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Carbon monoxide production is not enhanced by nitrogenase activity

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Abstract

A diverse group of nitrogen-fixing bacteria and two heme degraders were grown with and without fixed nitrogen sources under oxic and suboxic conditions, with and without addition of heme-containing compounds. Several of the strains produced carbon monoxide (CO) under one or more of these conditions, but nitrogenase activity did not stimulate rates of production relative to controls. Although nitrogenase can reduce CO_2 to CO in vitro in the absence of N_2, this process likely contributes minimally to CO production in soils under in situ conditions. In contrast, myoglobin or hematin addition under oxic conditions significantly stimulated CO production by the heme degraders. However, estimates of CO production from microbial heme turnover suggest that this too is likely to be only a small source of CO in soils in situ.

Keywords: Carbon monoxide; Nitrogenase; Heme; Soil

1. Introduction

The importance of carbon monoxide (CO) for tropospheric chemistry is well documented (e.g. [1–4]). Soils have also been established as potential sources and sinks for atmospheric CO (e.g. [5–11]). In addition, it is evident that CO is produced routinely within the matrix of many soils, even though net CO consumption is observed overall [5,9,11]. The magnitude of CO production in soils on a global basis is uncertain, but it can contribute significantly to total CO budgets [8,9]. Previously, soil CO production has been associated almost exclusively with abiological processes [12–14], mostly involving temperature- or pH-driven chemical decomposition of organic matter [12].

However, biological CO production is also feasible. Possible biological CO sources include aerobic degradation of heme compounds, especially chlorophyll but also other heme-containing compounds [15–17], CO production from non-heme protein turnover [18], and production of CO by anaerobes in anoxic microzones ([7] and references therein). Recently, an additional source has been identified, that is enzymatic reduction of CO_2 to CO [19,20]. Assays with cell-free preparations of *Azotobacter vinelandii* have shown that nitrogenase reduces CO_2 to CO [19]. Conditions favoring this activity include a low pN_2, a relatively high pCO_2 and anoxia.

Whether or not CO production by nitrogenase occurs routinely in vivo or in situ is unknown. However, due to the widespread distribution of *Azotobacter* and other nitrogen-fixing taxa, the potential role of nitrogenase as a CO source merits attention. Thus, we have undertaken a survey using whole cells to determine the extent to which nitrogen-fixing bacteria might produce CO. The results suggest that nitrogenase is not likely to be an important CO source in situ, presumably as a result of the high affinity of nitrogenase for N_2 and its low affinity for CO_2 [19].

2. Materials and methods

2.1. Culture and growth conditions

A. vinelandii, *Rhizobium leguminosarum* BV *trifolii* and *R. leguminosarum* BV *meliloti* were obtained from the American Type Culture Collection. *Methylosinus trichosporium* OB3b was obtained from Dr. R.S. Hanson (University of Minnesota, MN, USA), and a CO-oxidizing *Xanthobacter* species was obtained from the roots of an aquatic macrophyte (Rich and King, unpublished data).

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Two additional strains, He 02 and He 011 were isolated from the top 10 cm of soil in a field near the Darling Marine Center, Walpole, ME, USA. Enrichment cultures for these strains were established with a 30% soil inoculum in the minimal medium below with hemoglobin added at a final concentration of 1 g l^{-1}. One set of enrichments was treated initially for 10 min at 80°C to select for Gram-positive spore-forming strains. Enrichments were shaken at 120 rpm for 48 h, at which time hemoglobin was no longer visible in the medium. A second pasteurization was performed immediately prior to plating the originally pasteurized set on blood agar with 1% hemoglobin. Colonies with clear zones around them were considered heme degraders. After repeated subculturing, one Gram-positive bacterium, He 02, and one Gram-negative bacterium, He 011, were isolated. These strains were not characterized further.

All cultures other than *Xanthobacter* spp. were grown in a standardized minimal medium containing (g l^{-1}): K_2HPO_4 (0.8), KH_2PO_4 (0.2) at pH 7.2, with either NH_4Cl (0.8) or $NaNO_3$ (1.5), and vitamins and trace minerals [21]. *R. trifolii*, *R. meliloti*, He 02 and He 011 required 0.05% yeast extract for growth. Nitrogen-free carbon sources included mannitol (10 g l^{-1}) for *R. trifolii* and *R. meliloti*; glycerol (1 ml l^{-1}) for He 02 and He 011; sucrose (20 g l^{-1}) for *A. vinelandii*; and ethanol (1 ml l^{-1}) for *Xanthobacter* sp. *M. trichosporium* OB3b was grown in Higgins mineral salts as described previously [22] with a headspace containing 25% methane (v/v). *Xanthobacter* sp. was grown in mineral medium as above, but with a 2-fold dilution of the phosphate buffer. All cultures were incubated with shaking (120 rpm) at 30°C without illumination.

2.2. CO production assays

All cultures were assayed in both log (OD 0.2 to 0.4 at 600 nm) and stationary growth phases (OD > 1.0). Cultures were harvested by centrifugation at 10 000 × *g* at 4°C in a Dupont-Sorval RC-5B centrifuge for 10 min, then resuspended in fresh medium. Cultures were supplemented with either hematin (62 μM), myoglobin (1 g l^{-1}, 62 μM), or bovine serum albumin (BSA, a non-heme-containing protein; 1 g l^{-1}), all from Sigma Chemical Co. (St. Louis, MO, USA); controls were untreated. Hematin was dissolved in 0.5 ml of 0.1 N KOH prior to mixing, which elevated the final pH of the medium by 0.1–0.4 units. Aliquots (10 ml) of resuspended cultures were pipetted into 160-ml serum bottles and sealed with teflon-lined stoppers and crimp caps. Bottle headspaces were sampled at intervals with an air-tight syringe and analyzed by gas chromatography for CO. Prior to sampling headspaces, subsamples were collected for protein analysis using a bicinchoninic acid kit according to the manufacturer's instructions (Pierce, Inc., Rockford, IL, USA). All analyses were conducted in triplicate.

A. vinelandii and *M. trichosporium* OB3b were grown in media without fixed nitrogen for CO production assays. *R. meliloti* was grown in the presence of NH_4^+ and then washed and resuspended with NH_4^+-free medium under a headspace of 0.1% O_2 to induce nitrogenase activity [23]. These cultures were then assayed for CO and H_2 production under suboxic (0.1% O_2) and aerobic conditions. Nitrogenase activity was confirmed by formation of ethylene from added acetylene (10%) in the headspaces of active cultures [24], which were assayed by gas chromatography with flame ionization detection after scrubbing acetylene from the samples using ammoniacal silver nitrate as described by David et al. [25].

CO and hydrogen were determined using either an SRI

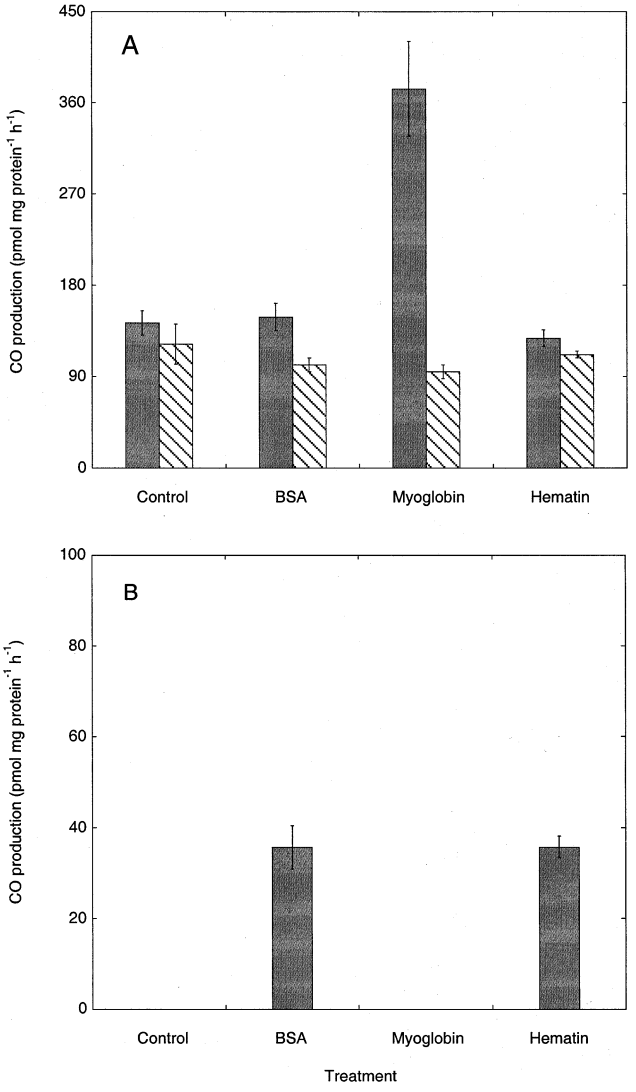

Fig. 1. A: CO production rates (mean ± 1 standard error) for oxic (closed bars) and suboxic (1% O_2; cross-hatched bars) *A. vinelandii* cultures grown with ammonium as a nitrogen source. All cultures contain sucrose as a carbon source with other treatments as indicated. B: CO production rates (mean ± 1 standard error) for suboxic (1% O_2) cultures of *A. vinelandii* grown without a fixed nitrogen source. All cultures contain sucrose as a carbon source with treatments as indicated. Note change in scale from A.

8610 gas chromatograph (SRI Instruments, Inc.; Torrance, CA, USA) fitted with a Trace Analytical RGA2 reduction gas detector or a Trace Analytical RGA3 reduction gas analyzer (Trace Analytical, Menlo Park, CO, USA). Instrument responses were calibrated using NOAA-CMDL certified standards (91.9 or 103.0 ppb). Initial CO production rates (ppb min^{-1}) were calculated using linear regression analysis; rates were corrected for production by uninoculated media.

3. Results and discussion

In the presence of ammonium, *A. vinelandii* produced 130 pmol CO mg $protein^{-1}$ h^{-1} under aerobic conditions (Fig. 1). CO production increased significantly (to 375 pmol CO mg $protein^{-1}$ h^{-1}) in the presence of myoglobin. However, no significant differences were observed between controls and either BSA or hematin treatments. In addition, CO was not produced at all under suboxic conditions with ammonium. Under aerobic nitrogen-fixing conditions, *A. vinelandii* controls produced CO at approximately the same rate (120 pmol CO mg $protein^{-1}$ h^{-1}) as aerobic controls grown on ammonium (Fig. 1), but there was no significant difference between treatments and controls under aerobic nitrogen-fixing conditions. CO was not produced in suboxic controls or in the presence of myoglobin (Fig. 1), but significant rates were observed for BSA and hematin treatments (approximately 40 pmol CO mg $protein^{-1}$ h^{-1}). During parallel assays for acetylene reduction, cultures incubated under suboxic conditions produced more ethylene than fully aerobic cultures (256–876 μmol mg $protein^{-1}$ h^{-1} versus 107 μmol mg $protein^{-1}$ h^{-1}).

In a series of similar assays, several other nitrogen-fixing taxa (e.g. *M. trichosporium* OB3b, *R. leguminosarum* and a *Xanthobacter* sp.) did not produce detectable levels of CO when grown with or without fixed nitrogen (not shown). Likewise these strains did not produce CO when supplemented with heme-containing compounds or BSA, with the exception of ammonium-grown aerobic cultures of *R. leguminosarum* BV *trifolii* (rates for myoglobin and hematin = 65 pmol CO mg $protein^{-1}$ h^{-1}).

Collectively, the results for *A. vinelandii* indicate that CO production by this organism under in vivo conditions is not closely coupled to nitrogenase activity. Indeed, the highest observed CO production rates occurred for an aerobic, ammonium-grown culture containing myoglobin. In addition, comparable levels of CO production by ammonium- and N_2-grown cells incubated with aerobic headspaces, and the lack of CO production in controls incubated under suboxic conditions further mitigate against nitrogenase-linked in vivo CO production. The absence of significant levels of CO production by other nitrogen fixers under nitrogen-fixing conditions, including *M. trichosporium* OB3b, *R. leguminosarum* and a *Xanthobacter* sp. (Table 1, data for *M. trichosporium* and *Xanthobacter* sp. not shown), suggests that nitrogenase is not likely a routine bacterial CO source.

These results differ from but do not contradict those of Seefeldt et al. [19], who have reported significant levels of CO production by nitrogenase in cell-free preparations of *A. vinelandii* incubated in an argon atmosphere. Results of kinetic analyses by Seefeldt et al. [19] reveal a much higher affinity of *A. vinelandii* nitrogenase for nitrogen than CO_2 (K_m = 0.08 mM and 23.8 mM for nitrogen and CO_2, respectively). Coupled with a higher V_{max} for nitrogen reduction and greater affinity for nitrogen, relatively high dissolved nitrogen concentrations under typical conditions likely preclude significant CO_2 reduction in most, if not all, nitrogen-fixing bacteria.

In contrast, heme-containing compounds appeared to support significant but variable levels of CO production among the taxa examined (Table 1). During log phase growth, the Gram-positive soil isolate He 02 produced 230 and 320 pmol CO mg $protein^{-1}$ h^{-1} for aerobic control and BSA-amended cultures, respectively. Similar cultures amended with myoglobin or hematin produced CO at rates of 1400 and 1100 pmol CO mg $protein^{-1}$ h^{-1}, respectively. Heme-dependent CO production required oxygen, since a reduction in headspace oxygen concentrations significantly decreased CO levels for myoglobin and hematin treatments (reductions of 62% and 25% respectively; Table 1), and completely inhibited CO production in controls and BSA-amended cultures. In addition, CO production from controls and heme-containing cultures decreased significantly during stationary phase. The Gram-negative soil isolate He 011 also produced CO primarily during log phase growth. Fully aerobic conditions promoted CO production over suboxic conditions, except in hematin-amended cultures (Table 1).

Results from assays with myoglobin and hematin suggest that heme degradation represents a more likely biological CO source in soils than does nitrogenase activity.

Table 1
CO production (pmol mg $protein^{-1}$ h^{-1}) during log phase by selected nitrogen-fixing and heme-degrading (He 02 and He 011) bacteria

Culture	Control		BSA		Myoglobin		Hematin	
	Oxic	1% O_2	Oxic	1% O_2	Oxic	1% O_2	Oxic	1% O_2
R. leguminosarum BV *trifolii*+NH_4^+	–	–	–	–	65	–	65	–
He 02+NH_4^+	230	–	320	–	1400	540	1100	820
He 011+NH_4^+	125	100	175	85	530	–	365	410

Data are CO production rates in pmol mg $protein^{-1}$ h^{-1}; –, not detectable.

Although the content and turnover of soil heme compounds have not been specifically documented, an estimate of the potential importance of heme degradation for CO production can be developed from some limiting assumptions. If soil heme is primarily microbial in nature and occurs in cytochromes with an average molecular mass of 100 000 Da that account for at most 3% of cell mass [26], then a 10-cm soil column with a bulk density of 1.5 gfw cm^{-3} and 1 mgdw bacteria gfw^{-1} soil will contain approximately 45 μmol heme m^{-2}. For a ratio of 1 mole CO per mole heme degraded [15,16] and turnover times of 1 week, 1 month and 1 year, CO production from heme could equal 120, 28 and 2.4 μg m^{-2} day^{-1}, respectively. Based on these assumptions only heme turnover times on the order of 1 week would likely contribute significantly to observed gross CO production rates for forest soils of 400–10 000 μg m^{-2} day^{-1} [9,11,14]. Rates of heme-related CO production would be further constrained by requirements for molecular oxygen ([15] and Fig. 1, Table 1) and an apparent requirement for bound iron [15].

Results from this study also indicate that the form of heme available, e.g. hematin versus myoglobin, can affect CO production (Fig. 1, Table 1) as does microbial growth state (e.g. CO production is greater in logarithmic than stationary phase growth). Although bacteria capable of producing CO from heme degradation may be diverse and widespread, heme turnover appears to be a minor component of the soil CO budget with abiological processes most likely dominating in oxic soils even at moderate temperatures. Heme turnover associated with leaf senescence may yet prove to be a significant process, but it is not closely coupled to activity in the soil matrix and therefore not a direct contributor to soil CO production.

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