

# CO Metabolism in the Thermophilic Acetogen *Thermoanaerobacter kivui*

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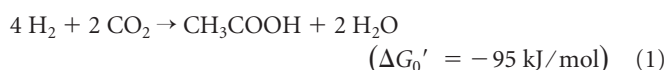
The thermophilic acetogenic bacterium *Thermoanaerobacter kivui*, previously described not to use carbon monoxide as a carbon and energy source, was adapted to grow on CO. This was achieved by using a preculture grown on H<sub>2</sub> plus CO<sub>2</sub> and by increasing the CO concentration in small, 10% increments. *T. kivui* was finally able to grow within a 100% CO atmosphere. Growth on CO was found in complex and mineral media, and vitamins were not required. Carbon monoxide consumption was accompanied by acetate and hydrogen production. Cells also grew on synthesis gas (syngas) with the simultaneous use of CO and H<sub>2</sub> coupled to acetate production. CO oxidation in resting cells was coupled to hydrogen and acetate production and accompanied by the synthesis of ATP. A protonophore abolished ATP synthesis but stimulated H<sub>2</sub> production, which is consistent with a chemiosmotic mechanism of ATP synthesis. Hydrogenase activity was highest in crude extracts of CO-grown cells, and carbon monoxide dehydrogenase (CODH) activity was highest in H<sub>2</sub>-plus-CO<sub>2</sub>- or CO-grown cells. The genome of *T. kivui* harbors two CODH gene clusters, and both CODH proteins were present in crude extracts, but one CODH was more prevalent in crude extracts from CO-grown cells.

Carbon monoxide is a colorless, odorless gas, which is toxic to most organisms in trace amounts. However, some organisms can use carbon monoxide as an electron and carbon source (1, 2). These organisms are aerobic carboxydrotrophic bacteria such as *Oligotropha carboxydovorans* (3), phototrophic purple sulfur bacteria such as *Rhodospirillum rubrum* (4), hydrogenogenic bacteria and archaea such as *Thermosinus carboxydovorans* (5) or *Thermococcus* sp. strain AM4 (6), or some organisms that employ the Wood-Ljungdahl pathway (WLP), such as methanogens (7–9) or acetogens (10–13). Acetogenic bacteria use the WLP for CO<sub>2</sub> fixation to acetate. This pathway is considered one of the most ancient biochemical pathways for CO<sub>2</sub> fixation, as it combines two essential features: CO<sub>2</sub> fixation and the synthesis of ATP (14, 15). The use of carbon monoxide as an electron donor for the WLP has the advantage of providing extremely low potential electrons for reducing cellular electron carriers with a CO<sub>2</sub>/CO reduction potential of –520 mV (16).

The production of third-generation biofuels from carbon dioxide, molecular hydrogen, and/or carbon monoxide as catalyzed by, for example, acetogenic bacteria is a promising alternative for existing biofuel production routes from renewable sources (17–19). However, it is still in its infancy with respect to knowledge on the biochemistry and bioenergetics of CO oxidation and CO<sub>2</sub> reduction in many acetogens. Several acetogenic representatives have been shown to use CO as the sole energy source, such as *Butyribacterium methylotrophicum* (CO strain) (20), *Eubacterium limosum* (21), *Blautia producta* (22), *Clostridium thermoautotrophicum* (11), *Moorella thermoacetica* (12), and *Clostridium ljungdahlii* (23). The extent of CO tolerance and consumption varies greatly between different species, and some closely related bacteria cannot utilize CO as the sole energy source at all (20) or can utilize it only in combination with H<sub>2</sub> plus CO<sub>2</sub>, as is the case, for example, for *Acetobacterium woodii* (24). The circumstances defining the constraint of using CO as a sole energy source are still widely unresolved.

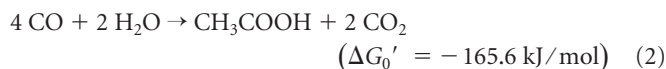
Acetogenic bacteria use the WLP to produce acetate as a primary end product. This can be through either heterotrophic

growth or autotrophic growth on H<sub>2</sub> plus CO<sub>2</sub> or on CO. During acetogenesis from CO<sub>2</sub> plus H<sub>2</sub> according to the equation



CO<sub>2</sub> is reduced in the methyl branch to formate, activated to formyl-tetrahydrofolate (THF) at the cost of 1 ATP molecule, and further metabolized to methenyl-THF, which is reduced stepwise to methylene-THF and finally to methyl-THF (14). The other CO<sub>2</sub> molecule is reduced in the carbonyl branch to CO, which is then combined with the methyl group and coenzyme A (CoA) to acetyl-CoA, catalyzed by a bifunctional carbon monoxide dehydrogenase (CODH)/acetyl-CoA synthase (ACS). The acetyl-CoA is then converted to acetyl-phosphate and finally to acetate, giving rise to 1 ATP molecule.

Acetogenesis from carbon monoxide according to the equation



involves, besides the WLP, an enzyme system that is able to oxidize CO to CO<sub>2</sub>. This enzyme system can be either the above-mentioned CODH/ACS or a monofunctional CODH. The CO<sub>2</sub> produced is then reduced in the WLP as outlined above.

Since the WLP requires the input of 1 ATP molecule for the activation of formate and gives rise to only 1 ATP molecule from

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substrate-level phosphorylation from the acetate kinase reaction, net energy conservation requires the generation of a chemiosmotic gradient established by a redox-driven ion pump. The gradient is then used by an ATP synthase to generate ATP. In acetogenic bacteria, this redox-driven ion pump is either an Rnf complex, as in *A. woodii* (25–27), or an Ech complex, as found in *M. thermoacetica* (28) or *Thermoanaerobacter kivui* (29). The coupling ions for Rnf and Ech can be either H<sup>+</sup> or Na<sup>+</sup>.

*T. kivui* is a thermophilic acetogenic organism that can sustain a heterotrophic or autotrophic lifestyle with H<sub>2</sub> plus CO<sub>2</sub> as the substrate (30). A genetic system is available for some *Thermoanaerobacter* strains, making *T. kivui* a good candidate for a strain to be used for the production of biocommodities from CO<sub>2</sub> at high temperatures (31, 32). Another interesting feedstock for third-generation biofuels is synthesis gas (syngas), which contains carbon monoxide, carbon dioxide, and molecular hydrogen in variable proportions and with trace gases whose nature depends on the source (33). Unfortunately, it was reported that *T. kivui* is unable to grow on carbon monoxide, which would exclude syngas as a feedstock for this bacterium (12). However, we describe here that *T. kivui* can be adapted to grow on CO or syngas. Not only does CO support growth as a single electron source, but CO oxidation is also coupled to energy conservation.

## MATERIALS AND METHODS

**Organism and cultivation.** *T. kivui* LKT-1 (DSM 2030) was routinely cultivated at 60°C. The media were prepared by using anaerobic techniques as described previously (34, 35). All growth experiments were performed with 120-ml serum flasks containing 20 ml medium. Mineral medium contained 3.6 mM KH<sub>2</sub>PO<sub>4</sub>, 50 mM KHCO<sub>3</sub>, 5.4 mM KCl, 6.3 mM NH<sub>4</sub>Cl, 1.6 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.35 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 1.5 mM cysteine-HCl, 10 ml/liter DSM 141 vitamin solution, 10 ml/liter trace element solution (DSM 141), and 4 μM resazurin. Complex medium contained 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 50 mM NaH<sub>2</sub>HPO<sub>4</sub>, 1.2 mM K<sub>2</sub>HPO<sub>4</sub>, 1.6 mM KH<sub>2</sub>PO<sub>4</sub>, 4.7 mM NH<sub>4</sub>Cl, 1.7 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 7.5 mM NaCl, 0.37 mM MgSO<sub>4</sub>, 42 μM CaCl<sub>2</sub>, 7.2 μM Fe(II)SO<sub>4</sub>, 54 mM KHCO<sub>3</sub>, 3 mM cysteine-HCl, 0.2% (wt/vol) yeast extract, 10 ml/liter vitamin solution (DSM 141), 10 ml/liter trace element solution (DSM 141), and 4 μM resazurin at pH 7.5. Growth was determined by measuring the optical density at 600 nm (OD<sub>600</sub>).

Growth experiments on CO were conducted with 0 to 100% CO as the substrate at a final pressure of 2 × 10<sup>5</sup> Pa. The remainder of the gas phase (makeup gas) was N<sub>2</sub> plus CO<sub>2</sub> (80:20 [vol/vol]). Growth with H<sub>2</sub> as the electron donor was carried out with H<sub>2</sub> plus CO<sub>2</sub> (80:20 [vol/vol]) at 2.5 × 10<sup>5</sup> Pa for the preparation of crude extracts or with H<sub>2</sub> plus CO<sub>2</sub> plus N<sub>2</sub> (50:20:30 [vol/vol]) at 2 × 10<sup>5</sup> Pa for growth experiments. Growth on glucose was carried out by using 25 mM glucose in a gas atmosphere of N<sub>2</sub> plus CO<sub>2</sub> (80:20 [vol/vol]) at 1 × 10<sup>5</sup> Pa. Growth on syngas was carried out by using a mixture of 30% CO, 50% H<sub>2</sub>, 14% CO<sub>2</sub>, and 6% N<sub>2</sub> at 2 × 10<sup>5</sup> Pa.

**Preparation of resting cells and experiments with cell suspensions.** For preparation of cell suspensions, *T. kivui* was cultivated in 1-liter flasks (Müller-Krempel, Bülach, Switzerland) in the above-mentioned growth media. Cells were cultivated on glucose (25 mM glucose with N<sub>2</sub> plus CO<sub>2</sub> [80:20 {vol/vol} at 1 × 10<sup>5</sup> Pa], CO [50% CO, 40% N<sub>2</sub>, and 10% CO<sub>2</sub> at 2 × 10<sup>5</sup> Pa], or H<sub>2</sub> plus CO<sub>2</sub> [80:20 {vol/vol} at 2.5 × 10<sup>5</sup> Pa]); harvested at an OD<sub>600</sub> of 1.8 to 2.2, an OD<sub>600</sub> of 0.10 to 0.15, or an OD<sub>600</sub> of 0.3 to 0.4, respectively, by centrifugation (11,500 × g for 10 min at 4°C); and washed twice with imidazole buffer (50 mM imidazole, 20 mM MgSO<sub>4</sub>, 20 mM KCl, 20 mM NaCl, 4 mM dithioerythritol (DTE), 4 μM resazurin [pH 7.0]). After the last centrifugation step, cells were resuspended in 1 ml of the same buffer and kept in a gas-tight Hungate tube. The gas phase was

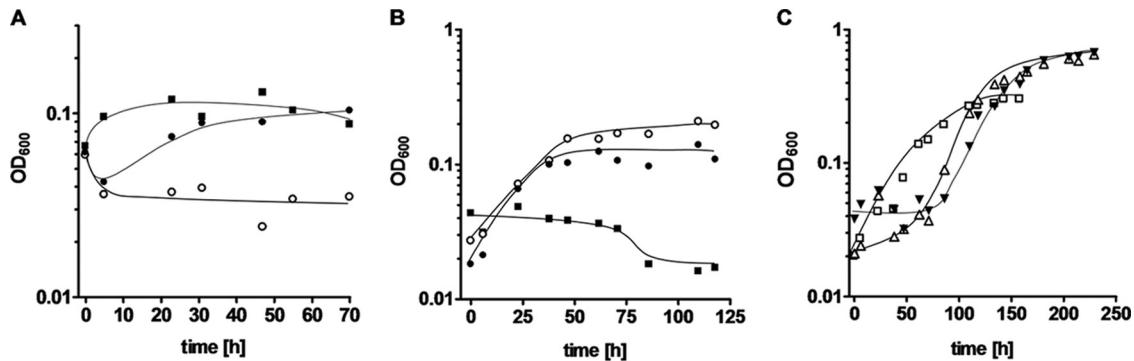
exchanged to 100% N<sub>2</sub>. The protein concentration was determined according to methods described previously by Schmidt et al. (36). Serum flasks (120 ml; Glasgerätebau Ochs GmbH, Bovenden-Lenglern, Germany) were filled with 20 ml imidazole buffer containing 50 mM KHCO<sub>3</sub>. If indicated, the protonophore 3,3',4',5-tetrachlorosalicylanilide (TCS) was added to a concentration of 30 μM from a 10 mM stock solution with 100% ethanol as a solvent. Subsequently, the gas phase of the serum flasks was changed to N<sub>2</sub> plus CO<sub>2</sub> (80:20 [vol/vol]) at 1.3 × 10<sup>5</sup> Pa, and resting cells were added to a final concentration of 1 mg/ml and then incubated at 60°C in a water bath. After preincubation for 15 min, the reaction was started by flushing the flasks with 100% CO for 20 s. At the times indicated, 50-μl gas samples were withdrawn and directly injected into a gas chromatograph. Liquid samples (400 μl) were withdrawn for ATP measurements and immediately added to 300 μl of ice-cold 3 M perchloric acid. The samples were incubated on ice for exactly 1 h, and the pH was adjusted immediately to 7.0 by the addition of 93 μl 0.4 M N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer (pH 7.4) and 57 μl of a saturated K<sub>2</sub>CO<sub>3</sub> solution. The precipitated KClO<sub>4</sub> was removed by centrifugation (13,400 × g for 5 min at 4°C), and the supernatant was immediately used for ATP measurements or frozen in liquid N<sub>2</sub> and stored at -20°C. The ATP concentration was determined by using a luciferin-luciferase assay (37).

**Preparation of crude extract, membranes, and cytoplasm.** Cells were grown to an OD<sub>600</sub> of 0.2 to 0.3 on CO (50% CO, 40% N<sub>2</sub>, and 10% CO<sub>2</sub> at 2 × 10<sup>5</sup> Pa), an OD<sub>600</sub> of 0.3 to 0.4 on H<sub>2</sub> plus CO<sub>2</sub> (80:20 [vol/vol] at 2.5 × 10<sup>5</sup> Pa), or an OD<sub>600</sub> of 1.9 to 2.4 on glucose (25 mM glucose with N<sub>2</sub> plus CO<sub>2</sub> [80:20 {vol/vol} at 1 × 10<sup>5</sup> Pa]) in 1-liter flasks (Glasgerätebau Ochs, Bovenden-Lenglern, Germany) filled with 200 ml complex medium for growth on gases or 500 ml complex medium for growth on glucose. Cells were harvested anaerobically by centrifugation at 17,000 × g for 10 min at 4°C. Cells were washed once in buffer A (50 mM Tris, 20 mM MgSO<sub>4</sub>, 20% glycerol, 2 mM DTE, 4 μM resazurin [pH 7.5]), and after another centrifugation step at 14,300 × g for 20 min at 4°C, cells were resuspended in 2 to 4 ml buffer A. Cells were disrupted by a single passage through a French press (110 MPa). Undisrupted cells and cell debris were separated by a centrifugation step at 14,300 × g for 20 min at 4°C. The supernatant contained the crude extract.

**Measurement of enzyme activities.** Hydrogenase or carbon monoxide dehydrogenase activity was measured at 60°C in 1.8-ml anaerobic cuvettes (Glasgerätebau Ochs, Germany) filled with 1 ml Tris buffer at pH 7.5 containing 4 mM DTE and 4 μM resazurin with H<sub>2</sub> or CO, respectively, at a pressure of 1.3 × 10<sup>5</sup> Pa. Methylviologen (MV) (10 mM) served as the electron acceptor for both reactions and was used to start the reaction. MV reduction was measured at 604 nm.

**Determination of acetate concentrations.** Samples were withdrawn with a syringe, and cells were removed by centrifugation (14,000 × g for 1 min at 4°C). The acetate concentration in the supernatant was determined by using a gas chromatograph (Clarus 580 GC; Perkin-Elmer, Waltham, MA, USA). The samples were injected at 250°C and separated on an Elite FFAP column (30 m by 0.25 mm; Perkin-Elmer, Waltham, MA, USA) with helium as the carrier gas at a flow rate of 30 cm/s and a split of 1:50. The oven was kept at 60°C for 3 min, followed by a temperature gradient to 150°C at 10°C/min. The samples were analyzed with a flame ionization detector at 250°C. 1-Propanol (10 mM) was used as an internal standard.

**Determination of H<sub>2</sub> and CO concentrations.** The concentrations of H<sub>2</sub> and CO were determined by using a gas chromatograph (Clarus 580 GC; Perkin-Elmer, Waltham, MA, USA). The samples were injected at 100°C and separated on a ShinCarbon ST 80/100 column (2 m by 0.53 mm; Restek Corporation, Bellefonte, PA, USA). Nitrogen or helium was used as the carrier gas for the determination of H<sub>2</sub> or CO concentrations, respectively, with a head pressure of 400 kPa and a split flow of 30 ml/s. The oven was kept at 40°C, and the samples were analyzed with a thermal conductivity detector at 100°C.



**FIG 1** Adaptation of *T. kivui* to growth on CO. (A) A culture adapted to growth on  $H_2$  plus  $CO_2$  served as the preculture for inoculation of fresh medium in the absence of CO (■) or in the presence of 10% CO (●) or 30% CO (○). (B) The culture cultivated on 10% CO from panel A served as the preculture to reinoculate fresh medium in the absence of CO (■) or in the presence of 10% CO (●) or 20% CO (○). (C) The 20% CO culture from panel B was used as the preculture for cultivation on 30% CO (□), which in turn served as the preculture for cultivation on 40% CO (△). The latter served as the preculture for growth on 50% CO (▼).  $N_2$  plus  $CO_2$  (80:20 [vol/vol]) was used as the makeup gas at a final pressure of  $2 \times 10^5$  Pa. All cultures were grown in complex medium, and growth was measured by monitoring the optical density at 600 nm. Shown are data from one representative experiment out of three independent replicates.

**Analytical methods.** The protein concentration was measured according to methods described previously by Bradford (38) or, for whole cells, according to methods described previously by Schmidt et al. (36).

For immunological detection of both CODH proteins, the encoding genes were fused to a sequence encoding a His<sub>6</sub> tag at the 5' end and cloned according to methods described previously by Sambrook et al. (39), and the corresponding proteins were produced in *Escherichia coli* BL21(DE3), purified by affinity chromatography according to the manufacturer's instructions (New England BioLabs GmbH, Frankfurt/Main, Germany), and used for immunization of a rabbit (performed by Davids Biotechnologie, Regensburg, Germany). For Western blot analysis, 10  $\mu$ g of each cell extract was separated by SDS-PAGE (12%). Proteins were transferred onto a nitrocellulose membrane (Protran BA 83; GE Healthcare, United Kingdom), followed by immunoblotting with a 1:2,000 dilution of the rabbit antiserum for both antibodies. Primary antibody detection was performed with a goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (dilution of 1:10,000; Bio-Rad).

**Chemicals.** Chemicals were purchased from AppliChem GmbH (Darmstadt, Germany), Merck KGaA (Darmstadt, Germany), Roche Diagnostics GmbH (Mannheim, Germany), Carl Roth GmbH & Co. KG (Karlsruhe, Germany), and Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Gases were purchased from Praxair Deutschland GmbH (Düsseldorf, Germany).

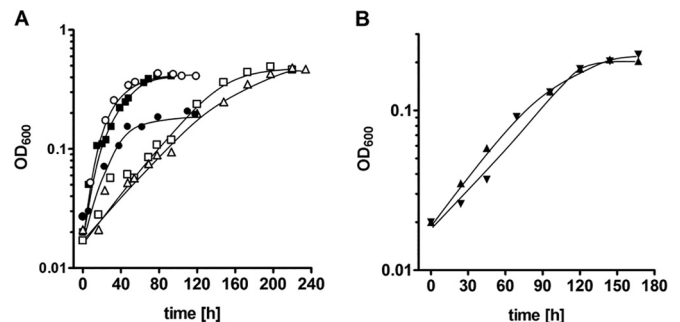
## RESULTS

**Adaptation of *T. kivui* to growth on CO.** When *T. kivui* was grown on glucose and transferred to complex medium containing CO (10%) as the carbon and energy source, growth was not observed. Next, cells were pregrown on  $H_2$  plus  $CO_2$  and transferred to media with different CO concentrations. There was no growth in the presence of 30% CO (or higher) but one doubling in the presence of 10% CO (Fig. 1A). However, cells transferred to medium containing no CO also showed one doubling. Nevertheless, the 10% CO culture was taken as the preculture to inoculate media containing 10 or 20% CO. As shown in Fig. 1B, cells started to grow, and the final optical density was dependent on the CO concentration. At 10% CO, the final OD was 0.1, and at 20% CO, it was 0.2 (Fig. 1B). Next, the CO concentration was increased gradually in 10% increments. Transfer of cells from a 20% CO preculture to media with 30, 40, or 50% CO led to growth only in the presence of 30% CO, and the final OD was higher than that at 20% CO. Upon transfer of cells from a preculture grown at 30%

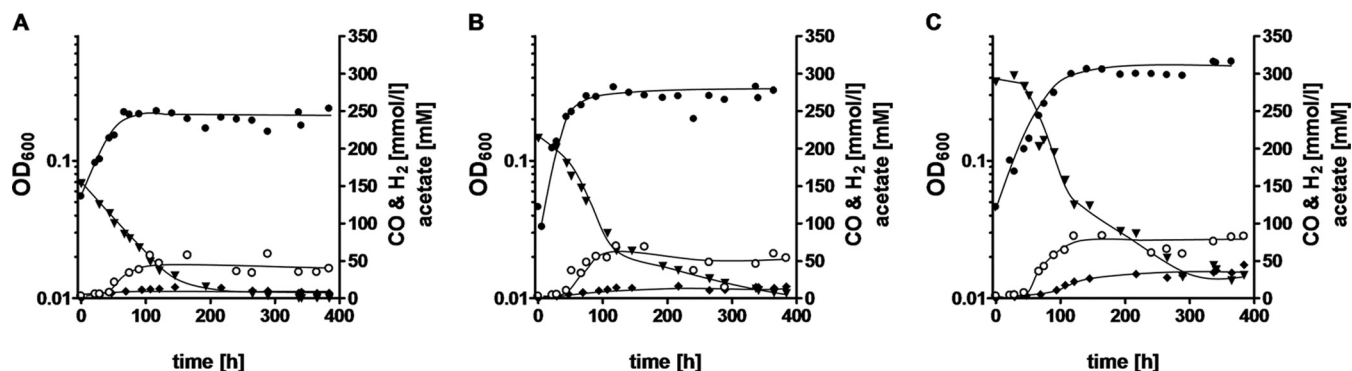
CO to 40% CO, there was a lag phase of  $\sim 72$  h (Fig. 1C). Again, the final OD increased. In the next step, from 40 to 50% CO, the final optical density increased again (Fig. 1C). By a subsequent increase of the CO concentration in 10% increments, it was possible to adapt the culture to grow on up to 100% CO. Each transfer to a higher CO concentration resulted in a lag phase. However, the final optical density did not increase substantially above 50% CO.

When cells were kept at the same CO concentration for at least three transfers, a lag phase was no longer observed (Fig. 2A). Adapted cultures grew with doubling times of 18.7, 15.4, 10.2, 34.7, and 33.0 h at 20, 50, 70, 90, and 100% CO, respectively. The corresponding final optical densities ( $OD_{600}$ ) were 0.21, 0.39, 0.41, 0.46, and 0.47, respectively (Fig. 2A).

Growth was also observed when cells were grown in mineral medium; the doubling time was 40.4 h ( $\mu = 0.017$  h<sup>-1</sup>), and the final optical density was 0.21 with 50% CO in the atmosphere (Fig. 2B). Omission of the vitamin solution had not effect on the growth rate or final optical density (Fig. 2B).



**FIG 2** Growth of *T. kivui* adapted to CO. All cultures were grown in complex medium with increasing concentrations of CO (●, 20%; ■, 50%; ○, 70%; □, 90%; △, 100%) (A) or in mineral medium with 50% CO in the absence (▼) or presence (▲) of additional vitamins (B).  $N_2$  plus  $CO_2$  (80:20 [vol/vol]) was used as the makeup gas at a final pressure of  $2 \times 10^5$  Pa. Growth was measured by monitoring the optical density at 600 nm. Cultures adapted to growth on CO (at least 3 transfers at the given concentrations) served as precultures for this experiment. Shown are data from one representative experiment out of three independent replicates.



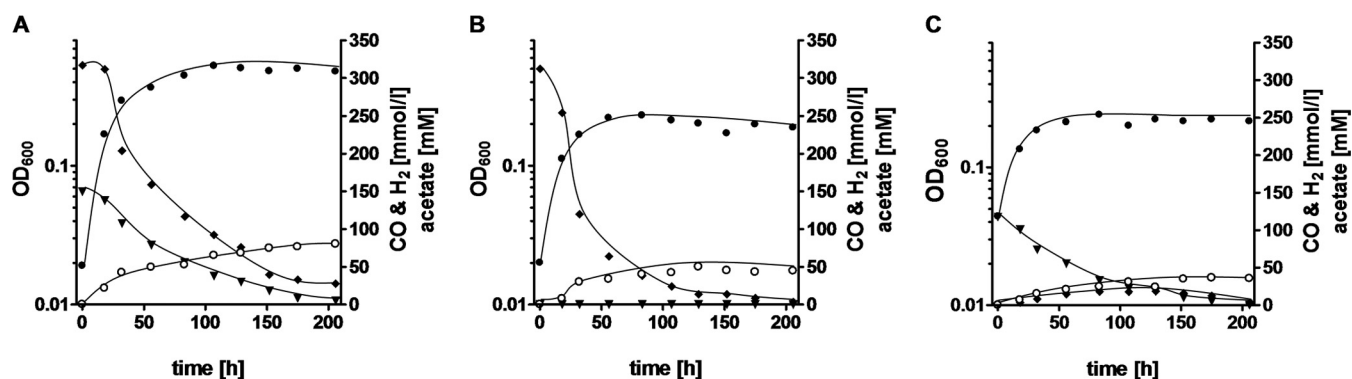
**FIG 3** CO consumption, acetate production, and H<sub>2</sub> formation in cells growing on increasing concentrations of CO. The gas phase consisted of 30% CO (A), 50% CO (B), or 70% CO (C). N<sub>2</sub> plus CO<sub>2</sub> (80:20 [vol/vol]) was used as the makeup gas at a final pressure of  $2 \times 10^5$  Pa. All cultures were grown in complex medium, and growth was measured by monitoring the optical density (●) at 600 nm. The concentrations of CO (▼) and H<sub>2</sub> (◆) in the gas phase and of acetate in the liquid phase (○) were determined by gas chromatography. Shown are data from one representative experiment out of three independent replicates.

**Consumption of CO and production of acetate and H<sub>2</sub>.** CO consumption and product formation were measured in cultures adapted to 30, 50, or 70% CO (Fig. 3). All cultures started to grow immediately and consumed CO at rates of 21  $\mu\text{mol/h}$  (30% CO), 30  $\mu\text{mol/h}$  (50% CO), or, after a short lag phase, 29  $\mu\text{mol/h}$  (70% CO) in 20-ml cultures with a 200-ml gas phase. All cultures produced acetate and H<sub>2</sub>. When the final optical density was reached, acetate was no longer produced. At this point, 39.6, 53.6, or 82.7 mM acetate was produced in cultures containing 30, 50, or 70% CO, respectively. At the same time, H<sub>2</sub> levels did not change anymore and stayed at 6.8, 15.8, or 44.9 mmol/liter medium in cultures containing 30, 50, or 70% CO, respectively. At the time when growth ceased and acetate and hydrogen production stopped, CO was still available in the cultures. Interestingly, it was consumed in the stationary growth phase but with much lower rates of 2, 4, and 9  $\mu\text{mol/h}$  in the cultures with initial CO concentrations of 30, 50, and 70% CO, respectively. Electron flow from CO in the stationary growth phase could not be accounted for experimentally; formate, ethanol, propanol, and H<sub>2</sub> were apparently not produced.

**Simultaneous consumption of CO and H<sub>2</sub> in a culture cultivated on syngas.** To determine whether *T. kivui* is able to grow on syngas and convert the gases simultaneously, cells were grown on

30% CO, 50% H<sub>2</sub>, and 14% CO<sub>2</sub> (and 6% N<sub>2</sub>). Upon transfer of cells from a CO-grown preculture, CO and H<sub>2</sub> consumption started immediately and continued simultaneously (Fig. 4A). The OD<sub>600</sub> reached was 0.5, and 80 mM acetate was produced. In controls that received only H<sub>2</sub> or CO as the electron donor, oxidation of the electron donor also started immediately, but the final optical density of the culture with H<sub>2</sub> plus CO<sub>2</sub> was only 46% of that of the syngas culture (Fig. 4B), and the final optical density of the CO culture was only 44% of that of the syngas culture (Fig. 4C). At the same time, acetate production was reduced.

**Energetics of CO-dependent acetate formation in cell suspensions.** To investigate whether CO consumption was accompanied by acetate production, cell suspension experiments were conducted. Suspensions of cells that had been cultivated on CO (50%) consumed CO at a rate of 1.5  $\mu\text{mol/mg/min}$ , and concomitantly, acetate was produced at a rate of 0.3  $\mu\text{mol/mg/min}$ , reaching 5.1 mM acetate after 6 h (Fig. 5). Next, the effect of CO on cellular ATP levels was investigated. The introduction of CO to cell suspensions pregrown on CO led to an immediate increase of cellular ATP levels (Fig. 6A). When cell suspensions were prepared from cells pregrown on glucose or on H<sub>2</sub> plus CO<sub>2</sub>, there was an immediate decrease in ATP levels upon the addition of CO, and acetate



**FIG 4** Simultaneous consumption of CO and H<sub>2</sub> in cells growing on syngas. The gas phase consisted of 30% CO, 50% H<sub>2</sub>, 14% CO<sub>2</sub>, and 6% N<sub>2</sub> (A); 50% H<sub>2</sub>, 20% CO<sub>2</sub>, and 30% N<sub>2</sub> (B); or 30% CO, 14% CO<sub>2</sub>, and 56% N<sub>2</sub> (C) at a pressure of  $2 \times 10^5$  Pa. All cultures were grown in complex medium, and growth was measured by monitoring the optical density (●) at 600 nm. The concentrations of CO (▼) and H<sub>2</sub> (◆) in the gas phase and of acetate in the liquid phase (○) were determined by gas chromatography. Shown are data from one representative experiment out of three independent replicates.

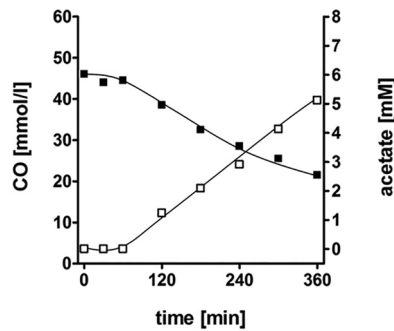


FIG 5 CO consumption and acetate production in resting cells. *T. kivui* was grown on CO; cells were harvested, washed, and resuspended to a protein concentration of 1 mg/ml in 20 ml buffer (50 mM imidazole, 50 mM  $\text{KHCO}_3$ , 20 mM  $\text{MgSO}_4$ , 20 mM KCl, 20 mM NaCl, 4 mM DTE, 4  $\mu\text{M}$  resazurin [pH 7.0]) in 120-ml serum bottles. The cell suspension experiments were carried out at 60°C in a shaking water bath. The gas phase contained 10% CO, and  $\text{N}_2$  plus  $\text{CO}_2$  (80:20 [vol/vol]) was used as the makeup gas at a final pressure of  $2 \times 10^5$  Pa. The concentrations of CO (■) in the gas phase and acetate (□) in the liquid phase were determined by gas chromatography. Shown are data from one representative experiment out of three independent replicates.

was not produced. When the protonophore TCS was present in the assay mixture, ATP was depleted, and there was no increase in ATP levels after the addition of CO (Fig. 6A). CO oxidation was coupled to  $\text{H}_2$  formation (Fig. 6B), with a hydrogen evolution rate of 0.7  $\mu\text{mol}/\text{mg}/\text{min}$ , and 0.98 mM acetate was formed after 160 min. Preincubation of cells with the protonophore TCS led to an inhibition of acetate formation but a stimulation of  $\text{H}_2$  production, with an  $\text{H}_2$  evolution rate of 1.4  $\mu\text{mol}/\text{mg}/\text{min}$ . The final amount of  $\text{H}_2$  produced was also doubled, with 7.3 mmol/liter buffer compared to 3.6 mmol/liter buffer in the assay containing no uncoupler. These data are fully consistent with a chemiosmotic mechanism of ATP synthesis coupled to CO oxidation and hydrogen formation. CO oxidation led to ion translocation and the generation of a membrane potential,  $\Delta\psi$ , which resulted in ther-

modynamic backup pressure on CO oxidation. The addition of the protonophore TCS led to uncoupling, which in turn relieved the thermodynamic backup pressure and stimulated  $\text{H}_2$  formation. ATP synthesis was inhibited, and consequently, acetate formation from CO/ $\text{CO}_2$ , which requires ATP, was also inhibited.

**Key enzyme activities in crude extracts.** The above-mentioned experiments revealed the production of molecular hydrogen from CO. Hydrogenase and carbon monoxide dehydrogenase activities were analyzed in crude extracts prepared from a culture in the exponential growth phase grown on CO,  $\text{H}_2$  plus  $\text{CO}_2$ , or glucose. The specific activity for  $\text{H}_2$ -dependent methylviologen reduction was by far the highest in crude extracts of CO-grown cells, at  $82.1 \pm 22.7$  U/mg, compared to  $25.1 \pm 3.1$  U/mg in crude extracts of  $\text{H}_2$ - and  $\text{CO}_2$ -grown cells and  $33.9 \pm 7.3$  U/mg in crude extracts of glucose-grown cells. CODH activity was highest in crude extracts prepared from  $\text{H}_2$ - and  $\text{CO}_2$ -grown cells ( $286.0 \pm 73.7$  U/mg), similar to that in crude extracts of CO-grown cells ( $233.3 \pm 58.5$  U/mg), and much higher than that in glucose-grown cells ( $91.0 \pm 19.9$  U/mg).

**Presence of carbon monoxide dehydrogenases in crude extracts.** The genome of *T. kivui* harbors two genes encoding two potential CO dehydrogenases. One gene product (CooS) is similar to monofunctional CO dehydrogenases (CooS), and the other (AcsA) is similar to the CODH subunit of the bifunctional CODH/ACS complexes. Western blot analyses revealed that both CODH enzymes were present in crude extracts prepared from cells grown on CO,  $\text{H}_2$  plus  $\text{CO}_2$ , or glucose. The amounts of bifunctional AcsA were more or less the same in cells grown on CO and those grown on  $\text{H}_2$  plus  $\text{CO}_2$  but were smaller in glucose-grown cells (Fig. 7A). In contrast, CooS levels were highest in CO-grown cells (Fig. 7B).

## DISCUSSION

It was reported previously that the thermophilic acetogen *T. kivui* does not grow on CO (12, 40, 41). This finding could be reproduced in this study: upon transfer of a glucose-cultivated preculture to medium containing CO, cells did not grow. However,

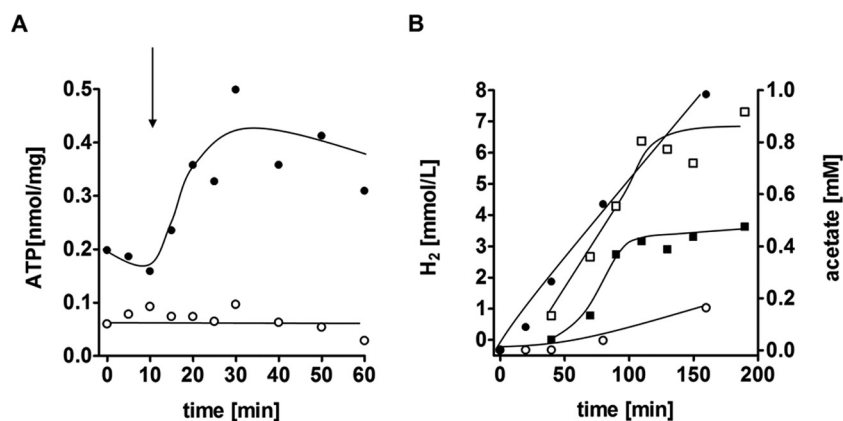
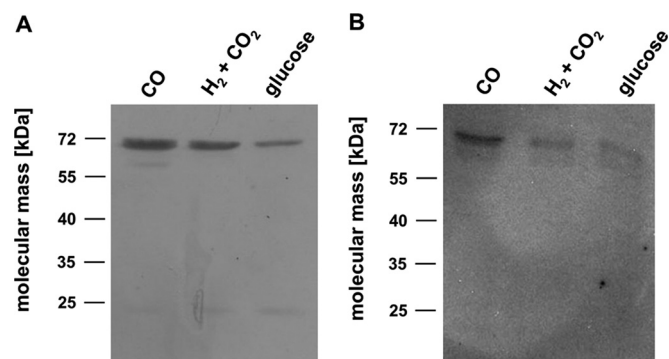


FIG 6 Influence of a protonophore on acetate,  $\text{H}_2$ , and ATP formation from CO in resting cells. *T. kivui* was grown on CO, cells were harvested, washed, and resuspended to a protein concentration of 1 mg/ml in 20 ml of buffer (50 mM imidazole, 50 mM  $\text{KHCO}_3$ , 20 mM  $\text{MgSO}_4$ , 20 mM KCl, 20 mM NaCl, 4 mM DTE, 4  $\mu\text{M}$  resazurin [pH 7.0]) in 120-ml serum bottles. Experiments were carried out at 60°C in a shaking water bath.  $\text{N}_2$  plus  $\text{CO}_2$  (80:20 [vol/vol]) was used as the makeup gas at a pressure of  $1 \times 10^5$  Pa. Cells and the protonophore were preincubated for 10 min at 60°C. Vials were flushed with CO for 20 s. (A) The ATP content was measured by using the luciferin-luciferase assay with an assay mixture containing no protonophore (●) or 30  $\mu\text{M}$  TCS (○). The arrow indicates the time point of CO flushing. (B) Hydrogen (squares) and acetate (circles) formation was measured by gas chromatography in an assay mixture in the absence of the protonophore (filled symbols) or in an assay mixture containing 30  $\mu\text{M}$  TCS (hollow symbols). Shown are data from one representative experiment out of three independent replicates.



**FIG 7** Detection of AcsA and CooS in crude extracts. Cells were grown on CO, H<sub>2</sub> plus CO<sub>2</sub>, or glucose and harvested in the exponential growth phase. The cell extract was prepared, and 10 μg protein from each sample was separated on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The presence of AcsA (A) or CooS (B) was determined immunologically with antibodies raised against heterologously produced proteins.

when inoculated from a preculture cultivated on H<sub>2</sub> plus CO<sub>2</sub>, cells grew in the presence of 10% CO but not in the presence of 30% CO or higher. When reinoculated with 10% increments of CO in the gas phase, *T. kivui* grew even in the presence of 100% CO. Apparently, the organism needs sufficient time to adapt its metabolism and has set metabolic changes upon autotrophic growth on H<sub>2</sub> plus CO<sub>2</sub> that are missing during heterotrophy. Apparent final optical densities did not increase substantially at concentrations of CO above 50%, indicating that a factor other than the CO concentration was limiting. The medium pH was not the limiting factor, since it remained constant at pH 7.5.

Acetogenic bacteria play an emerging role in biotechnological applications as organisms that convert industrial waste gases (syngas) to biocommodities (42, 43). The ability of *T. kivui* to use syngas is thus of great biotechnological interest. *T. kivui* is a thermophilic organism with an optimal growth temperature at 66°C and a temperature tolerance of 55°C to 70°C (30), which could be advantageous for certain applications at higher temperatures. Growth on CO was observed in mineral medium without yeast extract and without additional vitamins, which is a great advantage for potential biotechnological use due to a considerable cost reduction. Another interesting aspect of syngas utilization is the simultaneous consumption of CO and H<sub>2</sub>. While CO-tolerant H<sub>2</sub>-evolving hydrogenases have been described and characterized in several organisms (44, 45), the occurrence of an H<sub>2</sub>-consuming hydrogenase that is insensitive to CO inhibition is hitherto unprecedented (43). The syngas experiments were, however, conducted in a standing culture and thus are subject to the buildup of a gas gradient within the medium. Hence, it remains to be elucidated whether the simultaneous gas consumption is due to the different solubilities of CO and H<sub>2</sub> due to an established gas gradient or whether there is indeed a CO-tolerant H<sub>2</sub>-consuming hydrogenase.

The requirement of adaptation to CO is not only reflected by the growth experiments but also validated by cell suspension experiments with CO. Cell suspensions of *T. kivui* prepared from a culture adapted to CO showed an immediate increase in cellular ATP levels upon the addition of CO. In contrast, the addition of CO to cell suspensions of glucose- or H<sub>2</sub>-plus-CO<sub>2</sub>-grown cells resulted in an immediate decrease in cellular ATP levels and no

acetate formation. A requirement for adaptation to CO as an energy source was reported previously for another acetogenic bacterium. In 1982, a strain of *Butyrivibrio methylotrophicus* (Marburg strain), was adapted to grow vigorously on CO alone, which was termed the CO strain of *B. methylotrophicus* (20). The authors of that study selected the strain by transferring the Marburg strain twice to medium containing methanol with a 100% CO gas phase and subsequently transferring the strain four times to medium with 100% CO. While cells from the first transfers reached an OD<sub>660</sub> of 0.1 to 0.2 after 2 to 3 weeks, the fifth transfer resulted in an OD<sub>660</sub> of 0.5, and CO was completely consumed in 3 days. CODH levels in the methanol-grown strain were surprisingly higher than those in the CO-grown strain, which led to the conclusion that more than one CODH activity is required for growth on carbon monoxide. The genome of *T. kivui* harbors two genes coding for carbon monoxide dehydrogenases. One of these genes is *acsA*, which encodes the CODH that, together with the acetyl-CoA synthase, forms the central enzyme complex of the WLP, the CODH/ACS complex. The second CODH gene (*cooS*) presumably encodes a monofunctional CODH (29). CODH activity measured in crude extracts revealed that CODH activity was highest in cells grown on H<sub>2</sub> plus CO<sub>2</sub> and only marginally lower in CO-grown cells but much lower in glucose-grown cells. This finding is plausible, as during growth on CO, the latter must be scavenged as quickly as possible to prevent a possible inhibition of the soluble hydrogenases. Immunoblot analysis revealed that bifunctional AcsA was present during growth on all substrates but was clearly less abundant in glucose-grown cells. The monofunctional CODH CooS, on the other hand, was clearly more abundant in cells grown on CO than in cells grown on H<sub>2</sub> plus CO<sub>2</sub> or on glucose. Thus, the monofunctional CODH seems to play a superior role during CO metabolism, making it quite feasible that the ability to grow on CO is at least partly attributable to the monofunctional CODH. However, a monofunctional CODH besides the CODH/ACS is also encoded in the genome of *A. woodii*, and since this acetogen cannot grow solely on CO (24), it is more likely that a more elaborate adaptation is required.

During autotrophic growth, acetogenic bacteria rely on a chemiosmotic mechanism for energy conservation. In the case of *T. kivui*, the respiratory enzyme is an Ech complex (29) that, in principle, catalyzes ferredoxin oxidation and H<sup>+</sup> reduction. This is coupled to H<sub>2</sub> evolution and ion translocation. Here we show that the production of acetate and H<sub>2</sub> was coupled to the synthesis of ATP. Whereas acetate production was inhibited by a protonophore, H<sub>2</sub> production was stimulated, indicating a coupling of hydrogen production from CO with the synthesis of ATP by a chemiosmotic mechanism. Thus, the generation of the chemiosmotic ion gradient during autotrophic growth on CO is directly coupled to H<sub>2</sub> evolution. H<sub>2</sub> evolution from CO requires the presence of a CO-tolerant hydrogenase. This is reminiscent of the CO-oxidizing, H<sub>2</sub>-forming enzyme systems (Coo) found in *Methanosarcina barkeri* (46), *Rhodospirillum rubrum* (47), *Carboxydotherrmus hydrogeniformans* (48), and *Thermococcus onnurineus* NA1 (49). These organisms grow on CO by coupling the oxidation of CO to CO<sub>2</sub> by a proton-translocating and proton-reducing electron transport chain. Purification of the CO-oxidizing, H<sub>2</sub>-forming enzyme from *C. hydrogeniformans* revealed that a 6-subunit [NiFe]-hydrogenase forms a tight complex with a Ni-containing CODH (CooS) and an electron transfer protein (CooF) (45). It is feasible that a

very similar complex is present in *T. kivui* grown on CO, and the higher abundance of CooS in CO-grown cells consolidates this idea. This idea is consistent with our previous notion that *T. kivui* has two Ech hydrogenases (29) and the observation that hydrogenase activity is by far the highest in crude extracts prepared from CO-grown cells. The role of the different Ech hydrogenases in *T. kivui* and their biochemistry and bioenergetics remain to be established.

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