

Regulation of Caffeate Respiration in the Acetogenic Bacterium *Acetobacterium woodii*[∇]

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The anaerobic acetogenic bacterium *Acetobacterium woodii* can conserve energy by oxidation of various substrates coupled to either carbonate or caffeate respiration. We used a cell suspension system to study the regulation and kinetics of induction of caffeate respiration. After addition of caffeate to suspensions of fructose-grown cells, there was a lag phase of about 90 min before caffeate reduction commenced. However, in the presence of tetracycline caffeate was not reduced, indicating that de novo protein synthesis is required for the ability to respire caffeate. Induction also took place in the presence of CO₂, and once a culture was induced, caffeate and CO₂ were used simultaneously as electron acceptors. Induction of caffeate reduction was also observed with H₂ plus CO₂ as the substrate, but the lag phase was much longer. Again, caffeate and CO₂ were used simultaneously as electron acceptors. In contrast, during oxidation of methyl groups derived from methanol or betaine, acetogenesis was the preferred energy-conserving pathway, and caffeate reduction started only after acetogenesis was completed. The differential flow of reductants was also observed with suspensions of resting cells in which caffeate reduction was induced prior to harvest of the cells. These cell suspensions utilized caffeate and CO₂ simultaneously with fructose or hydrogen as electron donors, but CO₂ was preferred over caffeate during methyl group oxidation. Caffeate-induced resting cells could reduce caffeate and also *p*-coumarate or ferulate with hydrogen as the electron donor. *p*-Coumarate or ferulate also served as an inducer for caffeate reduction. Interestingly, caffeate-induced cells reduced ferulate in the absence of an external reductant, indicating that caffeate also induces the enzymes required for oxidation of the methyl group of ferulate.

Acetogens are strictly anaerobic bacteria that are key players in the anaerobic food web. They are nutritionally very versatile and ferment compounds such as sugars, alcohols, or C₁ compounds to acetate (and CO₂), which is then converted to methane by methanogenic archaea in nonmarine ecosystems. Acetogenic bacteria are characterized by a unique pathway, the Wood-Ljungdahl pathway, that enables the use of CO₂ as an electron acceptor during heterotrophic growth as well as during autotrophic growth. During heterotrophic growth, hexoses are oxidized to 2 mol pyruvate via the Embden-Meyerhof-Parnas pathway, and pyruvate is subsequently oxidized to acetyl-coenzyme A (acetyl-CoA), CO₂, and reduced ferredoxin. Acetyl-CoA is converted to acetate by the enzymes phosphotransacetylase and acetate kinase, and the latter reaction is coupled to substrate level phosphorylation. The electrons are funneled to CO₂, which is reduced to acetate in the Wood-Ljungdahl pathway according to:



In summary, 1 mol of sugar is converted to 3 mol of acetate. Due to the exclusive formation of acetate this fermentation is referred to as homoacetogenesis (14, 26, 33, 34, 37, 39, 40).

The Wood-Ljungdahl pathway not only uses electrons derived from organic compounds but also hydrogen and thus enables chemolithoautotrophic growth on H₂ plus CO₂ according to:



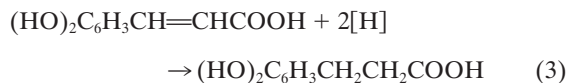
The Wood-Ljungdahl pathway is coupled to a chemiosmotic mechanism of ATP synthesis, but the way that the ion gradients are established across the cytoplasmic membrane varies in acetogens (37). Some organisms, the so-called “proton organisms,” represented by the model organism *Moorella thermoacetica*, have a cytochrome-containing, proton motive electron transport chain with methylene tetrahydrofolate as the electron acceptor (4, 19, 23–25). The other class is the “sodium ion organisms,” which are strictly Na⁺ dependent. The model organism *Acetobacterium woodii* couples the Wood-Ljungdahl pathway to the generation of a primary sodium ion potential, which in turn drives ATP synthesis via an Na⁺-translocating ATP synthase of the F₁F₀ type (2, 21, 22, 38, 41). The Na⁺-translocating reaction of the pathway is unknown.

In recent years evidence has accumulated that some acetogens can use not only CO₂ but also alternative electron acceptors, such as aromatic acrylate groups (the acrylate side chain of aromatic compounds), fumarate, dimethyl sulfoxide, or nitrate (3, 5, 10, 11, 17, 35, 43, 47). *A. woodii* is known to reduce caffeate, which is a degradation product of lignin and is readily available in its natural habitat. The electrons can be derived from various donors, such as fructose, methanol, or hydrogen,

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and are used to reduce the double bond in caffeate, yielding hydrocaffeate according to equation 3 (3):



Experiments with cell suspensions of *A. woodii* revealed that caffeate reduction with hydrogen as the electron donor is coupled to the generation of a primary sodium ion potential across the cytoplasmic membrane that in turn drives ATP synthesis (28). Therefore, this process is also referred to as caffeate respiration. The enzymes involved in hydrogen-mediated caffeate reduction, as well as the Na^+ -translocating step, remain to be identified.

The ability of *A. woodii* to reduce at least two different electron acceptors poses a question about the regulation of the utilization of the electron acceptors. Earlier work based on growth experiments suggested that caffeate reduction in *A. woodii* is not constitutive but is induced in the presence of caffeate (9). However, despite its ecological impact little is known about the regulation of caffeate and CO_2 reduction in *A. woodii*. Here, we addressed the regulation of the utilization of the two electron acceptors in *A. woodii* at the level of enzyme synthesis, as well as at the enzyme activity level.

MATERIALS AND METHODS

Organism and cultivation. *A. woodii* DSMZ 1030 was cultivated at 30°C in 1.2-liter flasks (Müller-Krempel, Bülach, Switzerland). The medium was prepared using anaerobic techniques, as described previously (7, 27). The medium contained (under an N_2 - CO_2 atmosphere [80:20, vol/vol]) (per liter) 1.76 g KH_2PO_4 , 8.44 g K_2HPO_4 , 1.0 g NH_4Cl , 0.5 g cysteine hydrochloride, 0.33 g MgSO_4 , 2.9 g NaCl, 2.0 g yeast extract, 6.0 g KHCO_3 , 0.001 g resazurin, 1.0 ml trace element solution SL 9 (47), 1.0 ml selenite-tungstate solution (47), and 2.0 ml vitamin solution DSMZ 141. The pH was adjusted to 7.1 to 7.2 with HCl. Fructose, methanol, and betaine were used as carbon and energy sources at final concentrations of 20, 60, and 50 mM, respectively. Growth was monitored by measuring the optical density at 600 nm (OD_{600}).

Preparation of cell suspensions. All manipulations were done under strictly anaerobic conditions in an anaerobic chamber (Coy, Grass Lake, MI). For analyzing the induction of caffeate reduction cells were grown to an OD_{600} of 1.8 to 2.0. Cultures were harvested anaerobically by centrifugation ($2,700 \times g$, 15 min, 4°C) and washed three times with imidazole-HCl buffer (50 mM imidazole-HCl, 20 mM MgSO_4 , 5 mM dithioerythritol, 1 mg/liter of resazurin; pH 7.0). Cells were resuspended in medium to a final protein concentration of 20 to 30 mg/ml under an N_2 - CO_2 atmosphere (80:20, vol/vol). The suspension was kept on ice and used immediately.

For experiments with caffeate-induced cells cultures were grown to an OD_{600} of 0.3 to 0.4. Then caffeate was added from a 0.2 M stock solution to induce the ability of the cells to reduce caffeate. Cultures were harvested anaerobically at the end of the exponential growth phase (OD_{600} , 1.8 to 2.0) by centrifugation ($2,700 \times g$, 15 min, 4°C) and washed twice with imidazole-HCl buffer (see above). The cells were resuspended in the same buffer to a final protein concentration of 11 to 16 mg/ml. This suspension was kept on ice under an N_2 atmosphere and used immediately for the experiments. The protein concentrations of cell suspensions were determined as described previously (42).

Induction of caffeate reduction in cell suspensions. Experiments were carried out in 120-ml serum bottles containing medium (final volume, 50 ml). The medium was incubated at 30°C under an N_2 , H_2 , or H_2 - CO_2 (80:20, vol/vol) atmosphere as specified below. When CO_2 was used, the buffer contained 60 mM bicarbonate, unless stated otherwise. Cells were added from the concentrated cell suspension to a final protein concentration of 1 mg/ml. Fructose, methanol, betaine, and H_2 - CO_2 were added as indicated below. After 15 min of incubation, caffeate was added (from a 1 M stock solution in dimethyl sulfoxide) as indicated below.

Experiments with caffeate-induced cell suspensions. All experiments were performed in 58-ml bottles under an N_2 or N_2 - CO_2 (80:20, vol/vol) atmosphere. The mixtures (final volume, 10.5 ml) contained 9.5 ml of imidazole-HCl buffer

(pH 7.0) and 1 ml of the concentrated cell suspension or 9 ml of buffer, 1 ml of the concentrated cell suspension, and 0.5 ml of caffeate, ferulate, or *p*-coumarate (from a 0.2 M stock solution). All suspensions were supplemented with 50 mM NaCl. After incubation of the bottles for 10 min at 30°C in a shaking water bath, the gas atmosphere was changed to H_2 or H_2 - CO_2 (80:20, vol/vol) by extensive gassing for 3 min. Then caffeate, ferulate, or *p*-coumarate was added.

Determination of caffeate concentration. Samples (0.2 ml) were withdrawn with a syringe, and the cells were removed by centrifugation at $19,000 \times g$ for 10 min. The caffeate concentration was determined photometrically at 312 nm using an extinction coefficient of $13.72 \text{ mM}^{-1} \text{ cm}^{-1}$.

Determination of acetate and fructose concentrations. Samples (0.5 ml) were withdrawn with a syringe, and the cells were removed by centrifugation ($19,000 \times g$, 10 min). Acetate and fructose concentrations were determined by coupled enzymatic assays using an acetic acid or fructose determination kit from R-biopharm according to the instructions of the manufacturer.

Electrophoresis and Western blotting. Samples (0.8 ml) were withdrawn and centrifuged at $19,000 \times g$ for 10 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (32). Western blotting was performed as described previously (46) using an ECL detection reagent that was either purchased from PerkinElmer Life Sciences (Boston, MA) or made by using the following solutions: 4 ml of solution A (200 ml containing 0.1 M Tris-HCl [pH 6.8] and 50 mg luminol), 1.2 μl of H_2O_2 (30%), and 400 μl of solution B (10 ml of dimethyl sulfoxide containing 11 mg of *para*-hydroxycoumaric acid). Blot membranes were incubated in the solutions for 2 min before exposure to WICORex film (Typon Imaging AG, Burgdorf, Switzerland).

Statistics. The data presented below are representative of at least three independent experiments, each performed using a different cell culture.

Chemicals and gases. Chemicals were purchased from Roth (Karlsruhe, Germany), Merck (Ismaning, Germany), or Sigma (Taufkirchen, Germany). The purity of caffeate as determined by high-performance liquid chromatography was >99%. Gases were purchased from Air Liquide (Krefeld, Germany).

RESULTS

Experimental setup. Cells of *A. woodii* grown on fructose in the presence of 60 mM bicarbonate were harvested and resuspended in medium containing 60 mM bicarbonate under an N_2 - CO_2 (80:20, vol/vol) atmosphere to a final protein concentration of 1 mg/ml. These cell suspensions were capable of electron donor-mediated reduction of caffeate (see below), but caffeate concentrations above 5 mM were inhibitory and led to long lag phases and reduced rates of caffeate reduction. In contrast, when used at concentrations up to 3 mM, caffeate did not have an inhibitory effect (data not shown). Therefore, the subsequent experiments were performed with caffeate at concentrations up to 3 mM.

Caffeate respiration is induced in the presence of bicarbonate and fructose. When fructose and caffeate were added to cell suspensions incubated in the presence of 60 mM bicarbonate, fructose consumption and acetate formation started immediately and continued for 240 min at a rate of $88 \text{ nmol fructose} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ until the fructose was consumed. At the end of the experiment, a stoichiometry of fructose to acetate of 1:2.75 was obtained. In contrast to acetogenesis, the caffeate concentration did not decrease immediately but remained constant for about 60 min (Fig. 1). Thereafter, caffeate was reduced at a constant rate of $7.7 \text{ nmol caffeate} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ for about 240 min.

The lag phase in caffeate reduction indicates that there is an induction process and may reflect the time required for induction of the enzymes catalyzing caffeate reduction. The fact that *de novo* synthesis of proteins is indeed required was shown by the inhibition of caffeate reduction in the presence of the antibiotic tetracycline (data not shown). At the same time, CO_2 reduction, which is accomplished by the constitutive Wood-

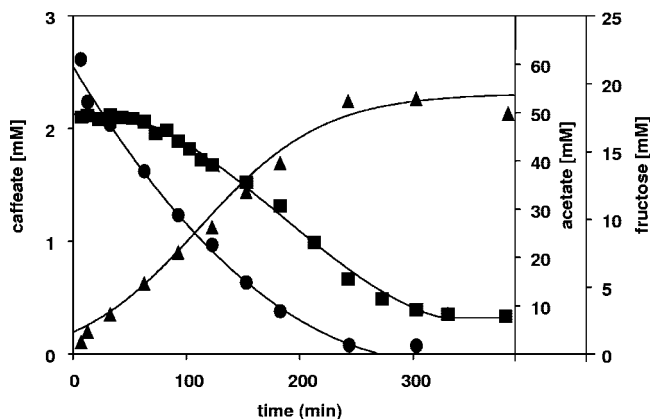


FIG. 1. Induction of caffeate respiration during acetogenesis from fructose by *A. woodii*. Cell suspensions of *A. woodii* (1.0 mg protein/ml) grown on fructose were incubated under an N_2 - CO_2 atmosphere at 30°C in a shaking water bath in the presence of 20 mM fructose and 2 mM caffeate. At the times indicated, samples were withdrawn and analyzed to determine the caffeate (■), fructose (●), and acetate (▲) concentrations as described in Materials and Methods.

Ljungdahl pathway, was not affected by tetracycline. Furthermore, the amounts of the flavoproteins EtfA and EtfB, which are found only in caffeate-grown cells of *A. woodii* (F. Imkamp and V. Müller, unpublished data), increased from nondetectable to the maximal level within 60 min after caffeate addition (data not shown).

These data demonstrate that caffeate reduction is induced during fructose consumption and acetogenesis. After the induction process was completed, caffeate consumption and acetogenesis occurred simultaneously, indicating that the cells did not discriminate between CO_2 and caffeate as electron acceptors. This can be concluded from the active acetogenesis during caffeate reduction and the rate of fructose consumption that exceeded by far the amount of fructose required to reduce the 2 mM caffeate present in the assay mixture. Furthermore, variation of the bicarbonate concentration from 0 to 60 mM had no effect on the length of time before caffeate reduction started or on the rates of caffeate reduction (data not shown).

Caffeate respiration is induced in the presence of carbon dioxide and hydrogen. Upon addition of H_2 plus CO_2 to cell suspensions prepared from fructose-grown cells, acetate formation started immediately (Fig. 2). As observed before with fructose as the electron donor, caffeate reduction was induced during acetogenesis from H_2 plus CO_2 , again indicating that CO_2 does not affect the cells' ability to induce caffeate respiration. This is also reflected by the fact that bicarbonate concentrations ranging from 10 to 60 mM had no influence on the length of time before caffeate reduction started (data not shown). After the ability to reduce caffeate was induced, caffeate was consumed at a rate of $5.1 \text{ nmol caffeate} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, and CO_2 and caffeate were used simultaneously as electron acceptors.

When cells were incubated in the presence of carbon dioxide and caffeate or in the presence of caffeate without an electron donor, neither acetogenesis nor caffeate reduction was observed at physiologically significant rates. However, addition of H_2 led to both acetate formation and caffeate reduction. The

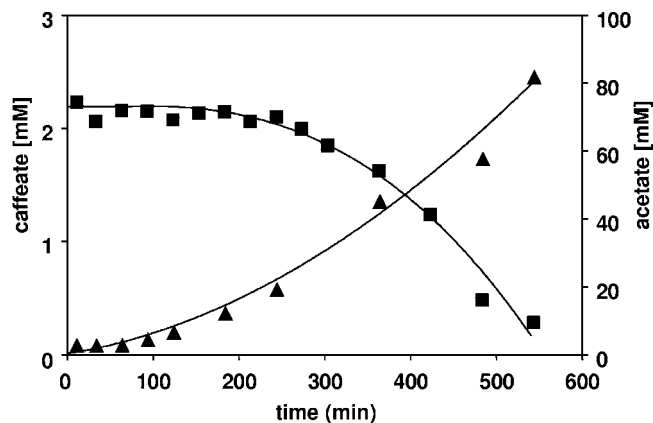


FIG. 2. Induction of caffeate respiration during acetogenesis from H_2 plus CO_2 by cell suspensions of *A. woodii*. Cell suspensions of *A. woodii* (1.0 mg protein/ml) grown on fructose were incubated under an H_2 - CO_2 atmosphere at 30°C in a shaking water bath. Caffeate was added from a stock solution. At the times indicated, samples were withdrawn and analyzed to determine the caffeate (■) and acetate (▲) concentrations as described in Materials and Methods.

time required for induction of caffeate reduction in the presence of H_2 plus CO_2 was much longer than the time required with fructose (Fig. 3). This could reflect the different energy status during homoacetogenesis from sugars ($\Delta G^{0'} = -311 \text{ kJ/mol}$) compared to acetogenesis from H_2 plus CO_2 ($\Delta G^{0'} = -105 \text{ kJ/mol}$). Interestingly, when cells were incubated in the absence of CO_2 but in the presence of H_2 , caffeate reduction was impaired (Fig. 3). To analyze this effect, substrate combinations were used. *A. woodii* is known to grow mixotrophically on fructose and H_2 plus CO_2 (6). Under these conditions, caffeate respiration was induced, as observed previously (Fig. 4). When CO_2 was omitted, fructose consumption and acetogenesis were largely inhibited. This was due to inhibition of

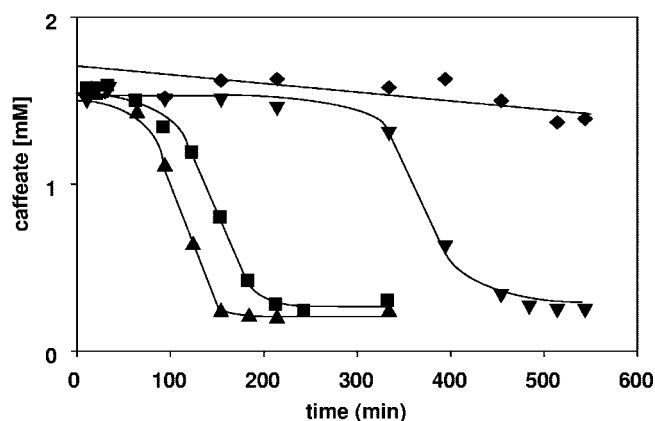


FIG. 3. Time course of caffeate reduction with fructose or hydrogen as the electron donor by cell suspensions of *A. woodii* incubated in the absence or presence of CO_2 . Cell suspensions of *A. woodii* (1.0 mg protein/ml) grown on fructose were preincubated either under an H_2 atmosphere (◆), under an H_2 - CO_2 atmosphere (▼), under an N_2 - CO_2 atmosphere with fructose (20 mM) (■), or under an N_2 atmosphere with fructose (▲) for 10 min. Caffeate (2 mM) was added from a stock solution. At the times indicated, samples were withdrawn and analyzed to determine the caffeate concentration as described in Materials and Methods.

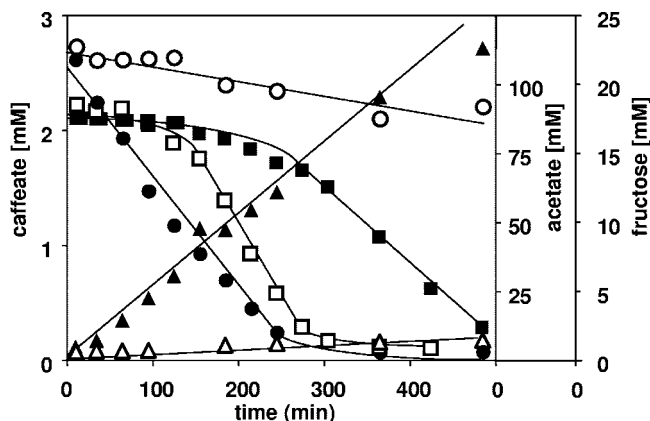
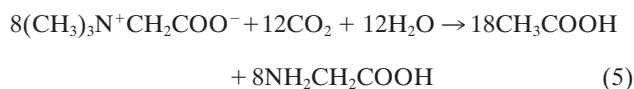


FIG. 4. Induction of caffeate respiration during acetogenesis with H₂, CO₂, and fructose or with H₂ and fructose by cell suspensions of *A. woodii*. Cells were grown on fructose and harvested, and cell suspensions (1.0 mg protein/ml) were incubated with fructose, H₂, and CO₂ (solid symbols) or with fructose and H₂ (open symbols) at 30°C in a shaking water bath for 10 min. Caffeate (2 mM) was added from a stock solution. At the times indicated, samples were withdrawn and analyzed to determine the caffeate (squares), acetate (triangles), and fructose (circles) concentrations as described in Materials and Methods.

fermentation by the presence of hydrogen (6, 8). However, the low rate of acetogenesis was apparently sufficient to induce caffeate respiration. Once caffeate reduction was induced, the rate was two times higher in the absence of CO₂ than in the presence of CO₂, again indicating that there is simultaneous use of CO₂ and caffeate as electron acceptors and competition in both systems for electrons (Fig. 4).

CO₂ is preferred over caffeate as an electron acceptor during methyl group oxidation. *A. woodii* is able to grow on methyl group-containing compounds, such as methanol or betaine, according to:



The methyl group of methanol or betaine is disproportionated. One mole is oxidized to CO₂, and the six electrons generated are used to reduce 3 mol of CO₂ to CO that is combined with three additional methyl groups and HS-CoA to give rise to 3 mol of acetyl-CoA.

When methanol-grown cells were incubated in the presence of methanol and CO₂, acetate formation started immediately and proceeded at a rate of 92.6 nmol · min⁻¹ · mg protein⁻¹ for about 7 h. In contrast to what was observed previously with fructose or H₂ (plus CO₂) as an electron donor, caffeate consumption started only after acetogenesis was complete (Fig. 5A), indicating that CO₂ is the preferred electron acceptor during methanol conversion and blocks the induction of caffeate reduction. The same phenomenon was observed with betaine as a substrate. Betaine was consumed much faster than methanol, but caffeate reduction started only after acetogenesis was complete (Fig. 5B).

Regulation of caffeate and CO₂ reduction in resting cells.

The experiments described above demonstrated that there was inhibition of caffeate reduction in the presence of hydrogen or methanol. To determine whether this was due to regulation of enzyme synthesis or activity, we analyzed the effect of the electron donor on caffeate reduction in resting cells of *A. woodii* that had been pregrown on fructose plus caffeate to induce the caffeate reduction machinery. These cells were harvested and resuspended in buffer.

Upon addition of caffeate to resting cells incubated with H₂, with H₂ and CO₂ (no bicarbonate in the buffer), or with H₂, CO₂ and bicarbonate, caffeate reduction started immediately in every case and proceeded at identical rates until caffeate was completely reduced (data not shown). At the same time that caffeate was reduced, cells produced acetate from H₂ and CO₂ or from H₂, CO₂, and bicarbonate. As expected, the rates of acetogenesis and the final yields were decreased in the presence of caffeate. These experiments clearly demonstrate that caffeate and bicarbonate are used simultaneously as electron acceptors during hydrogen oxidation.

When cells were pregrown on betaine plus caffeate, they produced acetate from betaine immediately after addition of betaine. In contrast, caffeate reduction started only after about 200 min, at a time when acetogenesis was complete. When CO₂

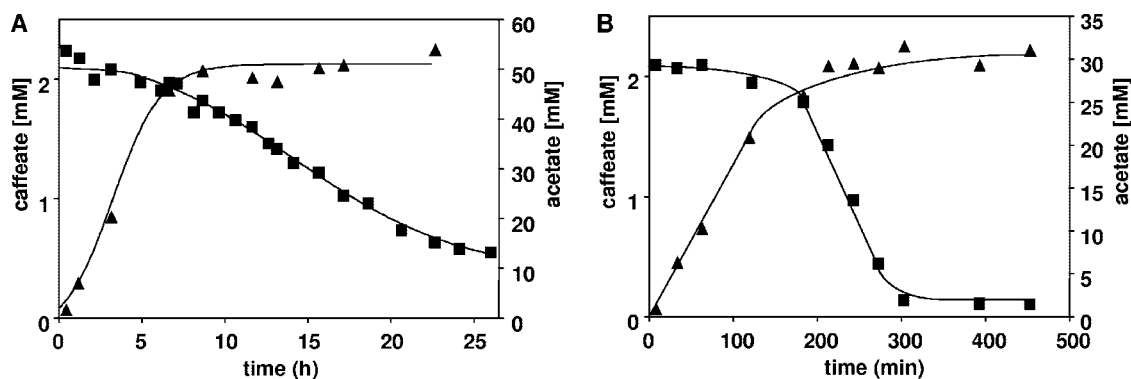


FIG. 5. Induction of caffeate respiration during acetogenesis from methanol (A) or betaine (B) by cell suspensions of *A. woodii*. Cell suspensions of *A. woodii* (1.0 mg protein/ml) grown on methanol or betaine were incubated at 30°C in a shaking water bath. Caffeate was added from a stock solution. At the times indicated, samples were withdrawn and analyzed to determine the caffeate (■) and acetate (▲) concentrations as described in Materials and Methods.

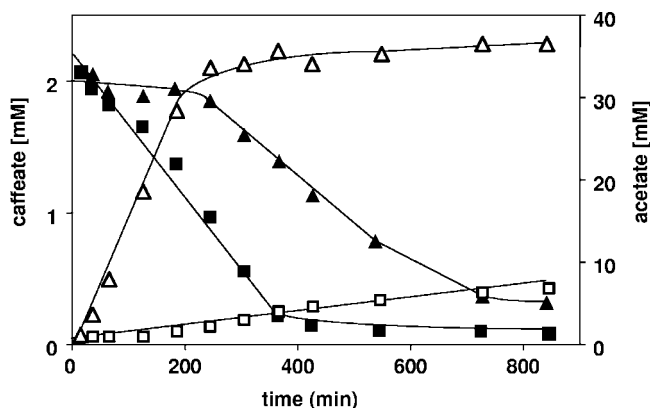


FIG. 6. Caffeate respiration with betaine as the reductant is impaired by CO_2 . *A. woodii* was grown with betaine plus caffeine to preinduce the caffeine reduction machinery and harvested. Cell suspensions (1.0 mg protein/ml) were incubated in medium containing 50 mM betaine in the absence (squares) or presence (triangles) of 60 mM bicarbonate at 30°C in a shaking water bath. After preincubation for 10 min, 2 mM caffeine was added from a stock solution. At the times indicated, samples were withdrawn and analyzed to determine the caffeine (solid symbols) and acetate (open symbols) concentrations.

(and bicarbonate) was omitted from the assay medium, caffeine reduction started immediately, while acetogenesis was largely impaired (Fig. 6). This experiment demonstrates that CO_2 is preferred over caffeine with betaine as the electron donor and indicates that CO_2 has a regulatory effect on enzymatic activities necessary for caffeine respiration.

Specificity of the caffeine-reducing system. Next, we analyzed whether caffeine-induced cells were able to reduce *p*-coumarate or ferulate, which are analogues of caffeine and also have the acrylate side chain. As Fig. 7A shows, caffeine-induced cells incubated under a hydrogen atmosphere in buffer were able to reduce *p*-coumarate or ferulate without a lag phase, and the rates of reduction were comparable to the caffeine reduction rates. In addition, *p*-coumarate-induced cells reduced *p*-coumarate, ferulate, and caffeine, and ferulate-induced cells reduced ferulate, caffeine, or *p*-coumarate (data not shown). Control experiments demonstrated that hydrogen is absolutely required as a reductant for caffeine and *p*-couma-

rate. However, much to our surprise, cells pregrown on fructose plus caffeine reduced ferulate in the absence of hydrogen (Fig. 7B). The reduction rate was 40% higher than the rate observed with hydrogen as the reductant. Since the only source for reducing equivalents was the oxidation of the methyl group of ferulate to CO_2 , this experiment indicated that caffeine also induces the enzymes required for oxidation of the methyl group of ferulate. A small amount of caffeine reduction in the absence of added reductant was observed quite often, but at different (low) rates. This might have resulted from endogenous reducing equivalents.

DISCUSSION

Here, we demonstrated that in *A. woodii* de novo synthesis of proteins is required to induce the ability to reduce caffeine. In a strict sense, induction cannot be differentiated from de-repression in our assays; this would require knowledge of genes and activators, as well as a detailed molecular analysis. However, it is clear that caffeine (directly or indirectly) activates expression of genes involved in caffeine reduction, such as a gene encoding a hypothetical caffeine reductase (and the electron transfer flavoprotein that probably mediates electron transfer from NADH to caffeine [Imkamp and Müller, unpublished]). Structural analogues of caffeine, such as ferulate and *p*-coumarate, also serve as inducers for caffeine reduction. This could be interpreted to result from isoenzymes that have limited catalytic specificity and which are induced by a cognate substrate (caffeine, ferulate, or *p*-coumarate). In contrast, it is also feasible that all inducers have the same effect. In addition to caffeine, ferulate, or *p*-coumarate, trimethoxycinnamate is able to act as an inducer for caffeine reduction in *A. woodii* (9).

As observed previously in *A. woodii* strain Nzva16, acetogenesis from H_2 plus CO_2 and H_2 -dependent caffeine reduction were catalyzed simultaneously by *A. woodii* DSM1030 (20). In addition, we show here that the induction of caffeine reduction is independent of the bicarbonate concentration. The same is true for induction of caffeine reduction during acetogenesis from fructose. Acetogens compete in their environment for CO_2 as an electron sink with, for example, methanogens. Thermodynamically, methanogenesis ($\Delta G^{\circ\prime} = -135$

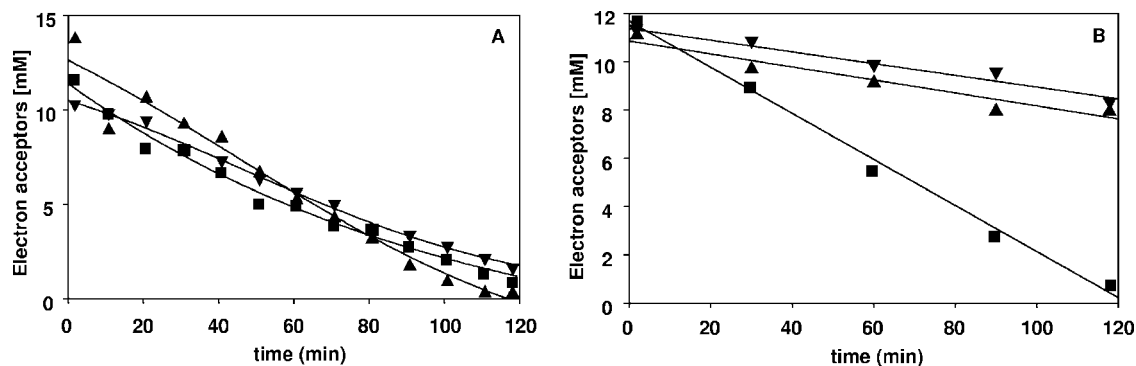


FIG. 7. Reduction of acrylate group-containing compounds by caffeine-preinduced resting cells of *A. woodii* in the presence of H_2 (A) or N_2 (B). Cell suspensions of *A. woodii* (1.35 mg/ml) were incubated in imidazole-HCl buffer at 30°C in a shaking water bath. Caffeine, *p*-coumarate, and ferulate were added from stock solutions to a final concentration of 10 mM. Samples were withdrawn at the times indicated and analyzed to determine the caffeine (▲), *p*-coumarate (▼), and ferulate (■) concentrations.

kJ/mol) is preferred over acetogenesis ($\Delta G^{\circ} = -105$ kJ/mol), and therefore, methanogens would outcompete acetogens (12, 13). The simultaneous use of different electron acceptors enhances the competitiveness of acetogens in their anaerobic environment (14). The simultaneous use of different electron acceptors has been observed before in acetogens. *Clostridium formicoaceticum* uses fumarate and CO₂ simultaneously in undefined medium as electron sinks (35). *Ruminococcus productus* is capable of growth with ferulate using the methyl group of ferulate as a sole carbon and energy source (36). The acrylate side chain of ferulate is used as a terminal electron acceptor. In the presence of CO₂, acetogenesis and ferulate reduction occur simultaneously.

For energetic reasons, the use of the electron acceptor that allows maximal energy conservation should be preferred. This is seen in a number of prokaryotes, and the underlying regulatory mechanisms have been studied best for the ability of *Escherichia coli* to switch from fermentation to anaerobic respiration with nitrate, fumarate, or dimethyl sulfoxide (48). The best-studied example in the acetogens is *M. thermoacetica*, which can use CO₂ or nitrate as an electron acceptor (17). Nitrate is the preferred electron acceptor for *M. thermoacetica*, and nitrate reduction is coupled to energy conservation (43, 44). The amount of energy conserved is larger during nitrate reduction than during acetogenesis with *O*-methyl groups. The redox potential of the CO₂/acetate couple is about -0.34 V, whereas the redox potential of NO₃⁻/NO₂⁻ is 0.42 V. Therefore, the Gibbs free energy change during acetogenesis from H₂ plus CO₂ is much smaller than the Gibbs free energy change for nitrate reduction (18, 43). Although the thermodynamic basis for the preference of nitrate over CO₂ is clear, the regulatory events involved are far from being understood. One detailed study revealed that nitrate blocks the acetyl-CoA pathway in *M. thermoacetica* at the level of the electron transport pathway (17). The enzymes of the acetyl-CoA pathway were present and their activity was not regulated by nitrate, but a cytochrome *b* was missing in nitrate-grown cultures. A second study revealed an additional effect of the CO₂ concentration. In CO₂-sparged cultures grown in the presence of nitrate, methyltransferase and CO dehydrogenase activities were 5- to 10-fold lower, and molecular studies revealed that the regulation of enzymes, as well as electron transport proteins, such as cytochrome *b*, was on a transcriptional level (1). Nitrate induced the nitrate reductase (1, 17).

The redox potential of the caffeate/hydrocaffeate couple is not known, but based on the assumption that it is similar to the redox potential of the fumarate/succinate couple ($E_0' = 0.032$ V), caffeate reduction is energetically favored over CO₂ reduction. This could lead to preferential use of caffeate over CO₂, as indeed was observed previously for *A. woodii* Nzva16 (47). However, we did not observe preferential use of caffeate. Either caffeate and CO₂ were used simultaneously (during hydrogen or fructose oxidation) or CO₂ was preferred over caffeate during methyl group oxidation. The differences cannot be explained easily but may be due to the different physiologies of the two strains of *A. woodii* or the culture conditions used. Tschech and Pfenning (47) cultivated *A. woodii* Nzva16 with 60 mM bicarbonate and limiting amounts of methanol (2 mM), and caffeate was added at a concentration of 6 mM, giving a methanol/caffeate stoichiometry of 1:3. The oxidation of one

methyl group yields six electrons, and Tschech and Pfenning observed that the electrons were funneled exclusively to caffeate under these conditions, with no acetate produced. We used 30 times more (60 mM methanol) or 75 times more (50 mM betaine) methyl groups than Tschech and Pfenning used, and it could be that the methyl group concentration plays a role in regulation. In line with this argument is the observation of Davies and Stephens (9), who observed an effect of the growth substrate and electron donor on caffeate reduction. Methanol-grown cells could not use fructose as an electron donor for caffeate reduction, which is surprising since the Embden-Meyerhof-Parnas pathway is constitutive. In addition, even small changes in the culture conditions can have a big impact on the regulation. For example, in undefined media, *C. formicoaceticum* uses CO₂ and fumarate simultaneously, whereas CO₂ is preferred in defined media (35).

The caffeate reductase system of *A. woodii* apparently does not discriminate between caffeate, ferulate, and *p*-coumarate. In addition, all three compounds were used as inducers for the system. A surprising finding, however, was that caffeate-induced cells were apparently able to demethylate ferulate. It is interesting that Davies and Stephens reported a similar phenomenon: cells grown with fructose plus caffeate were able to use methanol as a reductant for caffeate, and again, the methyltransferase system must have been induced by caffeate (9). *O* demethylation requires the concerted action of three proteins; methyltransferase I abstracts the methyl group and transfers it to the central cobalt atom in a corrinoid protein, from which it is transferred to tetrahydrofolate by the action of a second methyltransferase. The genes encoding these proteins are (often) organized in an operon, and the expression is induced by the *O*-methyl compound (15, 16, 29–31, 45). Here, the methyltransferase system was induced by caffeate, a compound that has no *O*-methyl groups. However, caffeate and *O*-methyl compounds are both derived from lignin and are present together in anaerobic soils that contain lignin. Therefore, the presence of caffeate might signal the presence of lignin and thus a *O*-methyl compound to the cells and induce a more global regulatory network involved in utilization of lignin derivatives.

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