Crawford 1985).

Colonial and cellular morphology

When grown on plates, colonies incubated about 15 d were about 1 mm in diameter with a smooth, circular edge. Colonies of R6H7 and LFH7 were cream to light yellow in colour after about a 15 d incubation on noble agar. R9H7 and R9H5 yielded deep yellowish pigmented colonies after a 21 d incubation. Further incubation of the R9H7 and R9H5 isolates showed smooth to rough edged colonies with a slightly raised centre. The viscosity of these colonies varied from mucoid to wet.

Microscopic examination of each strain showed non-motile and non-sporing rods of 1·5–3·6 μm in length and 0·2–0·5 μm in diameter as illustrated in Fig. 1. R6H7 isolates were medium-straight rods of about 0·3 μm in diameter and 2·1 μm in length. R9H7 cells were long, blunt rods that formed chains. They measured about 0·4 μm in diameter and 3·6 μm in

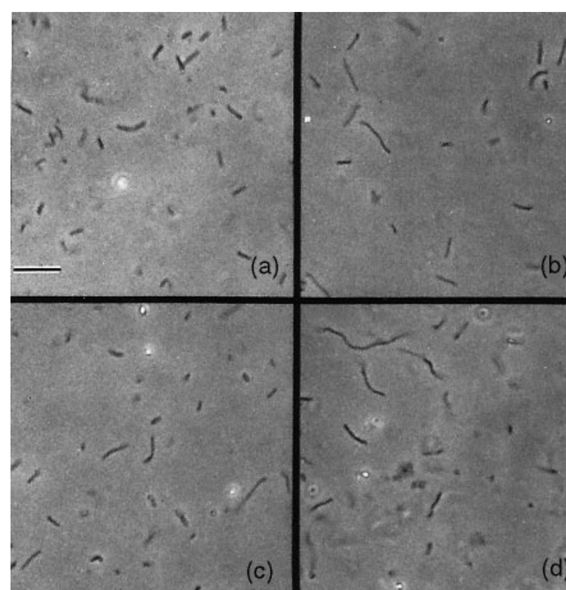

Fig. 1 Phase contrast micrographs of the isolates: (a) R6H7; (b) R9H7; (c) LFH7; (d) R9H5. Pictures were taken after growth for 8 d on H_2/CO_2. The bar represents 10 μm

length. LFH7 cells appeared as single short rods of about 0·2 μm in diameter and 1·6 μm in length. R9H5 cells were single-bent medium-sized rods of about 0·3 μm in diameter and 2·5 μm in length. All isolates stained as Gram-negative rods.

The wet preparations of isolates were also visualized by epifluorescence microscopy. The unstained preparation exhibited weak autofluorescent rods that faded quickly from field to field. Such autofluorescence has been a distinguishing characteristic of methanogens because of their unique cofactors (Doddema and Vogels 1978; Patel 1984; Kataoka *et al.* 1991). Examination of acridine orange-stained preparations of the isolates showed deep bluish-green fluorescence of varying intensity.

pH growth range

Isolates were checked for their ability to grow between pH 5·0 and 8·5 and their maximum growth rates over this pH range are presented in Fig. 2. R6H7 and LFH7 showed optimum growth at pH 7·0, while R9H7 showed an optimum growth at pH 7·5. The maximum growth rates were 0·018, 0·018 and 0·017 h^{-1} for R6H7, R9H7 and LFH7, respectively. They all grew between pH 6·0 and 8·0. Isolate R9H5 grew between pH 5 and 8·0, with a pH optimum at 6·5 and a μ_{max} of 0·016 h^{-1}.

Temperature growth range

The optimum temperature of isolates was determined in broth under H_2/CO_2 at pH 7·0 (Fig. 3). R6H7 grew between 25° and 45°C, with an optimum at 40°C where its maximum growth rate was 0·016 h^{-1}. The optimum growth temperature for R9H7 and LFH7 was 35°C and the maximum growth rates were 0·021 and 0·019 h^{-1}, respectively. Both isolates grew well between 30° and 45°C and R9H7 exhibited growth as low as 25°C. R9H5 grew well between 30° and 45°C, with an optimum at 40°C and a maximum growth rate of 0·018 h^{-1}.

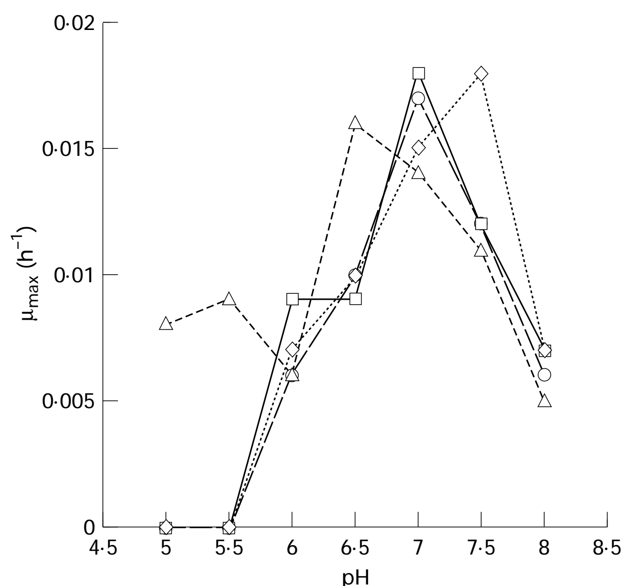

Fig. 2 Maximum growth rate of each isolate *vs* pH. Tests were initiated by inoculation of 0·1 ml of a log-phase culture into fresh media pressurized with H_2/CO_2. □, R6H7; ◇, R9H7; ○, LFH7; △, R9H5

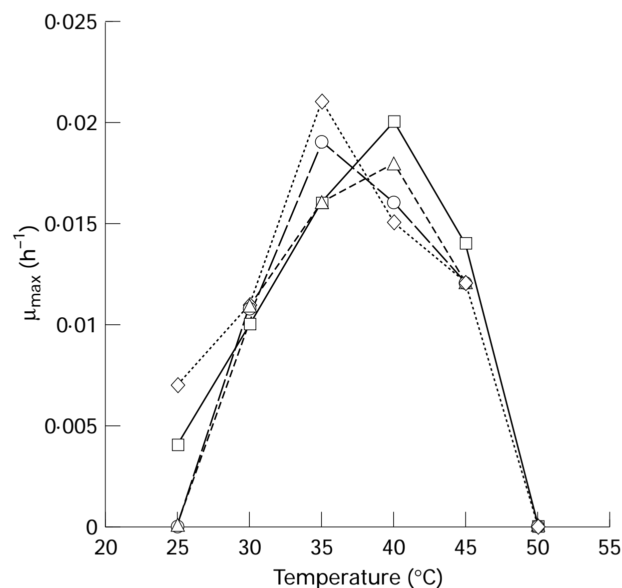

Fig. 3 Maximum growth rate of each isolate *vs* temperature. Tests were initiated by inoculation of 0·1 ml of a log-phase culture into fresh media pressurized with H_2/CO_2. □, R6H7; ◇, R9H7; ○, LFH7; △, R9H5

Sensitivity to sodium dodecyl sulphate

The isolates were not uniform in their sensitivity to SDS. Although none of the early stationary phase cultures showed any immediate lysis as evident in a proteinaceous cell wall (Harris *et al.* 1984; Boone and Whitman 1988), a delayed lysis of minor intensity was observed. Isolate R9H7 showed about 20% lysis within 1 h of SDS addition, while the other isolates showed about 20% lysis 3 h after SDS addition (data not shown). No isolate showed greater than 25% lysis after 24 h.

Substrate range and effects of media supplements on isolates

Isolates did not grow within 60 d on sodium formate, methanol, sodium acetate and trimethylamine (TMA). Experiments were also conducted to evaluate the effects of acetate, formate, TMA, yeast extract, anaerobic sludge supernatant and trypticase peptones on growth on H_2/CO_2. The maximum specific growth rate for each condition is presented

in Table 3. Yeast extract stimulated and anaerobic sewage sludge inhibited the growth rate of all isolates. The effects of other supplements were less consistent amongst isolates.

DISCUSSION

Comparison of the MPNs on acetate and H_2/CO_2 (Table 2) shows that H_2-utilizing populations were higher in all cases except the R6 inoculum at pH 5·5. Methanogen MPN data for fresh refuse samples have shown samples with equivalent acetate- and H_2-utilizing populations (Barlaz *et al.* 1989) as well as samples in which the H_2-utilizing population was greater (Qian and Barlaz 1996). Populations in excavated refuse samples contained H_2-utilizing populations 100 times greater than acetoclastic populations (Fielding and Archer 1986) and this is consistent with the results reported here.

Although most of the enrichments showed acetoclastic methanogens in broth, no pure acetoclastic isolates were obtained using either Difco or noble agar. This contrasts with Fielding *et al.* (1988), who isolated an acetoclastic methanogen (*Methanosarcina barkeri*) in one of their five landfill samples. Despite the high population of methanogens in the initial pH 5·5 MPNs (Table 2), it was not possible to isolate an acidophilic methanogen on plates. While acidophilic methanogens have been found in other niches (Williams and Crawford 1985; Patel *et al.* 1990; Maestrojuan and Boone 1991; Florencio *et al.* 1993), no report of a pure acidophilic isolate from the refuse habitat was found in the literature.

One explanation for the low morphological variation of the isolates was that these strains were prevalent during refuse decomposition in landfill and laboratory-scale reactors. Another explanation may be that the enrichment techniques used in this study favoured methanogenic rods over other morphological types (coccus, sarcinae). To confirm either situation, Gram stains of a refuse extract and highest positive MPN dilution tubes were prepared. The refuse extract showed as many rods and cocci on the slide. However, the MPN tube Gram stain reactions showed five times as many rods as cocci in almost every field. This suggests that the enrichment method may have supported the growth of rods over other morphological types. Alternatively, the cocci in the refuse extract and highest MPN dilution tubes may have been non-methanogens.

Table 3 Maximum specific growth rate of isolates in the presence of media supplements (h^{-1})

Metabolite added	Isolate R6H7	R9H7	LFH7	R9H5
None (control)	0·015	0·013	0·014	0·014
Sodium acetate	0·014	0·015*	0·015	0·014
Sodium formate	0·009*	0·012	0·014	0·013
Trimethylamine	0·012*	0·012	0·011*	0·014
Yeast extract	0·019*	0·018*	0·20*	0·016*
Anaerobic sludge	0·008*	0·010*	0·007*	0·008*
Trypticase peptone	0·016	0·016*	0·015	0·016

Experiments were conducted at pH 7 and 37°C in the presence of H_2/CO_2 as the growth substrate. Data marked with an asterisk are significantly different from the control at the 99% confidence level using the method of least significant differences (Milliken and Johnston 1992).

The pH behaviour of the isolates in this study was similar to that of earlier refuse-methanogen isolates (Fielding *et al.* 1988). Although R9H5 was enriched at pH 5·5, its growth behaviour indicates it to be acid tolerant rather than acidophilic. Production of methane at low pH is essential for landfills to progress from the anaerobic acid phase to the methane producing phase. Thus, the presence of an acid-tolerant organism is consistent with previous observations regarding methanogen population increases at low pH in laboratory reactors (Barlaz *et al.* 1989).

The optimal temperature for methane production from refuse has been reported to be 41°C (Hartz *et al.* 1982) and the laboratory-scale reactors were maintained at about 40°C. Thus, it is not surprising that the isolates exhibited temperature optima of 35–40°C.

Reduction of CO_2 to CH_4 with H_2 as the electron donor is the most common methanogenic reaction (Zinder 1993). Thus, that H_2/CO_2 served as the only growth substrate is not unique to these isolates. For example, other investigators have reported similar findings for *Methanocorpusculum labreanum* and *Methanococcus jannaschii* (Jones *et al.* 1983; Yizhang *et al.* 1989).

Anaerobic sewage sludge supernatant fluid was inhibitory to all isolates which is somewhat surprising given that the isolates originated in an analogous ecosystem with a wide variety of organic matter. In contrast, *Methanobacterium espanolae* and *Methanosaeta concilii* were stimulated by primary sludge supernatant fluids (Patel 1984; Patel *et al.* 1990). Metals have been shown to inhibit methanogenic consortia in other studies (Hickey *et al.* 1989; Lin 1992, 1993). Unfortunately, metal concentrations were not measured in the sludge supernatant fluids.

The complex nature of yeast extract does not permit speculation as to why it is more stimulatory than other supplements. It has been reported that distilled casamino acids, a less complex substrate, still contained 0·029–1·30 mmol l^{-1} acetate (Ladapo and Whitman 1990). Thus, a more complex undistilled substrate such as yeast extract will contain at least a similar amount of acetate. However, only one isolate was stimulated by acetate alone, suggesting that other components of yeast extract enhanced the growth of these isolates. These results contrast with the behaviour of *Methanobacterium espanolae*, which is inhibited by yeast

extract and tryptone (Patel *et al.* 1990). These authors did not speculate on the mechanism of this inhibition.

In summary, a limited characterization of four methanogens isolated from decomposing refuse shows the isolates to be similar to commonly characterized methanogens from other ecosystems. Further work is required to determine whether the isolates in this study possess unique physiological characteristics. This is only the second report of methanogens isolated from refuse and this work will lead to further investigation of the role of methanogenic bacteria and other microbes in the refuse ecosystem.

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