

## Primary Energy Metabolism in Geothermal Environments: The Role of Carbon Monoxide



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## ABSTRACT

Chemolithotrophic metabolism fuels primary production in many hydrothermal ecosystems, representing energy conservation strategies that may be independent of sunlight. Many autotrophic metabolic pathways in geothermal communities depend on H<sub>2</sub>; however, in this paper we focus on anaerobic carboxydrotrophs that are capable of using CO as a carbon and energy source, producing H<sub>2</sub> and CO<sub>2</sub>. We have recently completed the genome sequence of an autotrophic carboxydrotroph, *Carboxydotherrmus hydrogenoformans*, which appears to be a specialist in CO-oxidation, encoding five unlinked genetic loci capable of expressing CO dehydrogenase. Because of their global occurrence in geothermal environments, we propose that CO-utilizing organisms play important roles in anaerobic microbial consortia. To understand the role of CO-utilizing bacteria in Yellowstone hot springs we isolated and described a novel H<sub>2</sub>-producing bacterium (strain Nor1) from an Fe-rich site in Norris Geyser Basin, YNP. Strain Nor1 is a low-G+C Gram-positive bacterium belonging to the division Firmicutes, and grows chemolithotrophically at 75°C on CO (generation time 1.5 h), producing equimolar quantities of H<sub>2</sub> and CO<sub>2</sub>. Strain Nor1 is also capable of chemoautotrophic growth on Fe<sup>III</sup> and Se, as well as growing heterotrophically on glucose and several sugars, producing acetate, H<sub>2</sub>, and CO<sub>2</sub>. We have proposed that strain Nor1 be assigned to a new genus, *Thermosinus* gen. nov. The type species is *Thermosinus carboxydivorans* sp. nov. (type strain, Nor1T =DSM 14886T; Sokolova et al. 2004). It is evident that CO-dependent hydrogenogenic metabolism occurs in a diverse phylogenetic context and can be accompanied by a varied repertoire of alternative trophic strategies.

## Key Words

anaerobic  
carbon monoxide  
carbon monoxide dehydrogenase  
hydrogen  
iron reduction  
Norris Geyser Basin

## 1.0 INTRODUCTION

Over the past two decades, there has been an enormous increase in the number of new thermophilic isolates with diverse metabolic strategies, consistent with the diversity of geochemical energy sources associated with continental and submerged hydrothermal vents (reviewed in Reysenbach and Shock 2002). These metabolic pathways fuel chemolithoautotrophic primary production in many hydrothermal ecosystems such as hot springs in Yellowstone National Park (YNP) where opportunistic microbial metabolic adaptation is on display, often in brilliant color.

Carboxydrotrophic bacterial thermophiles capable of using CO(g) as their only source of energy and carbon have been isolated from diverse geothermal habitats worldwide (Sokolova et al. 2004; **Table 1**). This unique physiology is not new to microbiology. First described by Uffen (1976), many phototrophic purple nonsulfur bacteria, when cultured anaerobically in the dark, produce H<sub>2</sub> at the expense of CO, which is converted to CO<sub>2</sub>. Using tritiated water, Uffen showed that H<sub>2</sub> was the product in what is now known as the *water-gas shift reaction* [CO + H<sub>2</sub>O = CO<sub>2</sub> + H<sub>2</sub>]. In these phototrophs, the oxidation of CO appears to be a backup metabolic strategy allowing the cells to maintain a slow growth rate on CO. In effect, the capacity for using an alternative energy source represents an important adaptation that, in the case of phototrophs, appears to be justified by the reliable assumption that

darkness will ensue each day at sunset in the natural environment! The CO dehydrogenase and associated hydrogenase components are repressed by light and induced by CO and darkness, and growth proceeds at a slow rate in the absence of light with a relatively low growth yield coefficient of 3.7 g (dry wt.) of cells per mol of CO oxidized (Champine and Uffen 1987).

Thermophilic CO-utilizers produce H<sub>2</sub>, acetate, or methane as their primary end products, and it seems reasonable to assume that they are involved in cross-feeding other chemolithotrophs such as methanogens, acetoclastic methanogens, methanotrophs, H<sub>2</sub>-oxidizers, or S-reducers. This chapter reviews the distribution of several CO-utilizing thermophiles and describes the isolation of a CO-utilizing organism from Norris Geyser Basin, YNP.

## 2.0 MATERIALS AND METHODS

### 2.1 Strains

*Carboxydotherrmus hydrogenoformans* DSM 6008 and *Thermoterrabacterium ferrireducens* DSM 11255 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). *T. ferrireducens* was cultured under strictly anaerobic conditions on modified freshwater medium, containing 30 mM glycerol as an electron donor and carbon source, and 20 mM Na-fumarate as an electron

**Table 1.** CO-oxidizing hydrogenogenic microorganisms and their isolation locales.

Organism	GenBank Accession No.	Isolation Locale	Habitat	Reference
<i>Carboxydotherrmus hydrogenoformans</i>	NC_002972	Kunashir, Kuril Islands Kamchatka	Mud from hot swamp, 68°C, pH 5.5	Svetlichnyi et al. 1991
<i>Carboxydotherrmus restrictus</i>	Not Available	Raoul Island, Kermadec Archipelago	Littoral hydrothermal sample	Svetlichnyi et al. 1994
<i>Carboxydocella thermautotrophica</i>	AY061974	Gezyer Valley, Kamchatka	Terrestrial hot spring, 60°C, pH 8.6	Sokolova et al. 2002
<i>Thermosinus carboxydivorans</i>	AY519200	Norris Basin, YNP, USA	Terrestrial hot spring, 50°C, pH 7.5	Sokolova et al. 2004
<i>Thermococcus</i> sp. AM4	AJ583507	East Pacific Rise (13o N)	Hydrothermal vent	Sokolova et al. 2004
<i>Caldanaerobacter subterraneus</i> subsp. <i>pacificus</i>	AF120479	Okinawa Trough	Marine hydrothermal vent, 110-130°C	Sokolova et al. 2001; Fardeau et al. 2004
<i>Caldanaerobacter subterraneus</i> strain 2707	Not Available	Kunashir, Kuril Islands	Terrestrial hot spring	Subbotina et al. 2003

acceptor. *C. hydrogenoformans* strain Z-2901 (DSM 6008) was grown as previously described (Svetlichny et al. 1991). *T. ferrireducens* was mass cultured in a 200 L fermentor at the Center of Marine Biotechnology ExSUF facility, under a gas phase of 100% CO<sub>2</sub> at 65°C. Late exponential phase cells were harvested by centrifugation. The cell paste, containing 150 g of cells (wet wt.), was frozen at -80°C for purification of the Fe reductase.

## 2.2 Sequencing Studies

An earlier study presented results of a genome survey of *C. hydrogenoformans* strain Z-2901 (Gonzalez and Robb 2000). Genomic DNA was extracted from cultures of *C. hydrogenoformans* strain Z-2901 (DSM 60008), and a genomic library was prepared using lambda Zip-Lox, *EcoRI* arms (Gibco BRL). Gene walking using the Vectorette II system (Genosys) and the *CooS* specific primer 5'-CCA TCG ATA CTT TCA AAC GGC GC-3' was performed to complete the sequence of

the *cooS* gene from *C. hydrogenoformans*. Phylogenetic analyses were performed by the program Pileup (Genetic Computer Group) and CLUSTAL W. Bootstrap values were the percentages from a sampling of 1000. The genome sequencing project was conducted in collaboration with J. Eisen and colleagues at The Institute for Genomic Research (TIGR), and the primers devised by A. Lebedinski at the Russian Academy of Sciences in Moscow.

## 2.3 Sampling

Samples were collected from Norris Geyser Basin, within the One Hundred Spring Plain, during June 2000 (Figure 1). A sample of sediment and geothermal water was collected from a small geothermal pool containing visible organic matter, at ~ neutral pH (7.5) and a temperature of 50°C (lat 44°43'797"N, long 110°42'506"W). The samples were used as inoculum for the isolation of CO-utilizing organisms using cultivation strategies defined previously (Sokolova et al. 2004).



↑ **Figure 1.** Sampling locations in Yellowstone National Park used for enrichment of anaerobic CO-oxidizing strains. **A, B.** One Hundred Spring Plain, Norris Geyser Basin, showing the hot spring (80-95°C) containing Fe<sup>III</sup> oxides from which *Thermosinus carboxydovorans* was isolated. Sampling equipment shown in B includes temperature probe, pH meter, and altimeter/barometer. **C.** Sampling location at Potts Basin. **D.** High temperature sampling with telescopic probe at full extension.

## 3.0 RESULTS AND DISCUSSION

### 3.1 Insights from Sequencing Studies

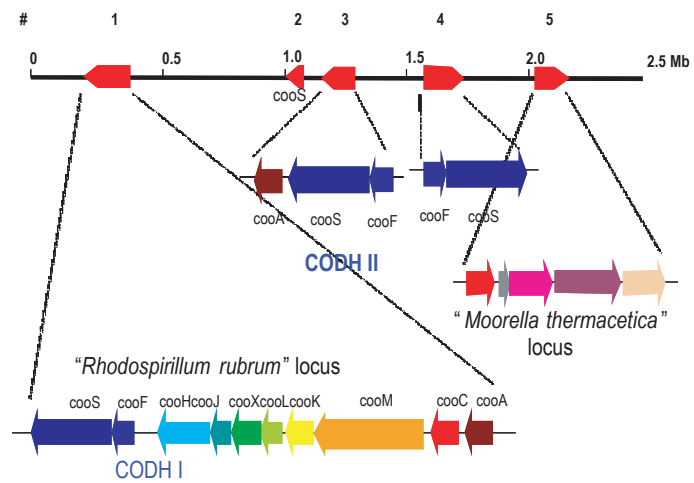
The complete genome sequence of *C. hydrogenoformans* has provided a gene inventory of a specialized thermophilic carboxydrotroph. *C. hydrogenoformans* was originally described as growing only on CO and pyruvate (Svetlichny et al. 1991). A recent study (Henstra and Stams 2004) indicates that *C. hydrogenoformans* displays more versatile substrate utilization than previously suspected. For example, 9,10-anthraquinone-2,6-disulfonate (AQDS) can act as an alternative to protons as an electron acceptor, where the strain utilizes CO and grows at the normal rate but produces no H<sub>2</sub>. One of the most unusual features of the annotated *C. hydrogenoformans* Z-2901 genome sequence is the presence of five dissimilar and apparently paralogous *cooS* genes (Figure 2). The operon encoding CODH I is homologous and similar in gene order and content to the locus in

*Rhodospirillum rubrum*, which is induced by growth in the dark in the presence of CO. The occurrence of a *cooA* gene that encodes a heme-containing transcriptional activator protein with marked similarity to a family of cAMP sensor proteins is of significant interest (Roberts et al. 2004). This operon has been shown to sense CO and to regulate CODH synthesis and H<sub>2</sub> production by *R. rubrum* and *Azotobacter vinelandii*. The *cooA* homolog from *C. hydrogenoformans* has been cloned and expressed in *Escherichia coli*, and shown to have similar sensing properties *in vivo* to the *cooA* from phototrophs (Youn et al. 2004).

Directional transfer of genetic information from *Archaea* to *Bacteria* is an area of active speculation. For instance, in the genome sequencing project of *C. hydrogenoformans* we have found various genes showing highest homology to members of *Archaea* suggesting the relatively recent transfer of genetic material between *Archaea* and the bacterium *C. hydrogenoformans*. For horizontal gene transfer to be feasible, we presume that anaerobic bacteria and potential archaeal donors occur in close physical proximity (Gonzalez and Robb 2000). *C. hydrogenoformans* is a strictly anaerobic thermophilic bacterium which utilizes CO and produces H<sub>2</sub> and CO<sub>2</sub> (Svetlichny et al. 1991). Methanogens in hydrothermal vents are known to produce CO under certain conditions, such as H<sub>2</sub> deficiency. These interspecies CO transfers in natural volcanic environments may be examples of mutualistic bacterial-archaeal interactions. Evidence supporting this hypothesis is forthcoming from the genome sequence data of carboxydrotrophic bacteria.

### 3.2 Isolation and Characterization Studies

The characterization of *Thermosinus carboxidovorans* (an extreme thermophile from Norris Basin), which exhibits a relatively high growth yield on CO and corresponding production of H<sub>2</sub> (Sokolova et al. 2004), confirms that the

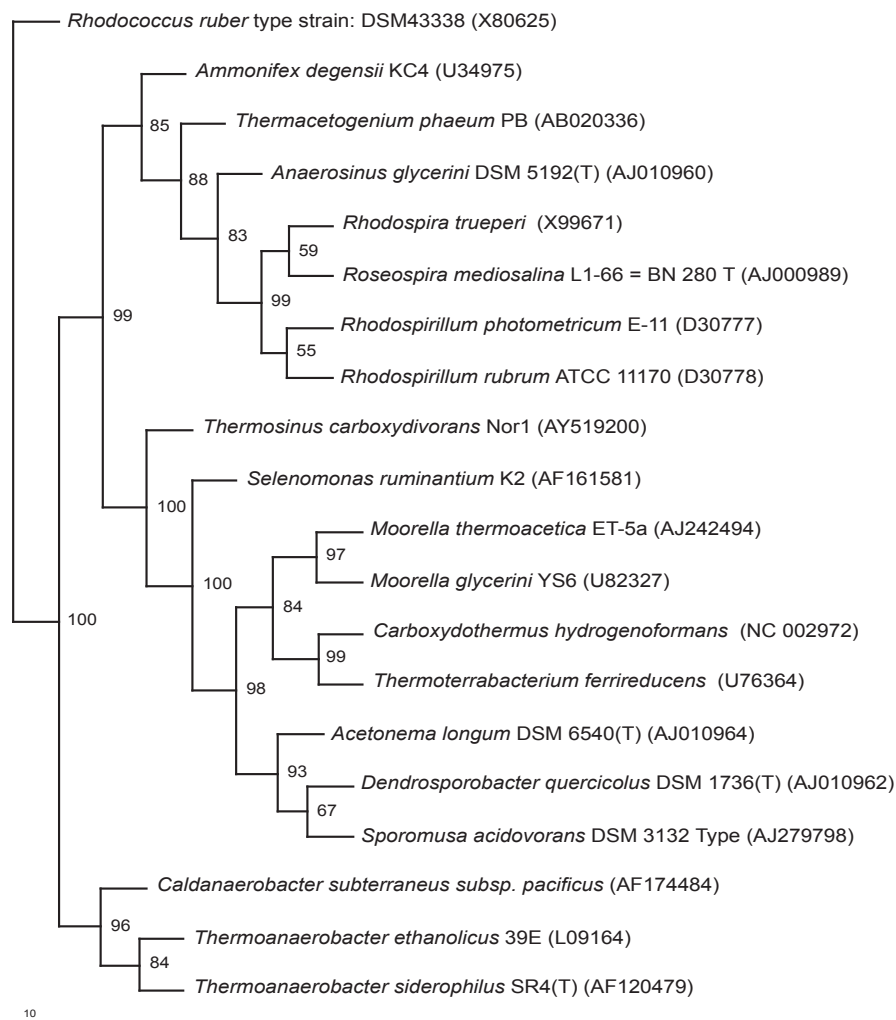


↑ **Figure 2.** Carbon monoxide dehydrogenase (CODH) encoding loci from the full genome sequence of *Carboxydotherrmus hydrogenoformans*. The CODH I locus corresponds to the *Rhodospirillum rubrum* dark- and CO-inducible operon that includes a *cooS* homolog and a multicomponent-membrane bound hydrogenase. CODH II is a three member putative operon including a *cooS* homolog with strong sequence similarity to the CODH from the archaeon, *Archaeoglobus fulgidus*. This CODH may function in generating reducing potential in the cell for biosynthesis (Svetlichnyi et al. 2000). The loci labeled 2, 4, and 5 have no assigned functions at present. Locus 5 has a putative five gene operon that is identical in gene order to the operon from the acetogenic Gram-positive bacterium, *Moorella thermoacetica*.

physiology of CO-oxidation is widespread, and probably global. This strain may also be capable of utilizing Fe<sup>III</sup> or selenite as additional electron acceptors, consistent with the relatively high abundance of Fe, S, and Se in the Norris Basin environment. Consequently, the Yellowstone isolate may demonstrate a new mechanism of energy conservation, namely CO oxidation with Fe<sup>III</sup> reduction. Indeed, Fe<sup>III</sup>-oxides were present in the hot spring from which this isolate was obtained (Figure 1). The isolation of a neutrophilic strain from this environment also highlights the potential variation of hot spring environments. For example, although many geothermal springs in Norris Basin are acidic, several springs exhibit pH values near neutral.

The widespread occurrence of anaerobic CO-oxidation begs the question as to the significance of these organisms in the “metabolic milieu.” CO is a key metabolite in methanogenesis and acetogenesis, major pathways in





↑ **Figure 3. Phylogenetic dendrogram of carboxydophilic bacteria and related strains.** The CO-utilizing strains are *Rhodospirillum rubrum*, *Thermosinus carboxydivorans*, *Moorella thermoacetica*, *Carboxydothemus hydrogenoformans* and *Caldanaerobacter subterraneus* subsp. *pacificus*. The maximum parsimony tree was created in PHYDIT and bootstrapped using 1000 replicates (bootstrap values over 50% are displayed).

global carbon cycling. There is also evidence that CO is important in microbial sulfate reduction. Recent studies by Voordouw (2002) indicated that, although CO is toxic to most sulfate reducing bacteria, metabolic cycling of CO is a critical feature for coupling electron transport in these organisms. Moreover, when the sulfate reducer *De-*

*sulfovibrio vulgaris* is grown on pyruvate, it produces CO as a metabolic end product. It is possible, therefore, that methanogens and sulfate reducers produce CO and, in return, are replenished with H<sub>2</sub> as a result of anaerobic CO oxidation. Interspecies CO transfers in natural volcanic environments may be examples of mutualistic bacterial-archaeal relationships along with subsequent genetic exchange, resulting in a more efficient utilization of available resources. Supporting this assumption, several associations among anaerobic bacteria and methanogens have been reported.

The reduction of Fe is a trait shared by *T. carboxydivorans* and *T. ferrireducens*, possibly a consequence of the Fe-rich locale from which they were both isolated (One Hundred Spring Plain, Norris Geyser Basin). *T. ferrireducens* is a thermophilic dissimilatory Fe<sup>III</sup>-reducing bacterium belonging to the Firmicutes (Slobodkin and Wiegel 1997). We are currently exploring the mechanism of Fe metabolism in *T. ferrireducens* and have purified a membrane-bound complex capable of Fe<sup>III</sup> reduction in vitro. Both *T. ferrireducens* and *T. carboxydivorans* are capable of organotrophic and lithoautotrophic growth with insoluble and soluble Fe<sup>III</sup> compounds; have a Gram-type positive cell wall surrounded by an s-layer; and contain c-type cytochromes. *T. carboxydivorans* is capable of growth

on a similar limited number of organic compounds and is very similar in growth physiology to *C. hydrogenoformans* and *T. ferrireducens* (Henstra and Stams 2004). The major exception is the inability of *T. ferrireducens* to grow on CO, and to produce H<sub>2</sub>. Conversely, *T. ferrireducens* appears to be specialized to reduce metals, using Fe<sup>III</sup> preferentially as a terminal electron acceptor. Since *T. ferrireducens* has no periplasmic space, the mechanism of Fe<sup>III</sup> reduction is expected to be different from that in Gram-negative bacteria, and indeed it appears that our preparation is able to carry out cytochrome reduction in response to Fe<sup>III</sup>, unlike Gram-negative bacteria (A. Slobodkin, unpublished results).

#### 4.0 SUMMARY

The phylogenetic context of *T. carboxydivorans* and *C. hydrogenoformans* is shown relative to several other Gram-positive bacteria and those related to *R. rubrum* (Figure 3). It is evident that CO-dependent hydrogenogenic metabolism occurs in a diverse phylogenetic context, and is accompanied by a varied repertoire of alternative trophic strategies. Chemoautotrophic pathways may have been present in ancestral lineages of *Archaea* and *Bacteria* (Reysenbach and Shock 2003). An important question that arises in consideration of the phylogenetic tree is whether carboxydrotrophy was widespread and subsequently lost by most lineages, or whether *cooS*-containing gene clusters were subject to widespread lateral gene transfer. The closest relative of *C. hydrogenoformans* is currently *T. ferrireducens*, a non-carboxydrotroph that was isolated from Norris Basin close to the location where *T. carboxydivorans* was found (Figure 1). The variety of alternative chemoautotrophic and organotrophic metabolisms in closely related microorganisms (based on 16S rDNA) suggests that horizontal gene transfer may be

common in hot spring communities. For horizontal gene transfer to be feasible, physical proximity and mutualistic or syntrophic relationships among bacteria and archaea would likely be important factors aiding DNA exchange between the domains. Continuous evolutionary pressure to gain a dominant position among the anaerobic organisms in a geothermal environment appears to be evident. Microorganisms that have obtained a competitive advantage through evolution may have sorted through combinations of physiological traits either by acquiring the required genetic material or by developing associations with microorganisms capable of performing complementary functions.

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