Establishment and Characterization of an Anaerobic Thermophilic (55°C) Enrichment Culture Degrading Long-Chain Fatty Acids

IRINI ANGELIDAKI AND BIRGITTE K. AHRING*

Institute of Environmental Science and Engineering, The Technical University of Denmark, DK-2800 Lyngby, Denmark

Received 12 December 1994/Accepted 28 March 1995

A thermophilic, long-chain fatty acid-oxidizing culture was enriched. Stearate was used as the substrate, and methane and carbon dioxide were the sole end products. Cultivation was possible only when a fed-batch system was used or with addition of activated carbon or bentonite. The enrichment culture consisted of a short rod and two bacteria antigenically related to Methanobacterium thermoautotrophicum ΔH and Methanosarcina thermophila TM-1.

At mesophilic temperatures, triglycerides are rapidly hydrolyzed to glycerol and long-chain fatty acids (LCFA) (3, 5). The LCFA are degraded via β oxidation by aceticogenic bacteria to acetate and hydrogen or acetate, propionate, and hydrogen (16). Only two cultures that degrade LCFA have been described (9, 12), and both are mesophilic.

LCFA inhibit anaerobic bacteria and exhibit acute substrate toxicity (1, 3, 4, 7). Several methods to reduce the toxicity of LCFA have been reported. Addition of cations, such as calcium, has been shown to reduce LCFA inhibition, probably because of formation of insoluble salts (2, 12). Addition of inert materials has also been shown to reduce inhibitory effects (2). Activated carbon decreased the acclimatization time of the anaerobic processes degrading wastewaters containing phenols (6).

In the present report, we describe the enrichment and characterization of a thermophilic, stearate-degrading culture. Different strategies for enrichment of LCFA-degrading bacteria were tested.

A stable, thermophilic, stearate-degrading culture was enriched from a laboratory scale biogas reactor operated at 55°C and fed with manure supplemented with 2% rapeseed oil. The culture converted stearate entirely to CH₄ and CO₂ and consisted of three morphotypes of bacteria.

Traditional enrichment methods, with repeated transfers of approximately 10% of the enrichment culture to the new medium (2) supplemented with 0.1 g of yeast extract per liter and 0.5 g of cysteine per liter with 0.7 to 1.8 mM sodium stearate as the substrate, were tried. However, although this stearate concentration was below the reported toxic level (1), viability was lost after two or three successive transfers. The inability to enrich the cultures on stearate by using 10% successive transfers to new medium indicated that bacteria become more susceptible to inhibitors when they are removed from their natural environments. Another reason for the loss of activity during successive transfers could be dilution of important components which were not included in the medium or elimination of particulate support material originally found in the digesting manure. A requirement for support material has previously been reported for lake sediment enrichment cultures that degraded 4-chlorophenol only when particulate matter was present (10).

Fed-batch cultivation. To keep the stearate concentration below toxic levels and at the same time obtain a high bacterial concentration, fed-batch cultivation was used. By using fed-batch cultivation, in which new cultivation medium was added but without withdrawal of the culture, it was possible to keep a steady, low stearate concentration below 0.2 mM. The feeding rate was kept at approximately 5%, which corresponded well to the stearate utilization rate. In this way, a stable enrichment culture could be maintained.

Batch cultivation with addition of inert material. Growth could also be obtained in batch cultures when the medium was supplemented with the following support material: either 0.2% granular activated carbon (14/60 mesh) or bentonite, a clay mineral composed of more than 60% montmorillonite. Activated carbon resulted in immediate growth, while a lag phase of approximately 1 week was observed in vials with bentonite (Fig. 1). Furthermore, the growth rate of the bentonite cultures was lower than that of cultures with activated carbon. Only slight growth was observed in vials without inert support material, even after several months of incubation (Fig. 1). When activated carbon in powder form (particle size was smaller than 40 μm) was added, no growth of the enrichment culture was observed (data not shown).

Addition of inert material may act by adsorption of LCFA, reducing the concentration of free LCFA. Particulate matter may also provide a substrate for immobilization of bacteria (see Fig. 3). This could be especially important for microbes in syntrophic relationships. Immobilization and closer contact between cells improve interspecies hydrogen transfer kinetics and could increase the overall growth rate of the culture (13). Stamset al. (15) obtained stable propionate-degrading enrichment cultures only after addition of FeCl₂ to the medium, which formed precipitations of FeS. Bacteria seemed to be embedded in the particles, and close contact between the propionate degraders and hydrogen scavengers could be maintained.

The reason for the superior effect of activated carbon compared with other inert material could be the high adsorbance capacity of activated carbon. Kiedzierski et al. (6) found that activated carbon exhibited a greater ability to adsorb phenolic compounds than other inert materials, such as synthetic ion-exchange resins.

Substrates. Degradation of stearate led to slight accumula-
tion of acetate before total conversion to methane and carbon dioxide (Fig. 2). Other intermediate products were not observed, except for trace concentrations of butyrate (below 0.05 mM).

Apart from stearate, a number of other fatty acids could be used by the enrichment culture. Fatty acids with even numbers of carbon atoms were totally degraded, while acids with uneven numbers of carbon atoms were converted to equivalent concentrations of propionate, in addition to methane and carbon dioxide (Table 1). Propionate was not further degraded by the culture. Branched-chain organic acids, such as i-butyrate or i-valerate, were not degraded (Table 1). These data indicated that β oxidation was the mechanism of LCFA degradation. This degradation pattern is in accordance with previous reports on the degradation of LCFA under mesophilic conditions (16).

The enrichment culture could also degrade oleate, a monounsaturated LCFA. A mass balance showed that 72 to 90% of the initial substrate could be accounted for by the products measured. Biomass synthesis and experimental variation could be an explanation for the remaining carbon.

**Morphological characterization of the enrichment culture.** Microscopic examination of the culture showed that three morphotypes of bacteria were present: a small, nonfluorescent rod; a longer, autofluorescent rod; and an autofluorescent cocccus. Figure 3A shows the enrichment culture immobilized on particles of activated carbon. Bacteria are not directly visible in this microscopic picture. However, with epifluorescence illumination (Fig. 3B), autofluorescent, rod-shaped bacteria became visible on the carbon granules. Examination with immunological probes showed that the long rods were immunologically related to *Methanobacterium thermoautotrophicum* ΔH, while the cocci were immunologically related to *Methanosarcina ther-

![FIG. 1. Methane production from the culture. Symbols: ○, without addition of inert material; ●, with granular activated carbon; ▽, with bentonite. The bars indicate standard deviations (n = 3).](#)

![FIG. 2. Time course of stearate degradation. Symbols: ▼, stearate; □, acetate; ●, methane.](#)

![FIG. 3. Photomicrographs of the enrichment culture immobilized on a particle of activated carbon. (A) Phase-contrast microscopy; (B) epifluorescence illumination and violet excitation showing the autofluorescence of methanogenic bacteria attached to an activated carbon particle.](#)

### Table 1. Fermentation balances of the enrichment culture with various organic acids as substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>No. of C atoms</th>
<th>Initial substrate concn (mM)</th>
<th>Fermentation product concn (mM)</th>
<th>Mass balance recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>2:0</td>
<td>9.2</td>
<td>8.2</td>
<td>89</td>
</tr>
<tr>
<td>Propionate</td>
<td>3:0</td>
<td>8.9</td>
<td>8.2</td>
<td>89</td>
</tr>
<tr>
<td>i-butyrate</td>
<td>4:0</td>
<td>6.3</td>
<td>6.2</td>
<td>90</td>
</tr>
<tr>
<td>Butyrate</td>
<td>4:0</td>
<td>8.1</td>
<td>6.5</td>
<td>72</td>
</tr>
<tr>
<td>Valerate</td>
<td>5:0</td>
<td>7.0</td>
<td>5.9</td>
<td>72</td>
</tr>
<tr>
<td>i-valerate</td>
<td>5:0</td>
<td>7.4</td>
<td>5.9</td>
<td>88</td>
</tr>
<tr>
<td>Caproate</td>
<td>6:0</td>
<td>4.2</td>
<td>4.2</td>
<td>90</td>
</tr>
<tr>
<td>i-caproate</td>
<td>6:0</td>
<td>4.9</td>
<td>4.9</td>
<td>90</td>
</tr>
<tr>
<td>Heptanoate</td>
<td>7:0</td>
<td>8.0</td>
<td>8.0</td>
<td>90</td>
</tr>
<tr>
<td>Caprylate</td>
<td>8:0</td>
<td>6.7</td>
<td>6.7</td>
<td>90</td>
</tr>
<tr>
<td>Caprate</td>
<td>10:0</td>
<td>2.9</td>
<td>2.9</td>
<td>90</td>
</tr>
<tr>
<td>Palmitate</td>
<td>16:0</td>
<td>1.2</td>
<td>1.2</td>
<td>90</td>
</tr>
<tr>
<td>Stearate</td>
<td>18:0</td>
<td>1.3</td>
<td>1.3</td>
<td>90</td>
</tr>
<tr>
<td>Oleate</td>
<td>18:1</td>
<td>1.2</td>
<td>1.2</td>
<td>90</td>
</tr>
</tbody>
</table>

*The values shown are means of duplicate cultures corrected for controls with no substrate added.*
mophila TM-1. The cocci were found both as single cells and as clumps. Calibrated polyclonal antisera against the following bacteria were also tested and showed negative reactions: *M. thermoautotrophicum* PLUK, *Methanoseta thermophila* CALS-1, *Methanoseta soehngenii* GP-6, and *Methanoseta soehngenii* Opfikon.

Gram staining of the culture showed that the fluorescent rods and cocci stained gram positive, while the shorter, non-fluorescent rods stained gram negative.

**Isotope test.** To investigate acetate degradation by the enrichment cultures, the fate of $^{14}$CH$_3$COOH was tested. $^{2-14}$C-labelled sodium acetate and unlabelled acetate at various concentrations were added to the vials from sterile stock solutions. The exact labelled and unlabelled acetate concentrations in the vials were determined at the start and end of the experiment. The experiment was stopped by addition of H$_3$PO$_4$. Table 2 shows the results obtained when radiolabelled acetate ($^{14}$CH$_3$COOH) was added at three different initial concentrations of unlabelled acetate. The $^{14}$CH$_3$/14CO$_2$ ratio was in all cases much higher than unity, indicating that acetate was utilized by aceticlastic cleavage and not indirectly via cleavage to H$_2$ and CO$_2$. Total counts of radioactive acetate, methane, and carbon dioxide at the end of the experiment did not vary from the counts at the start of the experiment in autoclaved controls, indicating that acetate was transformed by a biological process (data not shown).

LCFA degradation led to formation of acetate, which was then further converted to methane and carbon dioxide. Methanogenesis from acetate can occur via two different pathways, catalyzed by two different groups of microorganisms. The most common is the aceticlastic reaction, in which acetate is directly converted to CH$_4$ and CO$_2$ by acetate-utilizing methanogenic bacteria (14). The second type is syntrophic acetate oxidation (8), in which acetate is converted to CH$_4$ by a syntrophic consortium of acetate-oxidizing organisms which produce H$_2$ and CO$_2$ from acetate and H$_2$-utilizing methanogens. At very low acetate concentrations, acetate oxidation has been shown to be the major route for acetate catabolism in some systems (11). In our experiments with $^{2-14}$C-labelled sodium acetate, no difference in acetate metabolism was observed at low (below 1 mM) and high (12.7 mM) acetate concentrations, and nearly all of the label was found in the methane fraction in both cases. This indicated that methanogenesis in the culture proceeded solely via the aceticlastic reaction.

**Effect of addition of various pure cultures.** The effect of addition of different H$_2$ scavengers or acetate utilizers was tested with respect to the growth rates of the enrichment culture. The H$_2$ scavengers used were *M. thermoautotrophicum* ∆H, *M. thermoautotrophicum* Marburg, and the sulfate-reducing bacterium *Desulfotomaculum nigrificans* BA. In the last case, 10 mM Na$_2$SO$_4$ was further added to the medium. The acetate-utilizing bacterium used was *M. thermophila* TM-1.

Addition of different H$_2$-utilizing bacteria or acetate-utilizing methanogens did not increase the growth rate of the stearate enrichment culture significantly (Table 3). Furthermore, addition of a combination of H$_2$ utilizers together with acetate-utilizing methanogens had no significant effect, indicating that sufficient methanogens were available in the cultures. Addition of *D. nigrificans* BA, which can remove H$_2$ to an even lower concentration of hydrogen than methanogens (13), together with 10 mM sodium sulfate or of 10 mM sodium sulfate alone did not change the growth rate significantly. This could indicate that the LCFA-degrading cultures are not as sensitive to H$_2$ as other short-chain fatty acid-degrading organisms. Furthermore, *D. nigrificans* utilizes formate in addition to hydrogen, and the lack of influence could indicate that formate had no influence on the LCFA-degrading culture.

**Optimum temperature.** The enrichment culture grew best at a temperature of approximately 55°C (Fig. 4), as determined by comparing growth rates during the second cultivation of the culture at the corresponding temperature. Addition of *Desul-

![FIG. 4. Specific growth rate of the enrichment culture as a function of temperature. The bars indicate standard deviations ($n = 3$).](http://aem.asm.org/)
fobulus propionicus, an H₂ utilizer with a temperature optimum of 37°C, together with 10 mM Na₂SO₄ did not result in growth of the stearate-enriched culture at 37°C. This indicated that the stearate degrader is a true thermophile and that the enriched stearate-degrading bacteria were different from previously described mesophilic, LCFA-degrading bacteria (9, 12).

The wide range of short- to long-chain fatty acids degraded by the enrichment culture indicated that if an anaerobic reactor is adapted to one type of lipid, it is able to degrade other types of lipids as well.

The maximum growth rate determined for our thermophilic LCFA-degrading culture was 0.3 day⁻¹, which is low compared with those of many other bacteria present in biogas reactors. In thermophilic biogas reactors used to treat lipid-containing wastes, shock loads should therefore be avoided to prevent accumulation of toxic concentrations of LCFA.

We thank Anders Hay Sørensen for qualified help with the immunological probes and Hector Garcia for excellent technical assistance. This work was supported by grants from the Center for Process Biotechnology.

REFERENCES