Growth of *Eubacterium limosum* with Carbon Monoxide as the Energy Source

B. R. SHARAK GENTHNER¹ AND M. P. BRYANT^{1,2*}

Departments of Dairy Science¹ and Microbiology,² University of Illinois, Urbana, Illinois 61801

Received 1 July 1981/Accepted 28 August 1981

Eubacterium limosum grew with CO as the sole source of energy and formed acetate and CO₂ as the major products. The generation time on CO was 7 h. Uninhibited growth occurred in cultures containing 50% CO or less, but growth occurred at all concentrations tested (i.e., up to 75% CO). The pH optimum for growth was 7.0 to 7.2, whereas growth was poor at a pH below 6.7. CO₂ stimulated growth on CO. CO was preferentially utilized when both CO and H₂ were present.

A number of anaerobic bacteria oxidize CO to CO₂. These include *Clostridium aceticum* (16), *Clostridium formicoaceticum* (9), *Clostridium pasteurianum* (13), *Clostridium perfringens* (welchii) (21), *Clostridium thermoaceticum* (9), *Desulfovibrio desulfuricans* (18), *Rhodospseudomonas gelatinosa* (6, 25), and several methanogenic bacteria (5, 16). However, only *Methanobacterium thermoautrophicum* (5) and *R. gelatinosa* (6, 25) have been shown to utilize CO as the energy source.

The present investigation indicates that *Eubacterium limosum* RF grows quite well with CO as the energy source, forming acetate and CO₂ as fermentation products. This is the first report of acetate formation during growth on CO. Also, when CO, H₂, and CO₂ are present in the medium, *E. limosum* preferentially uses most of the CO before H₂ utilization is significant.

MATERIALS AND METHODS

Source of bacterial strain. *E. limosum* RF was isolated from the rumen of sheep fed a molasses-based diet (20).

Anaerobic methods and media. The anaerobic techniques employed were those previously described for growth of *E. limosum* on H₂-CO₂ (3, 20). The basal medium contained 5% rumen fluid, B-vitamins, minerals, NaHCO₃, cysteine-Na₂S-reducing solution, and 60% N₂-40% CO₂ gas phase (final pH, 7.2) (20). The amount of bicarbonate added was varied in the medium for determination of pH optima. Sterile 10% HCl was added to the medium instead of sodium bicarbonate to obtain pH's below 6.0.

To grow *E. limosum* on CO in the absence of CO₂, a medium under 100% N₂ was prepared in which sodium bicarbonate was replaced by 10 mM PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid); Sigma Chemical Co., St. Louis, Mo.] buffer. The pH of the medium was adjusted to 7.2 with 1 N NaOH before autoclaving.

Tubes containing 5 ml of medium each were inocu-

lated in triplicate with either 0.1 ml of an H₂-CO₂-adapted culture or 0.05 ml of an H₂-CO₂-adapted culture which had been grown under 50% CO before being used as inoculum. Cultures which were prepared as inocula were allowed to reach maximum absorbance before use.

Gases were added to the inoculated medium containing the 40% CO₂-60% N₂ (1 atmosphere [atm] [101 kPa]) storage gas phase, and the percent of gas added was based on 2 atm (202 kPa). Forty percent or less CO was added with a sterile syringe, and tubes were then pressurized to 2 atm (202 kPa) with N₂, which diluted the CO₂ to 20%. To add 50% CO, tubes were pressurized to 2 atm (202 kPa) with 100% CO, using a two-stage lecture bottle regulator (model 3320; Matheson Scientific, Inc., Elk Grove Village, Ill.). Approximately 60, 70, and 80% CO was added by pressurizing tubes with 100% CO to 2.1, 2.2, and 2.3 atm (212, 222, and 232 kPa), respectively.

Pressure in tubes was monitored with a manometer as described by Balch and Wolfe (3). The composition of the gas phase was determined on duplicate medium blanks for all growth conditions. Incubations were at 38 to 39°C. Growth was measured in 18- by 150-mm tubes by the change in absorbance at 600 nm with a Spectronic 70 (Bausch & Lomb, Inc., Rochester, N.Y.).

Analytical methods. Generation time was calculated and gas analysis was performed as described previously (17), except CO was determined by using helium carrier gas at a flow rate of 25 ml/min. If simultaneous quantification of H₂ and CO was desired, argon (25 ml/min) was used.

To determine utilization of CO or H₂ with time, growth in triplicate tubes was stopped by injection of 0.5 ml of 30% phosphoric acid at various timed intervals. The gas phase was then analyzed for CO or for both CO and H₂.

Volatile fatty acids were determined as described previously (20). The fermentation balance was calculated according to the method of Barker (4).

RESULTS

Growth of *E. limosum* with CO as the energy source. *E. limosum* cultures in basal medium

FIG. 1. Effect of increasing concentration of CO on the growth of *E. limosum* RF in basal medium with a gas phase containing 20% CO₂, CO as the energy source, and N₂ as the balance. Percent CO is based on 2 atm (202 kPa) and was determined in duplicate medium blanks. Tubes were inoculated with 0.1 ml of an H₂-CO₂-adapted culture grown to maximum absorbance. Each point is the mean value of triplicate cultures. Symbols: ●, 0%; ○, 5%; ■, 10%; □, 20%; ▲, 30%; △, 40%.

with increasing amounts of CO as the only energy source grew quite well, reaching maximum absorbance within 65 h (Fig. 1). Generation times were similar (about 11 h), and the maximum absorbance was approximately proportional to the initial amount of CO added. CO was not detected in the gas phase of cultures which had reached maximum absorbance, indicating that it had been completely utilized during growth.

E. limosum exhibited a high tolerance to CO, being able to grow uninhibitedly in up to 50% CO (Fig. 2). Generation times were calculated to be approximately 7 h, which was less than that indicated in Fig. 1. This was probably a result of using an inoculum which had been adapted to CO. At 57% CO, the doubling time increased to 9 h and the maximum absorbance was reduced, suggesting inhibition of growth at this level of CO. Growth rates and maximum absorbance continued to decline as the percentage of CO was increased. At 75% CO, the generation time increased 2.6-fold and the maximum absorbance decreased by 75%.

The time course of growth correlated with a concomitant decrease in CO (Fig. 3).

The pH optimum for growth in CO medium was 7.0 to 7.2 and was similar to that determined for growth on a methanol or H₂-CO₂ medium.

FIG. 2. Effect of increasing CO concentration to 50% and above on the ability of *E. limosum* RF to grow in a basal medium with a gas phase containing 20% CO₂ and N₂ as the balance. A total of 50, 57, and 75% CO was added by pressurizing tubes to 2.0, 2.07, and 2.25 atm (202, 209, and 227 kPa), respectively. Percent CO is based on 2 atm (202 kPa) and was determined in duplicate medium blanks for all CO concentrations. Tubes were inoculated with 0.05 ml of an H₂-CO₂-adapted culture grown to maximum absorbance in 50% CO before being used as inoculum. Each point is the mean value of triplicate cultures. Symbols: ●, 20%; ▲, 50%; ■, 57%; ○, 75%.

However, cultures grown on methanol grew fairly well down to an initial pH of 5.9, whereas those grown on H₂-CO₂ or CO did poorly at a pH of below 6.7 (data not shown).

Growth on CO was stimulated by the presence of CO₂. The generation time was 32 h for cultures in the CO medium buffered with PIPES but decreased to 14 h when 20% CO₂ was added to the gas phase. In bicarbonate-buffered medium containing 20% CO₂, the generation time was 11 h for cultures not previously grown under CO. Thus, bicarbonate was the preferred buffer (data not shown).

Fermentation products. Acetate and CO₂ were the major products during growth with CO. CO₂ was recovered in approximately twice the amount of acetate (Table 1). Other acids were not found in significant amounts.

Preferential utilization of CO compared with H₂. In cultures containing both CO and H₂,

FIG. 3. Decrease in CO concentration with time in cultures of *E. limosum* RF containing CO as the sole energy source. Cultures were prepared by adding 25% CO (based on 2 atm [202 kPa]) with a sterile syringe and pressurizing to 2 atm (202 kPa) with N_2 . Tubes were inoculated with 0.1 ml of an H_2 - CO_2 -adapted culture grown to maximum absorbance. Each point is the mean value of triplicate tubes.

growth occurred during the first 70 h of incubation (Fig. 4). During this time, the CO content declined, whereas the H_2 content remained unchanged for the first 44 h. When CO declined to 5% of the gas phase, the H_2 content began to

FIG. 4. Effect of CO on the utilization of H_2 by *E. limosum* RF during growth. Cultures were prepared by pressurizing basal medium to 1.5 atm (151.5 kPa) with H_2 and then adding CO to 2 atm (202 kPa). Initial CO concentration was determined on duplicate medium blanks.

decline at a rate of 5 μ mol/h. After CO was reduced to below 3% of the gas phase, the rate of H_2 utilization increased over sixfold, although the amount of further growth was relatively small.

DISCUSSION

Oxidation of CO to CO_2 has been reported for several *Clostridium* species (9, 13, 16, 21), *D. desulfuricans* (18, 26), *R. gelatinosa* (6, 25), and many methanogens (5, 16). Unlike *E. limosum*, the *Clostridium* species are incapable of using CO as the energy source because they require the presence of pyruvate or a precursor of pyruvate for the conversion of CO to CO_2 . Although cellular extracts of *D. desulfuricans* oxidize CO to CO_2 when supplied with an appropriate electron acceptor (26), this species has never been reported to grow on CO. Methanogenic bacteria remove CO from the gas phase in cultures grown on H_2 - CO_2 , but of these, only *M. thermoautotrophicum* has been reported to grow on CO (5). In contrast to *E. limosum*, the growth rate of *M. thermoautotrophicum* on CO is only 1% of that observed on H_2 - CO_2 (5). In the present study, we found that the generation time of *E. limosum* growing on CO or H_2 - CO_2 was similar, being 7 h with cultures previously adapted to the particular substrate. Thus, CO appears to support normal growth of *E. limosum*, but not of *M. thermoautotrophicum*.

It was also observed that *E. limosum* had a high tolerance of CO and could grow uninhibited when CO was 50% or less of the gas phase.

TABLE 1. Fermentation balance of *E. limosum* RF in basal medium containing two concentrations of CO^a

% CO	CO used as substrate	Amt (mmol/liter) of recovery ^b		% Recovery of:	
		Fermentation product		Carbon	Hydrogen
		Acetate	CO_2		
20	58.0	12.6	23.9	84.5	86.8
40	120.2	24.3	57.0	87.8	80.7

^a Numbers are the means of triplicate cultures incubated until maximum absorbance was reached.

^b Corrected for concentrations in uninoculated medium. CO was added with a sterile syringe to the desired percent CO (based on 2 atm [202 kPa]), followed by pressurizing to 2 atm (202 kPa) with N_2 . Initial percent CO was determined on duplicate medium blanks.

The growth of *Clostridium* species on pyruvate or other energy sources is inhibited when CO is greater than 5% of the gas phase (9, 13). Methanogens growing on H_2 - CO_2 in the presence of CO are inhibited if the CO content is greater than 10% (5). During growth on CO as the energy source, *M. thermoautotrophicum* shows a maximum growth rate at 30% CO, but at higher concentrations, the growth rate decreases drastically, reaching nearly zero at 60% CO and above (5). In contrast, *R. gelatinosa* (6, 25) was reported to grow routinely under 100% CO. We have observed normal growth of *E. limosum* with 50% CO as the energy source. Above 50% CO, inhibition was apparent as a decrease in maximum absorbance and an increase in generation time; however, growth did occur in 75% CO, which was the highest concentration tested.

Optimum growth of *E. limosum* on CO occurred at a pH of 7.0 to 7.2, which was similar to the pH optima found for H_2 - CO_2 and methanol; however, growth on methanol appeared to be more acid tolerant than growth on CO or H_2 - CO_2 . Optimum growth of *E. limosum* on CO, H_2 - CO_2 , and methanol also shared a requirement for CO_2 , it being stimulatory to growth on CO and required for growth on H_2 - CO_2 and methanol (20). Finally, the growth rates under optimum conditions for these three substrates were approximately 7 h.

The similarities in pH optimum, growth rate, and CO_2 requirement for growth of *E. limosum* on CO, H_2 - CO_2 , and methanol, together with characteristics of the enzyme systems involved in acetate formation by certain clostridia, support the hypothesis that these one-carbon compounds are metabolized by similar catabolic pathways. Diekert et al. (7) have proposed a scheme which couples the oxidation of CO with reduction of CO_2 to acetate and suggest that CO acts as an electron donor to form acetate from CO_2 and methyltetrahydrofolate. The final step in this scheme depends on a "corrinoid" enzyme (14). CO oxidation was reported to involve a corrinoid enzyme (9, 23) which might contain nickel (8, 10, 11). Finally, *Acetobacterium woodii*, which forms acetate during growth on H_2 - CO_2 , also contains high levels of tetrahydrofolate enzymes and corrinoids (22). Further study will be necessary to confirm common pathways during catabolism of C_1 compounds by *E. limosum*.

The formation of acetate by *E. limosum* during growth on CO has not been reported previously. *M. thermoautotrophicum*, as expected, forms CH_4 and CO_2 during growth on CO (5). *R. gelatinosa* growing at the expense of CO, both photosynthetically and anaerobically in the dark, converts CO to CO_2 and H_2 by an unknown mechanism (6, 25). *C. formicoaceticum*

(1) and *C. thermoaceticum* (12) form acetate from CO_2 in the presence of certain carbohydrates and oxidize CO to CO_2 but, as indicated above, are unable to utilize CO as the energy source. *A. woodii* can grow on H_2 - CO_2 and form acetate (2), but growth on CO has not been reported. Thus, *E. limosum* is apparently the only acetogenic anaerobe known to grow on CO as the sole energy source.

We recently reported that *E. limosum* formed acetate, butyrate, and caproate during growth on methanol and acetate and butyrate during growth on H_2 - CO_2 (20); however, fatty acids longer than acetate were not detected in cultures of *E. limosum* grown on CO. Butyrate may not be formed during growth on CO due to the inhibition of butyrate-forming enzymes by CO. The absence of longer-chain fatty acids may also be a result of the oxidation state of the substrate, as suggested by the formation of acetate, butyrate, and caproate during growth on methanol, which was the most reduced C_1 compound tested.

CO_2 was also a product during growth of *E. limosum* on CO and was formed in approximately twice the amount of acetate. This is the ratio expected if the following equation is accurate: expected if the following equation is accurate: $4CO + 4H_2O \rightarrow 2HCO_3^- + 1CH_3COO^- + 3H^+$.

Several experiments were done in which both CO and H_2 were present in cultures, and the results obtained suggested that H_2 uptake was low until CO was gone. To further investigate this phenomenon, the study presented in Fig. 4 was done. It is apparent that CO is preferentially utilized when both CO and H_2 are present. Inhibition of hydrogenase by CO is classical (15) and has been reported in other anaerobic bacteria known to use CO (5, 19, 24). The data suggest that it also occurred in *E. limosum*; however, further study will be required for confirmation.

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