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Carbon Monoxide-Dependent Growth of *Rhodospirillum rubrum*

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Under dark, anaerobic conditions in the presence of sufficient nickel, *Rhodospirillum rubrum* grows with a doubling time of under 5 h by coupling the oxidation of CO to the reduction of H^+ to H_2. CO-dependent growth of *R. rubrum* UR294, bearing a kanamycin resistance cassette in *cooC*, depends on a medium nickel level ninefold higher than that required for optimal growth of *coo*$^+$ strains.

Numerous microorganisms oxidize CO to CO_2: in aerobes the oxidation is catalyzed by an inducible molybdenum-containing oxidase and is coupled to the reduction of oxygen; in strictly anaerobic bacteria and archaea, CO oxidation occurs on a constitutively expressed nickel-containing carbon monoxide dehydrogenase (CODH) and is linked to a variety of reductions. These CODH enzymes are the key components of metabolic processes that interconvert single carbon units and acetyl coenzyme A, leading ultimately to the generation of acetate or methane or the reduction of sulfate. Aerobic and anaerobic CO oxidation and the fundamental role of CODH in anaerobic pathways of carbon metabolism have been reviewed previously (5, 13, 20, 23, 24, 29, 31, 33, 34).

The oxidation of CO to CO_2 ($E_0' = -0.52$ V) coupled to the reduction of protons to H_2 ($E_0' = -0.41$ V) under anaerobic conditions has been shown to support the growth of a few organisms and may be a component in the energetics of others. CO-tolerant photosynthetic growth of a bacterium was reported by Hirsch in 1968 (15), and dark CO-dependent growth and H_2 production by *Rhodocyclus gelatinosus* (formerly *Rhodopseudomonas gelatinosa*) was established by Uffen and coworkers (4, 6, 30–32). Dashekvicz and Uffen also suggested that *Rhodospirillum rubrum* was capable of slow growth under similar conditions (6, 31). More recently Svetlichny et al. demonstrated the CO-oxidizing and H_2-generating metabolism and rapid growth of a nonphotosynthetic thermophilic anaerobe, *Carboxydothermus hydrogenoformans* (27). *Methanosarcina barkeri* cultures (25) as well as cell suspensions of methanogenic (3, 28), acetogenic (8), and sulfate-reducing (21) organisms also catalyze this reaction with some evidence for coupling to ATP generation (2, 8) and formation of a transmembrane proton gradient (3, 8). Hence, CO-dependent H_2 production may be essential to a variety of anaerobic energy generation mechanisms (2, 8, 13, 25, 28).

Efforts in our laboratories have elaborated the biochemistry and molecular biology of the CO-oxidizing and H_2-producing system of *R. rubrum*. Under anaerobic conditions, regardless of the presence of light or other carbon sources, CO induces the synthesis of several proteins, including CODH, an associated Fe-S protein, and a CO-tolerant hydrogenase. The 67-kDa Ni-CODH and the 21-kDa Fe-S protein have been purified and characterized biochemically. In vivo as well as in vitro, electrons derived from CO oxidation, evidently at a Ni-Fe center of CODH (10), are conveyed via the Fe-S protein (and probably other intermediates) to the hydrogenase (11). The genes for these enzymes and additional components have been cloned and sequenced, and *R. rubrum* strains mutated in the genes encoding CODH (*cooS*), the Fe-S protein (*cooF*), and the probable hydrogenase large subunit (*cooH*) and a fourth open reading frame (now designated *cooC*; strain UR294) are available (16, 18). Although the organisms are evolutionarily distant, significant amino acid conservation exists between CooS and subunits of the Ni-CODHs of the acetogen *Clostridium thermoaceticum* and the methanogen *Methanothrix soehngenii* (18).

The purpose of this report is to demonstrate the rapid CO- and Ni-dependent dark anaerobic growth of *R. rubrum* in liquid and plate cultures. These procedures are used in our ongoing analyses of *coo* mutants.

Liquid cultures were cultivated in RRNCO medium containing (per liter of distilled tap water) 2 μg of biotin, 10 ml of a chelated iron-molybdenum solution (0.28 g of H_3BO_3, 2 g of Na_2EDTA, 0.4 g of ferric citrate, and 0.1 g of Na_2MoO_4 per liter of glass-distilled water), 250 mg of $MgSO_4 \cdot 7H_2O$, 132 mg of $CaCl_2 \cdot 2H_2O$, 1 g of NH_4Cl, 20 μM $NiCl_2$, 1.0 g of yeast extract, 2.1 g of morpholinopropanesulfonic acid (MOPS), and 0.82 g of sodium acetate as a nonfermentable carbon source. The medium, pH adjusted to 7.1, was prepared under strictly anaerobic conditions and dispensed under Ar (rendered oxygen free by passage through heated copper filings) to a volume of 10 ml per 100-ml serum vial (average volume, 121 ml; Wheaton, Millville, N.J.). Vials were sealed with butyl rubber stoppers (Bellco, Vineland, N.J.) prior to autoclaving. Anaerobic solutions of 0.05 ml of 1.91 M potassium phosphate (pH 7.0), 0.1 ml of 1% $Na_2S \cdot 9H_2O$, 0.25 ml of 0.5 M $NaHCO_3$ (pH 8.0), and filter-sterilized CO (Matheson; 99.99%; rendered oxygen free [10]) were added prior to inoculation. Vials were incubated horizontally on a reciprocal shaker (90 oscillations per min; 2.5-cm throw) at 30°C in the dark for CO-dependent growth. Photosynthetic growth conditions have been described previously (18).

The plate medium was agar-solidified (1.2%) SMN medium (18) supplemented with phosphate buffer (5 ml/liter of medium) and $NiCl_2 \cdot 6H_2O$ to the indicated levels. Plates (100 by 15 mm), which contained 30 ml of medium, were prepared and inoculated aerobically and then were incubated overnight in the dark in GasPak jars (BBL Microbiology Systems) under an H_2-CO_2 atmosphere prior to introduction of CO to approximately 40%.

Gas samples (0.25 ml) were removed with a 1-ml glass syringe fitted with a Mininert syringe valve (Supelco, Bellefonte, Pa.) and analyzed for H_2 by thermal conductivity gas chroma-

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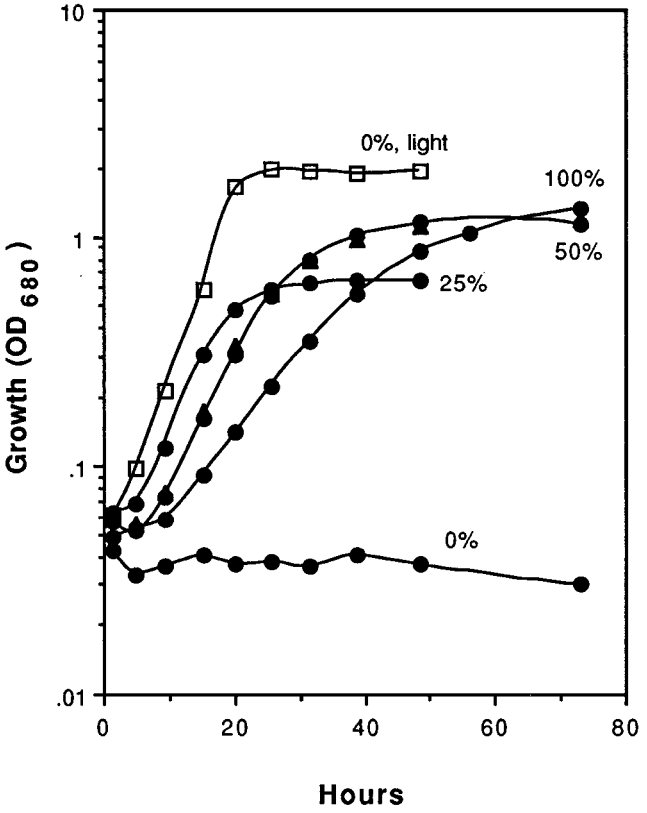

FIG. 1. CO-dependent growth of *R. rubrum* in liquid medium. Cultures were incubated photosynthetically in the absence of CO (□) or in the dark with an initial CO level of approximately 0, 25, 50, or 100% (vol/vol of gas phase) (●), as indicated. Results for cultures containing 50 mM DMSO in addition to an initial headspace CO concentration of 50% are also plotted (▲) but are nearly obscured by those for the cultures lacking DMSO. Plotted data represent averages from three cultures per condition, all of which were inoculated with a CO-grown inoculum (ca. 50% CO [initial]).

tography as previously described (18). CO was similarly analyzed except that He carrier gas was used in place of N_2. The H_2 and CO contents reported for vials include their calculated solubilized portions, based on partial pressures and gas solubilities, though these corrections are minuscule given the low solubilities and the large headspace-to-liquid ratio. CO_2 and CH_4 were similarly analyzed with a 15-ft (4.6-m) HayeSept DB column (Alltech Associates Inc., Deerfield, Ill.) and a He carrier gas flow of 30 ml/min. The dissolved and ionized CO_2 species were determined by headspace analysis of a sealed 25-ml Ar-flushed vial containing 2.5 ml of an acidified culture sample. The result, adjusted for the solubility of CO_2 and the total culture liquid volume, was added to the headspace CO_2 determined for the culture itself.

Soluble compounds were separated with an HPX-87H high-pressure liquid chromatography (HPLC) column (Bio-Rad, Hercules, Calif.) operated at 65°C with a 28 mM H_2SO_4 eluent and monitored at 210 nm. A Beckman DU-6 spectrophotometer was used for growth measurements. An optical density at 680 nm (OD_{680}) of 1 (1-cm path) against an RRNCO medium blank corresponds to 390 mg (dry weight) of cells per liter.

CO-dependent growth. Anaerobic CO-dependent growth of *R. rubrum* in the dark is shown in Fig. 1. Cell yields were approximately proportional to the initial headspace CO concentration: cultures with initial CO levels of 25 and 50% depleted the substrate and attained OD_{680}s of 0.64 and 1.15, respectively; cultures started with 100% CO attained an OD_{680} of 1.78 by 122 h and had consumed approximately 75% of the CO available. At the 50% initial CO level, the yield was unaffected by the presence of 50 mM dimethyl sulfoxide (DMSO), even though CO oxidation coupled to DMSO reduction (DMSO/dimethyl sulfide $E_0' = 0.16$ V [35]) is much more favorable than the CO-H_2 couple and *R. rubrum* is capable of using DMSO as an electron acceptor, albeit slowly (18-h doubling time on acetate-DMSO [26]). *R. rubrum* strains bearing mutations in *cooF*, *cooS*, or *cooH* (18) failed to grow (17).

TABLE 1. CO-dependent growth material balance[a]

Parameter	Initial value	Final value
OD_{680}	−0.01	0.75
pH	7.48	6.69
Dry wt (mg/vial)	0.14	3.16
Amt (μmol/vial) of:		
Cell C	6	132
CO	2,102	662
CO_2	126	1,646
H_2	18	1,586
Acetate	111	98

[a] Assays represent averages from triplicate vials per condition; vials initially contained approximately 50% CO in the headspace and were either analyzed immediately after inoculation or incubated for 46 h prior to analyses.

Under these batch growth conditions, the growth rate of *R. rubrum* was inversely related to the initial CO level, with doubling times, measured in this and a repeat experiment, of 4.8, 5.7, and 8.4 h observed for initial headspace CO levels of 25, 50, and 100%, respectively. For comparison, cultures incubated under photosynthetic conditions in the same medium lacking CO grew with a doubling time of 3.8 h (Fig. 1). Thus, the growth rate of *R. rubrum* with the CO-H_2 couple as a source of energy can reach 80% of the growth rate with an optimal energy source, namely, light.

The *R. rubrum* growth results are similar to those reported for *R. gelatinosus*, for which a doubling time of 6.7 h was observed in medium containing 0.1% tryptone under a 100% CO headspace (30). The nonphotosynthetic thermophilic anaerobe *C. hydrogenoformans* grows with a doubling time of 2 h in medium supplemented with 0.05% yeast extract (27). As with these organisms, CO-dependent growth of *R. rubrum* was enhanced by the inclusion of an undefined medium ingredient. In the absence of yeast extract yields were reduced threefold, and medium analyses indicated that an unidentified yeast extract component was utilized during CO-dependent growth (17).

Material balance. Material balance analyses resulted in the expected 1:1:1 ratio of CO consumed to H_2 produced to CO_2 produced, with carbon and electron balances of 107 and 113%, respectively, assuming that 50% of the cell dry weight is carbon (Table 1). The data indicate a yield of 2 g (dry weight) of cells per mol of CO, similar to the yields of other thermodynamically poor (ca. −20 kJ/mol of substrate) catabolic processes, including the disproportion of thiosulfate (1), succinate fermentation to propionate (9), and H_2- and CO_2-dependent acetogenesis (5). The reported yield for *R. gelatinosus* is 3.7 g (dry weight) of cells per mol of CO oxidized (4). We attribute the higher than expected carbon and electron balances to the CO-dependent consumption of a yeast extract component(s), one of which was noted during the HPLC analyses for acetate (17). No growth, CO consumption, H_2 and CO_2 accumulation, or pH changes were detected in vials inoculated with *R. rubrum* UR279 (*cooS*) in the presence of CO or in vials inoculated with *R. rubrum* UR2 (*coo*$^+$) in its absence. The lack of acetate

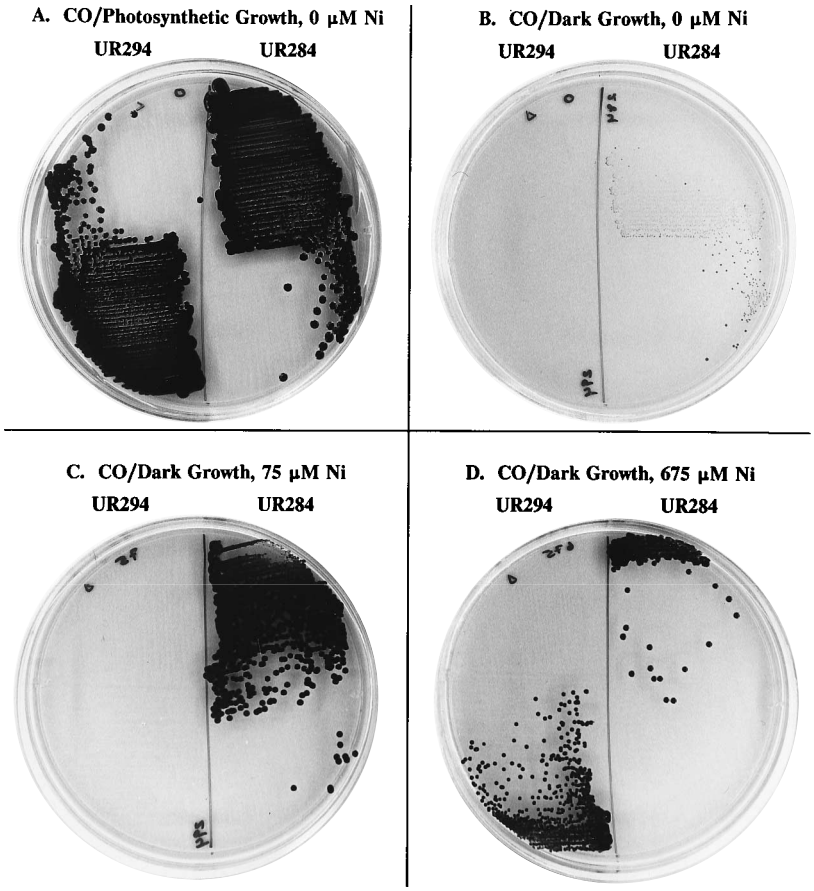

FIG. 2. CO- and Ni-dependent growth of *R. rubrum* on plates. SMN medium plates with the indicated additions of $NiCl_2$ were inoculated with photosynthetically grown (in the absence of CO) cultures of strains UR284 (*coo-6*::*kan*, phenotypically Coo^+) and UR294 (*cooC7*::*kan*) and incubated anaerobically under a CO-CO_2-H_2 atmosphere for 3 days in the light (A) or for 9 days in the dark (B to D).

accumulation during CO-dependent growth is consistent with the absence of acetyl coenzyme A synthase activity of the *R. rubrum* CODH. Methane (>10 μmol per vial) was not detected in cultures growing on CO (17).

Plate growth. CO-dependent growth on plates incubated anaerobically in the dark was observed when the medium was supplemented with appropriate levels of nickel. With rich SMN medium, Coo^+ *R. rubrum* strains, including UR284 (kanamycin resistance cassette located intergenically between *cooH* and *cooF* [18]), grew to 1.5-mm-diameter colonies after incubation at 30°C in the dark for 9 days. Growth was much poorer in the absence of added Ni (Fig. 2). In contrast, strain UR294, bearing the kanamycin resistance cassette in the putative nucleoside triphosphate-binding site of *cooC* (previously designated ORF4 [16, 18]), showed very slight CO-dependent growth unless the medium Ni concentration was increased to ≥600 μM (Fig. 2). On the basis of this observation, we expect that CooC and downstream gene products are involved in Ni insertion into CODH; this is consistent with sequence similarities of these proteins to gene products evidently involved in Ni incorporation into hydrogenase (7, 14, 22) and urease (19) in other organisms. Further mutational analysis of this region, encompassing 1.5 kb downstream of *cooS*, is under way (17).

This CO-dependent growth system may prove useful for thermodynamic analyses as the gaseous substrate and products, which potentially enter and exit the cell by simple diffusion, may be readily manipulated, measured, and correlated with growth rates. Furthermore, *R. rubrum* is amenable to genetic manipulation; while we are primarily interested in analyzing the CO-dependent growth of strains mutated in the *coo* region, one might expect altered CO-dependent growth in strains defective in electron transport, ATPase (12), or carbonic anhydrase, for example.

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