Minireview

Syntrophic Acetate-Oxidizing Microbes in Methanogenic Environments

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Acetate is one of the most important intermediates for methanogenesis in the anaerobic mineralization of organic materials. Methanogenic acetate degradation is carried out by either an aceticlastic reaction or an anaerobic acetate-oxidizing reaction. In contrast to the former reaction, the latter is energetically extremely unfavorable. However, the oxidation of acetate can occur with syntrophic interaction between certain bacteria and methanogenic archaea. The bacteria, namely syntrophic acetate-oxidizing bacteria, can oxidize acetate to produce hydrogen/CO₂ only when their products are subsequently utilized by the hydrogen-scavenging methanogens. Surprisingly, some of these bacteria can also axenically grow on hydrogen/CO₂ to produce acetate. This means that the bacteria can utilize both substrates and products reversibly. This review describes current studies of these curious and fascinating microbes.

Key words: syntrophic acetate oxidation, methanogenesis, reverse acetogenesis, CO dehydrogenase/acetyl-CoA synthase pathway, homoacetogen

Introduction

Methanogenic degradation of complex organic materials is a widespread process in anoxic environments where light or inorganic electron acceptors such as oxygen, nitrate, iron, manganese, and sulfate are absent. Examples of these environments are wetlands, freshwater sediment, and the digestive tracts of animals and insects. Under such conditions, the degradation of organic matter to methane requires at least four physiologically different microbial groups, namely primary fermenting bacteria, secondary fermenting bacteria (also called proton-reducing syntrophic bacteria), and two types of methanogenic archaea. The primary fermenting bacteria hydrolyze polymers including proteins, polysaccharides, nucleic acids, and lipids to monomers such as amino acids, sugars, purines, pyrimidines, and long-chain fatty acids. This microbial group further ferments monomers to reduced compounds (alcohols, short-chain fatty acids, organic acids, and certain aromatics), hydrogen and CO₂. The reduced products are oxidized to acetate, hydrogen and CO₂ by the secondary fermenting bacteria. Hydrogen and CO₂ are converted to methane by the hydrogenotrophic methanogens whereas methanogenic acetate degradation is carried out by the aceticlastic methanogens. Through the interactions of these microbial groups, the organic materials are eventually mineralized to methane and CO₂.

During the methanogenic mineralization process, oxidation of reduced compounds catalyzed by the secondary fermenting microbes is thermodynamically unfavorable (ΔG° > 0). For example, ethanol oxidation coupled with proton reduction is endergonic (ΔG° = +9.6 kJ/mol) under standard thermodynamic conditions (298 K, pH 7, solute at 1 M, gases at 10⁵ Pa). The oxidation of fatty acids is more unfavorable than that of ethanol. The ΔG° value for the oxidation of butyrate or propionate coupled with proton reduction is +48.1 kJ/mol and +61.1 kJ/mol, respectively. These reactions proceed only if hydrogen partial pressures are kept low by coupling with hydrogen-consuming methanogenesis (ΔG° = −135.6 kJ/mol). Thus, interspecies hydrogen transfer between the secondary fermenting bacteria and the hydrogenotrophic methanogens is considered to be essential for the oxidation of these substrates. To date, several syntrophic fatty acid-oxidizing bacteria including a butyrate oxidizer and a propionate oxidizer have been isolated and characterized. In addition to these syntrophs, it has been discovered that several bacteria can also oxidize acetate syntrophically when hydrogenotrophic and/or formate-utilizing methanogens are present. The microbes, namely syntrophic acetate-oxidizing bacteria, have quite unique physiological and biochemical features. This review briefly describes current studies of the syntrophic acetate-oxidizing bacteria, including physiology, taxonomy, biochemistry, energetics, and microbial ecology.

General concept of syntrophic acetate oxidation under methanogenic conditions

There are two processes for methanogenesis from acetate. The first is aceticlastic methanogenesis. In this reaction, acetate is cleaved to methyl and carboxyl groups. The methyl group is directly converted to methane via several biochemical reactions, whereas the carboxyl group is oxidized to CO₂. Aceticlastic methanogenesis (reaction 1, Table 1) is an exergonic reaction (ΔG° = −31.0 kJ/mol), thus theoretically it proceeds in the absence of other reactions such as hydrogenotrophic methanogenesis. At present, two genera of methanogens, Methanosarcina and Methanosaeta, are known to operate this biochemical process.

The second process consists of two reactions, syntrophic...
acetate oxidation and hydrogenotrophic methanogenesis. This process was originally proposed by Barker (1936)39. In syntrophic acetate oxidation, both methyl and carboxyl groups of acetate are oxidized to CO₂ with the production of H₂ (reaction 2, Table 1). Energetically, this reaction is extremely unfavorable (ΔG° = -104.6 kJ/mol). However, similar to alcohol or fatty acid oxidation, this unfavorable reaction can proceed if H₂-consuming methanogenesis (ΔG° = -135.6 kJ/mol) (reaction 3, Table 1) eliminate hydrogen. The overall reaction (reaction 4, Table 1) becomes exergonic (ΔG° = -31.0 kJ/mol), with the same stoichiometry as aceticlastic methanogenesis.

Syntropic acetate oxidation is catalyzed by syntrophic acetate-oxidizing bacteria, whereas the H₂-consuming methanogenesis is catalyzed by hydrogenotrophic methanogens. The two microbes obligately require each other since the bacteria require hydrogen scavengers (i.e., partner methanogens) and the archaea require hydrogen suppliers (i.e., syntropic acetate-oxidizing bacteria). Although this mutual syntrophy theoretically yields energy, the amount is quite small (ΔG° = -31.0 kJ/mol). Moreover, the syntrophic acetate-oxidizer and partner methanogens should share this small energy, because each microbe has to earn its own living. This energetical disadvantage may cause these syntrophs to be slow growers and to adapt rigid mutualism, both of which may explain why the isolation of syntropic acetate-oxidizing cocultures was long considered extremely difficult or even impossible.

**Isolation and characterization of the syntrophic acetate-oxidizing bacteria**

About a half century after Barker’s hypothesis, Zinder and Koch (1984)40 reported that methanogenesis from acetate via two-step reactions was actually catalyzed by syntrophic acetate-oxidizing bacteria and hydrogenotrophic methanogens. The coculture was obtained from a thermophilic (58°C) digester that converted lignocellulosic urban solid waste to methane. Morphologically, the coculture consisted of two different microbes i.e., rod-shaped bacteria and *Methanothrophic* (formerly *Methanobacterium*)-like methanogens. Aceticlastic methanogens such as *Methanoseta* and *Methanosarcina* were not observed in the coculture. The coculture stoichiometrically converted 1 mol of acetate to 1 mol of methane as shown in Table 1 (reaction 1 or 4). However, it did not convert [2-14C] acetate to 14CH₄ but mainly to 14CO₂, indicating that syntrophic acetate oxidation occurred in the coculture40. The coculture could also oxidize ethanol to methane via acetate. Lee and Zinder (1988)41 later isolated syntrophic acetate-oxidizing bacteria without partner methanogens by using energetically more favorable substrates such as ethylene glycol and pyruvate. The isolated syntrophic acetate-oxidizing bacterium, namely strain AOR (nick-named ‘Reversibacter’), was an anaerobic, thermophilic, gram-positive, non-spor-forming, rod-shaped bacterium. The bacterium could grow on ethylene glycol, pyruvate, formate, 1, 2-propanediol, and betaine in pure culture. In addition, the strain could also grow on H₂/CO₂ with production of acetate. The strain stoichiometrically converted 4 mol of H₂ to 1 mol of acetate as shown in Table 1 (reaction 5). This indicated that the strain belonged to a certain physiological group of bacteria, namely acetogens (also called homoacetogens). The acetogens are anaerobic bacteria that produce mainly acetate from autotrophic and/or heterotrophic substrates such as sugars, alcohols, amino acids, organic acids, methoxylated aromatics, CO₂ and H₂/CO₂.14 This group of bacteria can also utilize various electron acceptors such as nitrate, nitrite, fumarate, dimethylsulfoxide, pyruvate, thiosulfate, sulfate, and even protons14. As well as having physiological diversity, the acetogens are also phylogenetically very diverse, although many of the microbes belong to the class *Clostridiales within the phylum Firmicutes*. A hallmark of the acetogens is considered to be the presence of a CO dehydrogenase/acyt-CoA synthase pathway (Fig. 3) in which the acetogens operate (See section: Metabolism pathways in the syntrophic acetate-oxidizing bacteria). Partial sequencing of the 16S rRNA gene for strain AOR indicated this microbe likely belongs to the phylum *Firmicutes*. Unfortunately, there is no definitive phylogenetic position for the strain and determination of its phylogeny is impossible since the strain is currently unavailable.

**Clostridium ultunense** strain BS is a mesophilic syntrophic acetate-oxidizing bacterium that has been described as the second syntrophic acetate-oxidizing bacterium61. It was obtained from a syntrophic acetate-oxidizing triculture that was derived from a laboratory-scale digester (37°C) fed with swine manure in Sweden60. The source contained relatively high concentrations of ammonium (0.5 M) that generally inhibit the growth of aceticlastic methanogens. The strain is an anaerobic, gram-positive, spore-forming, rod-shaped bacterium. *C. ultunense* could oxidize acetate in the presence of a hydrogenotrophic methanogen, *Methanoculleus* sp. strain MAB1. The bacterium also utilized acetogenic substrates such as ethylene glycol, pyruvate, and betaine. *C. ultunense* did not grow on H₂/CO₂, most probably due to a low energy gain from this substrate. It should be also mentioned that H₂/
CO₂ was converted to acetate by resting cells of the culture. Phylogenetically, strain BS was placed in the clostridial cluster XII within the phylum Firmicutes.

The third acetate-oxidizing syntrophy described is *Thermacetogenium phaeum* strain PB, which oxidizes acetate in cocultures with both H₂- and formate-consuming methanogens (*Methanothermobacter thermautotrophicus* strain TM) (Fig. 1)⁹. It was isolated from an acetate-oxidizing enrichment culture that was obtained from a thermophilic methanogenic reactor treating wastewater from a kraft-pulp production plant in Japan¹⁰. The strain is an anaerobic, thermophilic, spore-forming, rod-shaped bacterium. The *T. phaeum/M. thermautotrophicus* strain TM coculture could degrade approximately 40 mM of acetate to a nearly stoichiometrically equal amount of methane within 20 days (Fig. 2).

The strain could also grow on acetate in the presence of H₂-utilizing methanogens (*M. thermautotrophicus* strain ΔH), although the acetate oxidation rate in the *T. phaeum/strain ΔH* coculture is about one third of that in the *T. phaeum/strain TM* coculture. Similar to the strain AOR/methanogen coculture, the *T. phaeum/methanogen* coculture could also oxidize ethanol to methane. Like the two syntrophic acetate oxidizers described above, strain PB is an acetogen. The strain could grow on alcohols, methoxylated aromatics, amino acids, organic acids, and H₂/CO₂, which are representative substrates for many acetogens. In addition to having metabolic traits, the strain could also oxidize acetate in the absence of partner methanogens when suitable external electron acceptors (sulfate or thiosulfate) existed. A dissimilatory adenosine-5'-phosphosulfate (APS) reductase gene that all sulfate-reducing bacteria harbor was recovered from this strain⁹. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that the strain belonged to the family *Thermoanaerobacteriaceae* within the phylum Firmicutes, in which a representative thermophilic acetogen *Moorella thermoacetica* (formerly *Clostridium thermoacetaticum*) is placed¹⁰.

*Thermotoga lettingae* strain TMO is the fourth isolate that can degrade acetate in syntrophic cooperation with *M. thermautotrophicus* strain ΔH. The strain was originally isolated from a thermophilic (65°C) sulfate-reducing bioreactor fed with methanol. Strain TMO is an anaerobic, thermophilic, non-spore-forming, rod-shaped bacterium. In the presence of partner methanogens, the strain could degrade 20 mM acetate in about 28 days. In the presence of thiosulfate, this microbe could axenically degrade acetate with the concomitant production of alanine and sulfate. The strain could also grow on complex substrates such as yeast extract, peptone, and gelatin. It could also utilize simple substrates including methanol, organic acids, and sugars. In addition, strain TMO could grow weakly on H₂/CO₂ only when thiosulfate was present. There has been no physiological data to support that this microbe is an acetogen. Phylogenetically, strain TMO belonged to the family *Thermotogaceae* within the phylum *Thermotoga*. Representative characteristics of these four syntrophic acetate-oxidizing bacteria are summarized in Table 2.

To date, only the above isolates (strain AOR, *C. ultunense*, *T. phaeum*, and *T. lettingae*) have been reported as syntrophic acetate-oxidizing bacteria that can cooperate with H₂-consuming methanogens. However, other syntops oxidizing acetate with the aid of non-methanogenic hydrogenotrophic partner microbes have been isolated and characterized. *Geobacter sulfurreducens* strain PCA is an iron-reducing, anaerobic, mesophilic bacterium isolated from hydrocarbon contaminated-sediment⁶. This strain can oxidize acetate in syntrophic cooperation with nitrate- or sulfate-reducing H₂-utilizing bacteria when suitable external electron acceptors such as iron and fumarate are depleted¹¹. It could rapidly grow on acetate with a doubling time of 6–8 hours (*G. sulfurreducens* and *Wolinella succinogenes* coculture) and 30 hours (*G. sulfurreducens* and *Desulfovibrio desulfuricans* coculture).

*Candidatus ‘Cotubemalis alkalaceticum’* is an anaerobic, mesophilic, alkaliphilic bacterium isolated from sulfidogenic soda lake sediment⁸. When cocultured with hydro-

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Fig. 1. Morphology of the syntrophic acetate-oxidizing coculture. (A) phase-contrast and (B) FITC epifluorescence micrograph of *Thermacetogenium phaeum* strain PB and *Methanothermobacter thermautotrophicus* strain TM. Arrows indicate *T. phaeum* cells. Scale bar=10 μm.

Fig. 2. Acetate degradation and H₂, formate, and methane production in the syntrophic acetate-oxidizing coculture (*T. phaeum/M. thermautotrophicus* strain TM). Symbols: ( ) acetate; ( ) H₂ (X) formate; (■) methane. Replotted from reference 20.
genotrophic alkalophilic sulfate reducers, it can oxidize acetate at pH 10 with the production of sulfide. Candidatus 'C. alkalaceticum' persists in pure cultures, although it can also grow syntrophically with partner microbes at the expense of several alcohols, serine, fructose, and isobutyrate. The 16S rRNA gene sequence indicated that G. sulfurreducens belongs to the family Geobacteraceae within the order deltaproteobacteria, whereas Candidatus 'C. alkalaceticum' is likely to belong to the family Syntrophomonadaceae within the phylum Firmicutes. It has not been reported whether G. sulfurreducens and Candidatus 'C. alkalaceticum' can oxidize acetate in cocultures with methanogens.

Metabolic pathways in the syntrophic acetate-oxidizing bacteria

As described above, the three syntrophic acetate-oxidizing bacteria (strain AOR, C. ulturnense, and T. phaeum) are acetogens that have an ability to synthesize acetate from H2/CO2. In acetogens, the synthesis of acetate from H2/CO2 proceeds via the carbon monoxide dehydrogenase/acyetyl-CoA synthase (CODH/ACS) pathway (also referred to as the Wood/Lojungdhal pathway)60). In this review, to avoid confusion, the term “reductive CODH/ACS pathway" will be used. The acetogens are known to use the reductive CODH/ACS pathway for both dissimilation and assimilation. In addition to the acetogens, several anaerobic microbes such as sulfate reducers, methanogens, and syntrophic propionate-oxidizing bacteria employ an analogous pathway.52,55,56,74,78

A principle of the reductive CODH/ACS pathway is the reduction of 2 moles of CO2 to 1 mol of acetate by using 8 reducing equivalents [H] (Fig. 3). In this pathway, 1 mol of CO2 is reduced to CO by a bifunctional enzyme, carbon monoxide dehydrogenase/acyetyl-CoA (CODH/ACS) synthase, that is known as the key enzyme in this pathway. The other molecule of CO2 is reduced to formate, which is activated to formyl-tetrahydrofolate (formyl-THF) in the presence of ATP and THF (carrier for the methyl group of acetyl-CoA). The formyl-THF is further dehydrated to methenyl-THF followed by successive reductions of methylene-THF and methyl-THF. Subsequently, the methyl group is transferred

![Fig. 3. Carbon monoxide dehydrogenase/acyetyl-CoA synthase pathway](image-url)
to the corrinoid/iron-sulfur protein (corrinoid/Fe-S) followed by the CODH/ACS. The bifunctional enzyme condenses the methyl group, CoA, and CO (derived from another molecule of CO$_2$) to acetyl-CoA. Finally, the acetyl-CoA is converted to acetate via acetyl-phosphate.

The three syntrophic acetate-oxidizing bacteria (the strain AOR, *C. ultunense*, and *T. phaeum*) grown in pure cultures exhibited CO dehydrogenase and formate dehydrogenase activities$^{18,38,62}$. In the cases of *C. ultunense* and *T. phaeum*, hydrogenase, ATP-dependent formyl-THF synthetase, methylene-THF dehydrogenase, and methylene-THF reductase activities were also detected. In addition, none of the three isolates showed 2-oxoglutarate dehydrogenase activity, the key enzyme of citric acid cycles, indicating operation of the reductive CODH/ACS type pathway in these pure cultures. In the case of strain AOR, formyl-THF synthetase activity was not detectable (<0.01 μmol$^{-1}$ min$^{-1}$ mg$^{-1}$), although a folate derivative was detected from a cell extract of this strain$^{38}$. The cell extract of *T. phaeum* in pure culture exhibited activity of this enzyme, but at a very low level (approximately 0.01 μmol$^{-1}$ min$^{-1}$ mg$^{-1}$)$^{18}$. These results imply that the syntrophic acetate oxidizer operates this pathway using a different or modified methyl carrier.

Under standard reaction conditions, the synthesis of acetate from H$_2$/CO$_2$ (reductive acetogenesis) is an exergonic reaction ($ΔG^\circ$=-104.6 kJ/mol). During this pathway, energetically 1 mol of ATP is consumed by substrate level phosphorylation (SLP) in the formyl-THF synthetase reaction, whereas 1 mol of ATP is gained by SLP in the acetate kinase reaction. Since no other reactions catalyzing SLP are concerned with this pathway, the acetogens that grow autotrophically must gain ATP via ion gradient-driven phosphorylation$^{42}$. Several acetogens contain membrane-associated electron transport carriers and energy-conserving apparatus such as cytochromes$^{17,25}$, menaquinone$^{32}$, H$^+$/ATPase$^{27}$, Na$^+$/ATPase$^{31}$, hydrogenase$^{26}$, CO dehydrogenase$^{25}$, methyltransferase$^{49}$, and methylene-THF reductase$^{25}$. Energetically, the last enzyme is considered a suitable candidate that couples to the conservation of energy in the reductive CODH/ACS pathway$^{75,80}$. Methyltransferase is also a candidate for sodium ion-dependent energy conservation in some acetogens$^{42,43}$. It is still unclear if these two enzymes are actually involved in energy conservation in acetogens. Likewise, it has not been determined which reaction is coupled to the synthesis of ATP in the syntrophic acetate-oxidizing bacteria. However, a cell extract of *T. phaeum* grown on methanol exhibited a significant level (approximately 60%) of methylene-THF reductase activity in the membrane fraction (Hattori, unpublished data). Thus, this enzyme might participate in electron transport in syntrophic acetate-oxidizing bacteria during the synthesis of acetate.

Although the reductive CODH/ACS pathway can generate energy by reducing CO$_2$ to acetate, reversal of the pathway, here termed the oxidative CODH/ACS pathway, is operated by several anaerobic microbes including sulfate reducers$^{55,56,66}$ and methanogens$^{15,78}$. In the oxidative CODH/ACS pathway, theoretically 1 mol of acetate is oxidized to produce 8[H] and 2 moles of CO$_2$ (opposite direction in Fig. 3). Similar to the pure cultures, the mixed cell extracts of the strain AOR/methanogen coculture exhibited hydrogenase, formate dehydrogenase, and CO dehydrogenase activities$^{38}$. In addition to these enzymes, formyl-THF synthetase, and methylene-THF dehydrogenase were detected in the mixed cell extracts of the *C. ultunense*/methanogen coculture or selective cell extract of *T. phaeum* pre-grown with partner methanogens$^{38,60}$. Likewise, 2-oxoglutarate dehydrogenase, a key enzyme of the citric acid cycle for the oxidation of acetate, was not detected in the cell extracts of the respective microbes. These results indicate that the three syntrophic acetate oxidizers also operate the oxidative CODH/ACS type pathway for oxidation of acetate. However, the coculture of strain AOR/methanogens, production of $^{14}$CO$_2$ from [2-$^{14}$C]acetate immediately ceased on the addition of cyanide, a potent inhibitor of CO dehydrogenase$^{38}$, also supporting the involvement of the oxidative pathway.

No biochemical study regarding *T. lettingae* has been reported so far. However, the complete genomic sequence of *T. lettingae* (GenBank accession no. CP000812) contained no gene encoding CO dehydrogenase. Therefore, *T. lettingae* is unlikely to operate the CODH/ACS pathway. *G. sulfurreducens* strain PCA, another syntrophic acetate-oxidizing bacterium coupled with non-methanogenic hydrogenotrophic microbes, is known to operate the citric acid cycle in the oxidative direction$^{16}$. This trait indicates that the syntrophic acetate-oxidizing bacteria possess at least two different biochemical mechanisms for the oxidation of acetate. Compared with the reductive CODH/ACS pathway, it is also possible to gain energy during operation of the oxidative pathway. However, the energy conservation process is likely to differ between the two pathways, since reversal of the pathway also makes their reactions energetically reversed. For example, the free energy change for the methylene-THF reduction ($ΔG^\circ$=-42.0 kJ/mol, $[H]$=H$_2$) to acetate oxidation ($ΔG^\circ$=-21.0 kJ/mol, $[H]$=H$_2$) becomes very unfavorable ($ΔG^\circ$=-42.0 kJ/mol, $[H]$=H$_2$) in the oxidative CODH/ACS pathway. In contrast, several reactions including the oxidation of CO ($ΔG^\circ$=-21.0 kJ/mol, $[H]$=H$_2$) and oxidation of formate ($ΔG^\circ$=-3.4 kJ/mol, $[H]$=H$_2$) become energetically favorable. In the oxidative pathway, the oxidation of CO catalyzed by the CO dehydrogenase is one candidate for a reaction to conserve energy. In the presence of CO, the generation of a proton motive force occurred in membrane vesicles of *Moorella thermoautotrophica* or in cell suspensions of *Acetobacterium woodii*. It has also been reported that *Methanosarcina barkeri*, known as an acetatic methanogen, likely gains ATP through coupling with the oxidation of CO to CO$_2$ via electrochemical membrane potential$^{4,5}$. In the case of syntrophic acetate-oxidizing bacteria, again, it is not known which reaction(s) could couple with the synthesis of ATP during the oxidation of acetate. However, a selective cell extract of *T. phaeum* pre-grown on acetate exhibited a small amount of CO dehydrogenase activity in the membrane fraction (Hattori, unpublished data). Although no definitive conclusion can be drawn, this enzyme might participate in energy conservation during the oxidation of acetate.

Results of the enzyme activities in the three syntrophic acetate-oxidizing bacteria commonly indicated that they operate both the reductive and oxidative CODH/ACS path-
way for their metabolism. These findings seem quite exciting since they may even operate the pathway reversibly using the same biochemical apparatus. Resting cells of *T. phaeum* pre-grown on acetate with the partner methanogens could rapidly oxidize acetate, whereas the same resting cells also produced acetate in the presence of an excess amount of H$_2$/CO$_2$[18]. This simply indicated that *T. phaeum* uses at least a major part of the CODH/ACS pathway's enzymes both in the oxidative and in the reductive direction. However, a shift-back reaction, i.e., from acetogenesis to acetate oxidation, did not occur when resting cells of each of axenically grown *T. phaeum* and the partner methanogens were combined[19]. This indicates that in this strain, some biochemical apparatus required for the oxidation of acetate may be inducible. Shift-back experiments with the strain AOR or *C. ultunense* have yet to be reported. However, it has been reported that these syntrophic acetate oxidizers require a significant lag period for reconstruction of the coculture with the partner methanogens[20]. The lag time required was approximately 20 days in *T. phaeum*[18] and approx. 60 days in *C. ultunense*[21]. From a biochemical point of view, the CO dehydrogenase active stain in strain AOR grown on either acetate or H$_2$/CO$_2$ exhibited two different forms[22]. SDS-PAGE analysis of *T. phaeum* indicated two dense bands in the syntrophic growth condition, although it exhibited basically the same protein profile in both the syntrophic and axenic conditions[23]. In *C. ultunense*, NAD$^+$-dependent methylene-THF dehydrogenase was active in the pure culture whereas NADP$^+$-dependent methylene-THF dehydrogenase was active in the syntrophic coculture[24]. These results indicate that the three syntrophic acetate oxidizers (strain AOR, *C. ultunense*, and *T. phaeum*) are likely to operate the CODH/ACS pathway using different components.

To date, only three acetogens (strain AOR, *C. ultunense*, and *T. phaeum*) are known to operate the CODH/ACS pathway for syntrophic acetate oxidation. Although the other acetogens (e.g. *M. thermoacetica* and *A. woodii*) harbor the reductive CODH/ACS pathway, it has not been reported whether they can operate it in the reverse direction for syntrophic acetate oxidation. Likewise, there are no reports that acetate-oxidizing sulfate reducers (e.g., *Desulfotomaculum acetoxidans* and *Desulfobacterium autotrophicum*) can oxidize acetate in a syntrophic relationship with hydrogenotrophic methanogens, although they harbor the oxidative CODH/ACS pathway[25,26,67]. Similar relationships are also likely to be found between the other syntrophic acetate oxidizers (e.g., *T. lettiformis* and *G. sulfurreducens*) and their relatives (e.g., *Thermotoga aestifera* and *Geoheacter metallireducens*). Although it remains unknown why only these acetate oxidizers can establish a syntrophic relationship with partner microbes, they might harbor crucial biochemical component(s) required for the oxidation. To answer the question, further studies (e.g., a comparative genome analysis of syntrophic and non-syntrophic acetate-oxidizing microbes) will be needed.

### Energy conservation in the syntrophic acetate-oxidizing bacteria

Among fatty acid-oxidizing reactions, the oxidation of acetate coupled with proton reduction (reaction 2, Table 1) is energetically an extremely difficult reaction ($AG^\circ=-104.6$ kJ/mol). However, similar to other fatty acid-oxidizing reactions, the oxidation becomes exergonic if the levels of H$_2$ are kept extremely low by the hydrogen-consuming reaction (reaction 3, Table 1). The $AG^\circ$ value for the all over reaction, i.e., syntrophic acetate oxidation to methanogenesis, is only $-31.8$ kJ/mol (reaction 4, Table 1). If the syntrophic acetate oxidizer and partner methanogens share the energy equally, then the $AG^\circ$ value for each reaction is only $-15.5$ kJ/mol. To maintain the syntrophic relationship, these microbes must maintain free energy changes with negative values ($AG^\circ<0$). Thermodynamically, the energy available to both microbes is affected by changes of temperature and concentrations of substrates and products. For example, the Gibbs free energy change ($AG'$) for syntrophic acetate-oxidation becomes more favorable under low H$_2$ partial pressure and high temperature, while that of the H$_2$-consuming methanogenesis becomes unfavorable under the same conditions (Fig. 4).

![Fig. 4. Effects of temperature and hydrogen partial pressure on the Gibbs free energy change ($AG'$) for the oxidation of acetate to H$_2$/CO$_2$ or methanogenesis from H$_2$/CO$_2$. Acetate oxidation to H$_2$/CO$_2$ at (a) 25°C and (b) 55°C. Methanogenesis from H$_2$/CO$_2$ at (c) 25°C and (d) 55°C. Arrows represent the H$_2$ partial pressure (Pa) at which $AG'$ for the reactions is zero. The stoichiometry of the reactions is given in Table 1. The $AG'$ values for respective reactions were calculated for pH 7, 20 mM acetate, 30 mM bicarbonate, and 35 kPa methane. The $AG'$ values were corrected using both Nernst and van't Hoff equations. The standard Gibbs free energy changes at pH 7 ($AG^\circ$), the standard enthalpy changes ($AS^\circ$) and the standard entropy changes ($AH^\circ$) were calculated using published data from references 39 and 75.](image-url)
This is because the C. ultunense/methanogen coculture involves mesophiles (37°C), and the two reactions catalyzed by these microbes require a much lower H₂ partial pressure than those reactions at high temperature (55°C or 60°C). In the C. ultunense/methanogen coculture, the theoretical limit of H₂ partial pressure was between 0.8 and 18 Pa (calculated under the following conditions: 25 mM acetate, 100 mM HCO₃⁻, and 31 kPa methane). Also, the ΔG' value at which each of the two microbes can fully gain energy was calculated to be -17 kJ/mol. This value is far lower than that for H₂-consuming acetogenesis (ΔG''=-104.6 kJ/mol) or for H₂-consuming methanogenesis (ΔG''=-135.6 kJ/mol). Furthermore, the ΔG' value (-17 kJ/mol) is even below the energy required for the synthesis of 1 mol of ATP (ΔG'' for the synthesis of ATP in the living cell is +60 to 70 kJ/mol)\(^{58,79}\). However, assuming that the hydrolysis of 1 mol of ATP is coupled to the translocation of three (or four) protons across the charged cytoplasmic membrane\(^ {69,77}\), and that one proton is considered the minimum quantum equivalent to the energy required for the synthesis of one third of ATP (ΔG''=+20 KJ/mol)\(^ {38}\), this small amount of energy may even support their growth. Supporting this idea, it has been reported that cocultures of syntrophic butyrate- (or benzoate-) oxidizing bacteria and H₂-consuming partner microbes yielded quite small amounts of energy (below ~20 kJ/mol) when they were grown under some stressed conditions\(^ {29}\). The C. ultunense/methanogen coculture exhibited extremely slow growth (doubling time=20 to 25 days)\(^ {62}\), indicating that they may be actually thriving at near the thermodynamically equilibrium level.

Formate as well as H₂ is likely to be an important factor for energy conservation in syntrophic acetate oxidation. Since the standard redox potential of CO₃/formate (E°= -432 mV) is nearly equivalent to that of H₂/H₂ (E°=+414 mV)\(^ {93}\), acetate oxidation with bicarbonate reduction (acetate+2HCO₃⁻→4HCOO⁻+H₂, ΔG°=+99.1 kJ/mol) is energetically close to that with proton reduction (ΔG°=+104.6 kJ/mol). In the coculture of T. phaeum and M. thermautotrophicus strain TM (H₂ and formate utilizer), a very low level of formate (5 to 9 μM) was detected during syntrophic acetate oxidation (Fig. 2)\(^ {20}\). It was calculated that the theoretical limit of the formate concentration in the T. phaeum/strain TM coculture was 0.7 to 20 μM, which gave a maximum ΔG' value (-15.2 kJ/mol) for each of the two microbes (calculated under the following conditions: 20 mM acetate, 55 mM HCO₃⁻, and 20 kPa methane). Since the acetate oxidation rate was significantly higher in the T. phaeum/strain TM coculture than T. phaeum/M. thermautotrophicus strain ΔH (H₂ utilizer) coculture\(^ {20}\), formate may also play an important role in an alternative electron shuttle between T. phaeum and formate-utilizing methanogens. In contrast, no formate was detected in the strain AOR/methanogen coculture during syntrophic acetate oxidation\(^ {37}\). Also, the acetate oxidation rate with AOR/strain ΔH was not distinguishable from that obtained with AOR/M. thermautotrophicus strain TH (formate utilizer). Thus, the energetic situation might be different among each syntrophic acetate-oxidizing species at the biochemical level.

The question remains of how much available energy is shared between the syntrophic acetate oxidizer and the partner methanogens. It seems quite difficult to quantify the amount of energy shared by each microbe. However, comparing cell numbers and/or cell protein levels between the acetate syntroph and partner methanogens, it might be possible to roughly estimate the amount of energy. Strain AOR comprised 50 to 60% of the total cell number (i.e., the acetate oxidizer and methanogens) when the coculture was grown on ethanol\(^ {36}\). Also, a selective cell extract of T. phaeum and M. thermautotrophicus strain TM grown on acetate showed that the protein/protein ratio between the bacteria and methanogens is approximately 1:1.2 (Hattori, unpublished data). Although these observations do not provide an answer to the question above, the syntrophic acetate oxidizer and the partner methanogens might actually equally share the extremely small energy exploited from the oxidation of acetate.

**Ecology of the syntrophic acetate-oxidizing bacteria**

Although pure cultures of the syntrophic acetate-oxidizing bacteria provided us relatively much information (e.g., physiology, biochemical pathway, and energetics), the ecological nature of their habitats is still unclear. Assuming that the syntrophs are actually active as syntrophic acetate oxidizers, their main competitors are the aceticlastic methanogens including the genera Methanosarcina and Methanosaeta. The aceticlastic methanogens generally have the following properties. Methanosaeta utilize exclusively acetate while Methanosarcina can grow on several substrates other than acetate. The former methanogens have a low growth rate but high affinity for acetate\(^ {53}\). Thus, the growth of aceticlastic methanogens is affected by the concentration of acetate. Peterson and Ahring (1991)\(^ {51}\) reported that the recovery of \(^{14}\)CO₂ from \(^{2-14}\)C acetate was increased in a genus Methanosaeta-free thermophilic (60°C) acetate chemostat when the acetate concentration was less than approximately 1 mM. This indicates that the syntrophic acetate oxidizer dominates over aceticlastic methanogens at low concentrations of acetate under thermophilic conditions. Actually, strain AOR consumed acetate below 0.1 mM\(^ {59}\), which is comparable to that of the genus Methanosaeta. Since syntrophic acetate oxidation becomes energetically favorable at elevated temperatures (Fig. 4), these conditions (a low concentration of acetate with a high temperature) are likely to provide a suitable environment for syntrophic acetate oxidizers. In addition, ammonia and volatile fatty acids are considered important factors for acetate metabolism. Aceticlastic methanogens are also known to be more sensitive to these compounds than hydrogenotrophic methanogens\(^ {32,67}\). It has been reported that in several mesophilic biogas reactors, the recovery of \(^{14}\)CO₂ from \(^{2-14}\)C acetate was enhanced by increases of ammonium and potassium concentrations\(^ {93}\). A similar observation was made in another mesophilic and thermophilic reactor in which Methanosaeta was absent but a hydrogenotrophic methanogen species was present\(^ {11}\). Changes in the archaeal community from the genus Methanosarcina to hydrogenotrophic methanogen species were also observed in an anaerobic sequencing batch reactor after the concentration of ammonium increased\(^ {11}\). These results indicate that the syntrophic acetate oxidizer become able to cooperate with hydrogenotrophic methanogens, and domi-
nate the acetoclastic methanogens at high ammonium concentrations at which the acetoclastic methanogens are inhibited. It should be also considered that the acetate dilution rate is an important factor for syntrophic acetate oxidation. Shigematsu et al., (2004) reported that the recovery of $^{13}$CO$_2$ from [2-13C] acetate was higher at a low dilution rate (0.025 day$^{-1}$) than high dilution rate (0.6 days$^{-1}$) in an anaerobic mesophilic (37°C) acetate-fed chemostat. Since C. ultunense (mesophilic syntrophic acetate oxidizer) is considered to be an extremely slow grower (µCH$_4$<0.035 days$^{-1}$), a static environment is likely to be required for the mesophilic syntrophs to increase their cell material sufficiently using acetate. Although the above observations were made in artificial environments (methanogenic reactors), recently it has been reported that syntrophic acetate oxidation could also occur in natural environments. Nüsslein et al. (2001) reported that a significant proportion (30–60%) of [2-14C] acetate was converted to 14CO$_2$ in subtropical lake sediment (15°C) in which sulfate and other suitable electron acceptors were absent. Although such a low temperature is thermodynamically unfavorable for syntrophic acetate oxidation, syntrophs may be thriving in these natural habitats. It should be mentioned that T. lettingae was originally obtained from a thermophilic sulfate-reducing bioreactor fed with methanol. Thus, this type of acetate syntroph may actually live as a methanol degrader in nature rather than as a syntrophic acetate oxidizer.

Since the previously isolated syntrophic acetate oxidizers (except T. lettingae) coupled with partner methanogens turned out to be acetogens, one may expect that they are actually likely to survive by using autotrophic or heterotrophic acetogenic substrates, both of which many acetogens use. In the case of autotrophic substrates, these may have to compete with hydrogenotrophic microbes such as methanogens and sulfate reducers. In general, acetogens are often outcompeted by these competitors in many habitats due to thermodynamic disadvantages or low affinity for hydrogen. However, some exceptional environmental conditions (e.g., low temperature, slightly acidic pH, and/or sulfate depleted conditions) could allow the acetogens to dominate over these competitors. Although the three previously isolated acetate syntrophs (strain AOR, C. ultunense, and T. phaeum) have the ability to utilize hydrogen/CO$_2$, they may not grow on this substrate in nature, since it supports very little if any growth in vitro. In the case of heterotrophic substrates, these may have to compete with primary and secondary fermenting bacteria. However, they may dominate over competitors since some genera of acetogens are known to be able to utilize two or more substrates simultaneously. Referring to previously described syntrophic acetate oxidizers, it has not been reported whether syntrophs support mixotrophic growth (i.e., simultaneous utilization of acetogenic substrates) or not. However, considering that the isolated acetate syntrophs can use various heterotrophic substrates, they may utilize various acetogenic substrate(s) to survive in environments in which primary and secondary fermenters are present.

Among the syntrophic acetate-oxidizing bacteria, it is still unclear which lifestyle (axenic or syntrophic manner) predominates in the in-situ environment. In any case, the syntrophs may increase their survivability in natural environments by using their special physiological trait, i.e., the ability to utilize various substrates in pure culture, or an ability to utilize acetate in syntrophic interactions with partner methanogens.

**Concluding remarks**

Recently, several culture-independent molecular studies have indicated that microbes which are phylogenetically related to previously isolated syntrophic acetate-oxidizing bacteria are present in some environments including methanogenic reactors, leachate, and an oil reservoir. Also, applications of a functional gene for formyl-THF synthetase implied the presence of phylogenetically diverse acetogens in several natural habitats. Considering that many previously isolated syntrophic acetate-oxidizing bacteria are acetogens, these acetate syntrophs may potentially be distributed more widely in various environments than previously thought. Moreover, very recently, several studies using a stable isotope probing (SIP) method indicate that syntrophic acetate oxidation in some environments (soils and lake sediment) is likely to be carried out by non-acetogens (e.g., the genera Geobacter, Anaeromicrobacter, and Syntrophus). Thus, it should be considered that not only acetogens but also other trophic groups of bacteria including T. lettingae may play a significant role in syntrophic acetate oxidation. To know more about the nature of these syntrophs, more isolates are needed, since they will provide valuable information such as complete genome sequences. For example, elucidation of complete genome sequences for syntrophic acetate oxidizers will enable us to compare their sequences with genomic sequences of “non-syntrophic acetate-oxidizing” acetogens (e.g., M. thermoacetica), which may lead to the discovery of genes specific for syntrophic acetate oxidation. Various isolation strategies with the aid of ecophysiological techniques (e.g., microautoradiography with FISH and DNA-SIP) and/or other molecular-based techniques may achieve successful isolation of this fastidious but fascinating physiological microbial group.

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