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Inference of Methanogenic Bacteria in Wastewater Digestor Sludges by Lipid Component Analysis

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The presence of methanogens of the genera *Methanosarcina* and *Methanosaeta*, and the family *Methanomicrobiaceae*, and the absence of the family *Methanobacteriaceae* were inferred from the analysis of lipid component parts in three kinds of sludge from two anaerobic digestors by comparison with the distribution of the lipid component parts among 31 species of methanogens previously reported.

[**Key words:** methanogen, ether lipid, wastewater treatment digestor, *Methanosarcina, Methanosaeta, Methanomicrobiaceae*]

One of the most important aspects in studying the ecology of methanogens in natural or artificial methanogenic consortia is to determine the variety of methanogens present in the ecosystem. For this purpose the immunologic method developed by Conway de Macario *et al.* (1) and the method using group-specific oligonucleotide probes to detect the 16S rRNA gene have been applied to the analysis of methanogen florae in wastewater digestor sludge consortia (2) and in natural samples (3; Torsvik, T. *et al.*, Abstr. International Workshop on Molecular Biology and Biotechnology of Extremophiles and Archaebacteria, p. 131, 1993). The present note reports the first attempt to identify methanogen groups in sludge samples based on the application of lipid component parts analysis. The presence of ether polar lipids is one of the most distinctive characteristics that distinguishes methanogens from eubacteria and eukaryotes. After the complete structures of lipids of seven species of methanogens were determined by several groups (4), we reported the distribution of the component parts of ether polar lipids in 31 species of methanogens and concluded that methanogens of a family or in some cases a genus have a particular combination of lipid component parts (5). This method could, in turn, be applied to inference of methanogenic groups present in an ecosystem. The nomenclature of archaebacterial lipids proposed by Nishihara *et al.* (6) is used in this text.

Three sludge samples were removed from two bench-scale fixed-bed digestors [one mesophilic (37°C, digestor A) and one thermophilic (55°C, digestor B)] for the anaerobic treatment of beet sugar-plant Steffen wastewater discharged after extraction of sucrose from beet molasses. The digestors (5 *l*) contained a number of fiberglass cages (60 cm length × 2 cm diameter) as supports on which methanogens and other microorganisms were fixed. The hydraulic retention times in digestors A and B were 24 h and 12 h, respectively. Sample I was a mixture of fluid phase and sludge scraped from the immobilized phase from the digestor A. Sample II was from the fluid fraction of digestor B. The microorganisms fixed on the cages in digestor B were scraped off with a toothbrush with the aid of a small amount of water (sample III). Lipids were extracted from the pellets collected by centrifugation of the sludge samples as described (7) with a trichloroacetic acid-acidified solvent. Lipids (plasmalogen-type phospholipids and diacyl polar lipids) from anaerobic eubacteria in anaerobic sludges were easily broken down by the acidic extraction and mild alkaline methanolysis (8) of the extract. Because the ether lipids of methanogens are resistant to both treatments, we can assume the lipids remaining intact after both treatments to be ether lipids from methanogens. Two-dimensional thin-layer chromatography was performed as described previously (7). Ether lipid components were analyzed as described previously (5). α-Hydroxyarchaeol and β-hydroxyarchaeol found in *Methanosaeta* (formerly *Methanothrix*) *concilii* and *Methanosarcina barkeri*, respectively, were not distinguished in this report. Sludge samples were observed with a scanning electron microscope JSM-T-300 (Japan Electron Optics Laboratory, Tokyo) operated at 10 kV after fixation, dehydration, critical-point drying and vacuum evaporation of platinum for scanning electron microscopy.

The results of lipid component parts analysis of the three samples are shown in Table 1 together with the distribution of the component parts in the relevant known methanogen groups (5). The limit of detection of this analysis (150 μg per glycerophosphoester spot on a thin-layer cellulose plate) was 10 mg of cells from one species. We inferred the presence of particular methanogen groups in each sample sludge from the sum of the results.

Sample I The presence of glucosaminylinositol strongly suggested the presence of *M. barkeri* in this sludge. The detection of hydroxyarchaeol core and the polar head groups (inositol, ethanolamine, and serine) supported it. Because of the detection of mannose in glycolipids, we could conclude that *M. concilii* was a component of this methanogenic consortium. The presence of inositol, ethanolamine, and galactose, which are found in *M. concilii*, also supported the above conclusion. The presence of the mesophilic filamentous methanogen *M. concilii* is consistent with the fact that sample I was taken from a mesophilic fixed-bed digestor. Because aminopentanetetrol is characteristic to the members of the family *Methanomicrobiaceae*, we could infer

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TABLE 1. Composition of ether lipid cores, glycolipid sugars, and phospholipid polar head groups of ether polar lipids of methanogens in sludge samples and known methanogen groups (5)

	Sample I	Sample II	Sample III	*Methanobacteriaceae*	*Methanococcus* (mesophilic)	*Methanococcus* (thermophilic)	*Methanomicrobiaceae*	*Methanosarcina barkeri*	*Methanosarcinaceae*[a]	*Methanosaeta concilii*	*Methanosaeta thermophila*
Core lipid											
Archaeol	+	+	+	+	+	+	+	+	+	+	+
Caldarchaeol	+	+	+	+	−	+	+	−	−	−	+
Hydroxyarchaeol	+	+	+	−	+	±	−	+	+	+	−
Glycolipid sugar											
Glucose	+	+	+	+	+	+	+	+	+	+	−
Galactose	+	+	+	−	−	−	+	−	−	+	+
Mannose	+	−	+	−	−	−	−	−	−	⊕	−
Phospholipid polar head group											
Inositol	+	+	+	+	−	−	−	+	+	+	+
Ethanolamine	+	+	+	+	−	+	−	+	+	+	+
Serine	+	+	+	±	+	+	−	+	±	−	−
Glycerol	+	+	+	−	−	−	+	+	+		−
Aminopentanetetrol	+	−	+	−	−	−	⊕	−	−	−	−
Glucosaminylinositol	+	+	+	−	−	−	−	⊕	−	−	−
Glucosyl caldarchaetidylinositol	−	−	−	⊕	−	−	−	−	−	−	−

[a] Except *M. barkeri* and methanogens of the genus *Methanosaeta*.
+, Present; −, absent; ⊕, signature lipid (component part) of the methanogen group.

FIG. 1. Scanning electron micrographs of mesophilic sludge A (sample I). Packet-shaped cells characteristic to methanogens of the genus *Methanosarcina* (A) and filamentous cells that resemble *Methanosaeta* cells (B) can be seen. Bars in A and B indicate 5 μm and 10 μm, respectively.

the presence of the methanogens of this family from the presence of this polar group of ether lipids. This inference was supported by the presence of galactose and caldarchaeol. Caldarchaeol is also a major core lipid of the members of the families *Methanobacteriaceae* and *Methanothermaceae*, and the thermophilic members of genera *Methanococcus* and *Methanosaeta*. Because we did not detect in this sludge gentiobiosyl caldarchaetidylinositol which is a signature phosphoglycolipid of *Methanobacteriaceae* (9), the presence of methanogens of this family was excluded. The other candidates for the origin of caldarchaeol were all thermophilic methanogens, which were not likely present in this mesophilic digestor. The presence of hydroxyarchaeol, glucose, and serine may suggest the presence of the mesophilic members of *Methanococcus*. These lipid component parts, however, were explained by the presence of other methanogens as described above and there is no specific evidence for the presence of the mesophilic methanogens of the genus *Methanococcus*. The presence of *Methanococcus* species in this sludge was not consistent with their original habitat (marine environment) from which all the methanogens of this genus have been isolated so far. The presence of *Methanosarcina* and *Methanosaeta* species was confirmed by electron microscopic observation. Figure 1 shows scanning electron micrographs from different fields of view of this sludge, which show cell shapes characteristic to *Methanosarcina* (packet-shaped cells, Fig. 1A) and *Methanosaeta* (filamentous cells, Fig. 1B) species.

Sample II The presence of methanogens of the genus *Methanosarcina* was inferred from the same basis as for sample I. We have found that the thermophilic species of *Methanosaeta thermophila* contains galactose as the sole glycolipid sugar and a caldarchaeol core in its cellular lipids, and we have found the absence of mannose in the species in contrast to the mesophilic *Methanosaeta* species (5). The presence of galactose and caldarchaeol, and the absence of mannose in sample II from a thermophilic fixed-bed digestor could be reasonably explained by the presence of *M. thermophila*. The presence of inositol and ethanolamine was consistent with the presence of a member of the genus *Methanosaeta*. Although caldarchaeol and galactose are components of lipids of the members of the family *Methanomicrobiaceae*, it was concluded that methanogens of the group were absent from the lack of aminopentanetetrol in this sample. Considering this situation, it was concluded that galactose was only derived from *Methanosaeta*. As in sample I, the lack of gentiobiosyl caldarchaetidylinositol suggested the absence of any members of the family *Methanobacteriaceae*.

Sample III The presence of the members of *Methanosarcina* was presumed from the same 5 kinds of lipid component parts as in sample II. *M. thermophila* was also assumed to be present in this sample based on the similar results of lipid component analysis as in sample II except for the presence of mannose. Because

mannose is the signature lipid component of mesophilic *M. concilii* as mentioned above, we might conclude either that mesophilic *M. concilii* was present in this thermophilic sludge or that a new thermophilic methanogen containing mannose in its glycolipids was present. The members of the family *Methanomicrobiaceae* were presumed to be present because of the presence of aminopentanetetrol in sample III (immobilized fraction) in contrast to sample II (the fluid fraction). As in samples I and II, the absence of the members of the family *Methanobacteriaceae* was suggested in this sample by the lack of gentiobiosyl caldarchaetidylinositol.

Possible methanogenic florae of three samples from two fixed-bed Steffen wastewater treatment digestors were inferred mainly from lipid component parts analysis. The results showed the presence of very similar methanogen florae in the two digestors but also showed slight differences, for example, the presence or absence of the members of the family *Methanomicrobiaceae* in the immobilized and fluid fractions of the thermophilic digestor, and the presence of mesophilic and thermophilic members of the genus *Methanosaeta* depending on the operating temperature of the digestors. The similarity might be mainly due to use of the same feedstock. The differences between the two digestors might be due to the differences in the operating temperature and the hydraulic retention time.

This is the first report to our knowledge stating that the characteristic distribution of polar ether lipids of methanogens can be applied to the analysis of the methanogen flora in an ecosystem composed of many kinds of microorganisms. In principle this method can be used to infer the possible presence of methanogen groups in a sludge from discretionary combinations of the detected lipid component parts. However, some components that are specifically found in only one group of methanogens, for example, aminopentanetetrol, glucosaminylinositol, and mannose (5, 9) could be characteristic marker lipid components, each of which could indicate the presence of a specific methanogen group.

Because lipid components are characteristic to the family or genus of methanogens, the present method can be used to infer the presence of methanogen groups at the family or genus but not at the species level. Direct immunologic analysis of methanogen florae (2, 3) can be used to detect methanogens at the species level but no antiserum can recognize an entire family. A polyclonal antiserum of restricted specificity spectrum will detect methanogens that are not identical but that share antigens with that used for immunization. The same antiserum will not detect methanogens that are related to the immunizing strain, but with which they do not share any of the antigens recognized by the antiserum. The immunologic analysis, detection of a signature sequence of 16S rRNA using a polymerase-chain-reaction amplification method, and the present method with low specificity may complement each other.

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