# Acetate oxidation Is the dominant methanogenic pathway from acetate in the absence of Methanosaetaceae

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# **Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of *Methanosaetaceae*.**

Acetate oxidation dominates without *Methanosaetaceae*

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## ABSTRACT

Oxidation of the acetate to hydrogen, and subsequent combination of hydrogen with carbon-dioxide to methane, has largely been regarded as a niche mechanism at high temperatures or under inhibitory conditions. In this study, 13 anaerobic reactors, and sediment from a temperate anaerobic lake were surveyed for dominant methanogenic population using fluorescent *in situ* hybridisation, and degree of acetate oxidation relative to aceticlastic conversion, using radiolabelled 2-[$^{14}$C] acetate in batch incubations. When *Methanosaetaceae* was not present, acetate oxidation was the dominant methanogenic pathway. Aceticlastic conversion was only observed in presence of *Methanosaetaceae*.

Acetate is the main precursor for methane production during anaerobic digestion of organic matter. Two mechanisms for methane formation from acetate have been described. The first one is aceticlastic, carried out by *Methanosarcinaceae* or *Methanosaetaceae* (2). *Methanosarcinaceae* generally have a higher acetate threshold, but higher growth rate and yield compared to *Methanosaetaceae* (2). .The second mechanism encompasses a two-step reaction in which acetate is first oxidised to $H_2/CO_2$ and with these products subsequently converted to methane (15). This reaction is performed by acetate-oxidising bacteria (often *Clostridium*) in a syntrophic association with hydrogenotrophic methanogens (often *Methanomicrobiales* or *Methanobacteriales*) (4, 10, 12).

Some important environmental factors influencing the rate of anaerobic aceticlastic activity are temperature, organic acid concentrations and ammonia concentration (9). At temperatures between 50$^{o}$C and 65$^{o}$C, acetate oxidation is favoured at low acetate concentrations, while aceticlastic methanogenesis is favoured at high acetate concentrations (15). The dominance of acetate oxidation at lower concentrations increases with increased temperature. Syntrophic acetate oxidation is the main mechanism for acetate degradation in the presence of inhibitors, particularly ammonium, and volatile fatty acids (VFAs) (13). Syntrophic acetate oxidation has been reported for natural anoxic environments in subtropical lake sediments at temperatures down to 15$^{o}$C (8).

It is relatively straightforward to detect acetate oxidation activity by measuring the production of $^{14}CH_4$ and $^{14}CO_2$ from acetate labeled in the methyl group (C-2). When aceticlastic methanogens degrade acetate, the labeled methyl group will form only

49 labeled methane (2). During syntrophic acetate oxidation, both carbon atoms of acetate
50 are converted to carbon-dioxide, and some of the carbon dioxide is subsequently reduced
51 to methane (13). Therefore, significant levels of labeled carbon dioxide from [2-$^{14}$C]-
52 acetate will only be formed during oxidation of acetate.

53 The diversity of environments in which syntrophic acetate oxidation has been found
54 indicates it may also be important for commercial gas production in biogas reactors,
55 digesting wastewater sludge and manure. Aceticlastic activity has generally been
56 considered to be the dominant pathway, with either *Methanosarcinaceae* or
57 *Methanosaetaceae* dominating (9, 15). If a second pathway such as acetate oxidation
58 dominates, it is necessary to re-evaluate reactor operation and optimisation, currently
59 based on maintaining *Methanosaetaceae* populations. The objective of this work was to
60 assess the degree of acetate oxidation relative to aceticlastic conversion in a wide range
61 of industrial anaerobic digesters, fed either with manure or wastewater sludge. A low-
62 temperature environmental sample was also evaluated.

63 **Sampling.** Thirteen Danish full-scale anaerobic digesters were sampled (Table 3) as
64 described in (5). organic waste from abattoirs or food industries. An anaerobic sediment
65 sample was collected from a lake situated in Orholm (Sollerod municipality, Denmark) at
66 0.2 m depth with a gravity corer (6).

67 **Analysis of the samples.** The samples were analyzed for volatile fatty acids (VFAs)
68 and ammonia by standard methods (1). Microbial ecology was evaluated using
69 fluorescent *in-situ* hybridisation FISH with established probes, and method as previously
70 reported (5). Methanogenic populations not identified by FISH were assessed using
71 polymerase chain reaction coupled to temperature gradient gel electrophoresis (PCR-

72 TGGE). Sequence data for identified microbes have been submitted to the Genbank
73 database under the accession numbers DQ409324 to DQ409326.

74 **Medium.** Basal anaerobic (BA) medium was used for acetic oxidation batch tests as
75 described previously (5). The medium was dispensed anaerobically under a $N_2/CO_2$ (80
76 %: 20 %) headspace in 100 ml incubation bottles, amended with labeled 2-[$^{14}$C]-sodium
77 acetate and non-labeled sodium acetate. The medium was reduced with $Na_2S.9H_2O$ and
78 supplemented aseptically with a sterilely filtered anaerobic vitamin solution as described
79 previously. After inoculation with raw sample the bottles were closed hermetically and
80 incubated until methane production ceased. This was treated as the end of the test, and
81 analysis followed.

82 **Radioisotope analyses.** The liquid and headspace was sparged with approx 2L $N_2$,
83 through a 5M NaOH trap to collect the $^{14}CO_2$. The $^{14}CH_4$ collected after trapping was
84 combusted to $^{14}CO_2$ in a tube furnace at 800$^{o}$C. $^{14}CO_2$ generated in this furnace was then
85 trapped in Carbosorb-E (carbon dioxide absorber for liquid scintillation counting,
86 Packard Bioscience Company, USA). Radioactivity measurements of liquid samples were
87 performed on a liquid scintillation counter (Tri-Carb 1600, Perkin Elmer, England).

88 **Simulation of Methane Production Rates.** A simple kinetic batch model, based on
89 Monod kinetics with zero order lag, for conversion of acetate to methane was
90 implemented in Aquasim 2.1d (11). The maximum acetate removal rate and lag phase
91 were estimated by fitting measured cumulative methane to modeled cumulative methane.
92 The Secant method, with an objective function of residual sum of squares was used to fit
93 the data.

94 An overview of the results from the acetate oxidation survey experiment is given in
95 Table 1.

96 **Rates of methane production and acetate removal.** Methane production rates varied
97 considerably, with fast samples (such as Lundtofte and Hillerød) stopping methane
98 production in 3 days, and slow samples (e.g., Nysted and Vegger) requiring more than 10
99 days. The anaerobic lake sediment sample (Orholm) had a lag phase of 31.5±0.8 days.
100 Acetate removal rates also varied within a factor of approximately 10 (Table 1). These
101 rates were higher (>4 $mM.day^{-1}$) in cultures with low acetate oxidation degree in
102 comparison with cultures with high acetate oxidation degree (the acetate utilisation rates
103 lower than 4 $mM.day^{-1}$). Our rates compare with acetate removal rates in pure culture for
104 mesophilic (12) and a thermophilic acetate oxidising cultures (4).

105 **Anaerobic acetate conversion pathways and environmental conditions.** In all cases,
106 populations dominated by *Methanosaetaceae* had minimal acetate oxidation degree
107 ($^{14}CO_2$ / $^{14}CH_4 < 0.1$), while populations dominated by other methanogenic *Archaea*, and
108 without *Methanosaetaceae* had a high degree of acetate oxidation ($^{14}CO_2$ / $^{14}CH_4 > 1$)
109 (Table 1). Results obtained clearly showed a strong correlation between the absence of
110 *Methanosaetaceae* and involvement of acetate oxidation pathway. Other factors (e.g.,
111 source and inoculum temperature) had no influence. Acetate cleavage has been generally
112 regarded as a bimodal system, dominated by *Methanosarcinaceae* at high acetate
113 concentrations, and *Methanosaetaceae* at low acetate concentrations (2, 14). From the
114 data presented here, we propose instead a different bimodal system in mixed cultures,
115 with aceticlastic methanogenesis in the presence of *Methanosaetaceae*, and acetate
116 oxidation in its absence. The absence of this methanogenic phylogenetic group has been

117 previously investigated in the systems analysed here, and was linked to the presence of
118 high ammonia and VFA levels (5). Most probably the high ammonia concentrations
119 inhibit the aceticlastic methanogens much more than the hydrogenotrophic methanogens,
120 and methane formation is mainly by hydrogen-utilising methanogens. This is supported
121 from previous studies (3) indicating that acetate-utilising methanogens are more sensitive
122 to ammonia than are hydrogenotrophic methanogens. The high degree of acetate
123 oxidation in digested manure at high ammonia and VFA levels is also in agreement with
124 other results (13). However a large potential for syntrophic acetate oxidation was also
125 observed at low acetate concentrations (in the Orholm sample). It is likely that inhibition
126 or other factors prevent growth of *Methanosaetaceae,* and allow dominance by acetate
127 oxidation by default.

128 The bimodality of the system is also highlighted in Figure 1, which shows two distinct
129 groups, with hydrogen-utilising methanogens (*Methanobacteriales, Methanococcales*,
130 *Methanomicrobiales*, possibly *Methanosarcinaceae,* uncultured archae (Hashøj and
131 Lemvig) and unidentified archae (Studsgard) in syntrophic cooperation with acetate
132 oxidisers at low maximum acetate removal rates, and the strict aceticlastic methanogen
133 *Methanosaetaceae* at maximum acetate removal rates. The presence of
134 *Methanosarcinaceae* as a hydrogen utilising syntrophic partner in the acetate oxidising
135 cultures is not surprising. In contrast to the *Methanosaetaceae,* which is a strict aceticlast,
136 most *Methanosarcinaceae* species are mixotrophic, utilising not only acetate but also
137 hydrogen and carbon dioxide, methanol and methylamines (2). In addition,
138 *Methanosarcinaceae* are capable themselves of acetate oxidation (7), and could be

139 therefore be mediating the entire process of acetate oxidation, and subsequent
140 methanogenesis, rather than acting as an acetate sink via aceticlastis.

141 **Methanogenic populations.** The FISH observations showed that dominant
142 methanogenic population of wastewater sludge samples was consistently
143 *Methanosaetaceae*, as previously reported (5), while manure samples were
144 phylogenetically more diverse. In every case, dominance of specific groups as observed
145 by FISH was clear, and they constituted more than 90% of the archaeal population, as
146 described previously (5)). Diversity in subdominant methanogens was limited, except in
147 the Orholm (sediment sample), where archaea belonging to *Methanosaetaceae*,
148 *Methanomicrobiales*, and *Methanococcales* were observed. Methanogenic population
149 changes were observed during growth on acetate in the incubations. For the
150 *Methanosaetaceae* dominated samples, the only changes observed during incubation
151 were elimination of subdominant populations. In the other samples, there was a shift to
152 known hydrogen consumers (*Methanobacteriales*, *Methanomicrobiales*, or
153 *Methanococcales*) or uncultured archae (samples M2 and M6).

154 Methanogenic communities in several samples (M2 and M5 before incubation and M2
155 and M6 after incubation) were not identified by FISH. This was due to the limitations of
156 visual *in situ* hybridisation. FISH is very convenient for the rapid analysis of a large
157 number of environmental samples but is limited when carried beyond the limits of
158 oligonucleotide probes. ARC915 is an effective general probe, and order-level probes
159 have been used in a wide range of systems, however in complicated systems such as
160 manure, they might fail to detect all methanogens. Therefore, unidentified methanogens
161 were phylogenetically characterised by PCR-TGGE. Samples not identified by FISH

(e.g., M5, Studsgard before inoculation), were found by PCR-TGGE to be far outside known phylogenetic groupings for methanogens. It is likely that these microbes are still methanogens, since bacterial methanogenesis is unknown. These unknown microbial groups are interesting scientifically, and deserve further investigation.

Thanks are due to Lene Kirstejn Jensen, Birthe Ebert and Hector Garcia for the technical assistance with the experiments. This work was supported by Danish Government Scholarship and Danish Research Programme (EFP 05).

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210 Table 1: Results from acetate oxidation survey

| Sample Id | Reactor name | Feed type | Temp. (type)[1] | Incubation period (days) | Dominant Methanogen[3] (nondominant) | | Level (mean ±SD[2]) of: | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Before incubation | After incubation | VFA (gHAc $L^{-1}$) | Ammonia (gN $L^{-1}$) | Maximim acetate removal rate (mM $day^{-1}$) | $^{14}CO_2/^{14}CH_4$ | Recov of $^{14}C$ |
| M1 | Nysted | manure | 38°C (M) | 9 | MS (NO) | MG (MS) | 2.7 ± 0.11[2] | 5.6±0.12 | 0.5[4]± 0.1 | 2.9 ± 0.18[2] | 96 ± 5 |
| M2 | Hashøj | manure | 37°C (M) | 13 | Uncultured archae DQ409324 (MC) | Uncultured archae DQ409325 (NO) | 1.9 ± 0.07 | 4 ± 0.1 | 1.8 ± 0.1 | 2.75 ± 0.11 | 97.5 ± |
| M3 | Lemvig | manure | 52.5°C (T) | 8 | MS (NO) | MB, MC (MS) | 0.6 ± 0.01 | 2.6±0.08 | 5.3 ± 0.6 | 2.45 ± 0.15 | 92.5 ± |
| M4 | Fangel | manure | 37°C (M) | 10 | MB (MC) | MB (MC) | 2.33 ± 0.12 | 4.5 ± 0.10 | 1.5± 0.1 | 2.2 ± 0.14 | 97.4 ± |
| M5 | Studsgard | manure | 52°C (T) | 10 | UnIDd filaments (NO) | MG (MS) | 0.22±0.003 | 2.20 ± 0.09 | 2.7 | 2.11± 0.16 | 99 ± |
| M6 | Vester Hjermitslev | manure | 37°C (M) | 16 | MS (NO) | Uncultured archae DQ409326 (MS) | 1.81± 0.06 | 4.4±0.11 | 0.7± 0.03 | 1.9 ± 0.18 | 92.2 ± |
| M7 | Vegger | manure | 55°C (T) | 8 | MS (NO) | MS (NO) | 0.77 ± 0.04 | 2.34±0.07 | 3.5± 0.4 | 1.61± 0.11 | 94 ± 3 |
| LS1 | Orholm | lake sediment | 4°C (P) | 49 | MB (MX, MG, MC) | MC (NO) | 0.01 | 0.03 | 1.4[4]± 0.2 | 1.4 ± 0.09 | 96.2 ± |
| M8 | Sinding | manure | 55°C (T) | 8 | MS (NO) | MS (NO) | 0.41 ± 0.01 | 2.5 ± 0.06 | 3.4 | 1.1± 0.06 | 101 ± |
| S1 | Hillerød | WW sludge | 55°C(T) | 5 | MX (MS) | MX (NO) | 0.13±0.001 | 1.44±0.05 | 10± 0.5 | 0.11 ± 0.007 | 97 ± |
| S2 | Lundtofte | WW sludge | 37°C (M) | 10 | MX (NO) | MX (NO) | 0.02 | 1.00±0.04 | 9.9± 0.6 | 0.08 ± 0.003 | 98.1 ± |
| S3 | Fakse | WW sludge | 37°C (M) | 6 | MX (NO) | MX (NO) | 0.04 | 1.50±0.03 | 7.1 | 0.065 ± 0.004 | 91 ± 2 |
| S4 | Sydkyst | WW sludge | 37°C (M) | 8 | MX (NO) | MX (NO) | 0.13 | 0.50±0.001 | 4.8[4]± 0.4 | 0.04 ± 0.002 | 92 ± 3 |
| S5 | Helsingør | WW sludge | 37°C (M) | 6 | MX (MB, MG) | MX (NO) | 0.06 | 0.84 ± 0.03 | 6.1± 0.7 | 0.025 ± 0.001 | 95.4 ± |

211 [1.] P: Psychrophilic (<20°C), M: mesophilic (35°C-40°C), T : thermophilic (>50°C) ; [2]. SD based on triplicate analysis ; [3]. MS,
212 *Methanosarcinaceae*; MX, *Methanosaetaceae*; MB, *Methanobacteriales*; MG, *Methanomicrobiales*; MC, *Methanococcales*; NO, not
213 observed; UnIDd, unidentified (both by FISH and TGGE). The term "dominant methanogens" was used in the sense of more than 90 % of
214 the total number of methanogenic cells (Archae responding to ARC915). The term "nondominant methanogens" was used in the sense of 1
215 to 10 % of the total number of methanogenic cells. Cells in 20 fields were countered. [4]. Long lag phase observed before acetate removal

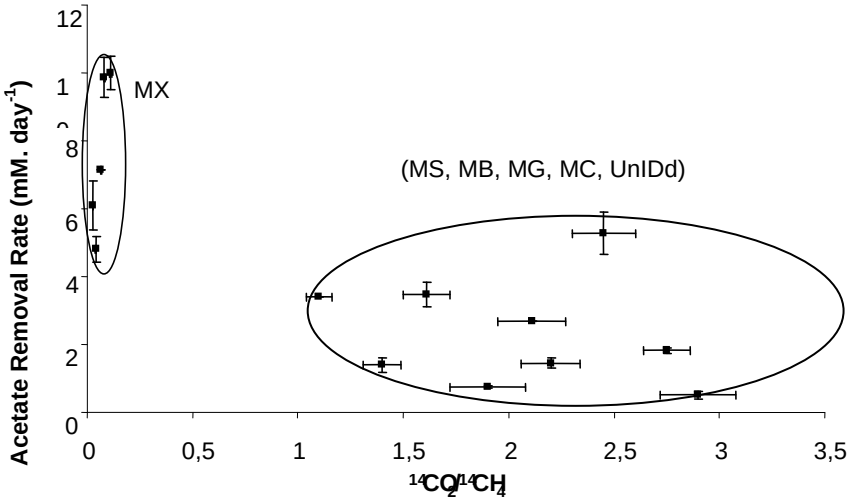


Figure 1. Distribution of the dominant methanogens observed in the samples as a function of the acetate oxidation degree versus acetate utilisation rates. Error bars indicate standard deviations. MX indicates *Methanosaetaceae* while other abbreviations indicate *Methanosarcinaca* (MS), *Methanobacteriales* (MB), *Methanomicrobiales* (MG), *Methanococcales* (MC), and Unidentified (UnIDd).