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Anaerobic Extremely Thermophilic Carboxydotrophic Bacteria in Hydrotherms of Kuril Islands

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Abstract. A new group of extremely thermophilic, obligately anaerobic, carboxydotrophic eubacteria is described. The organisms are characterized by a novel type of chemotrophic metabolism in thermophilic environments. They grow at temperatures up to 80–85°C chemolithotrophically with 100% CO in the gas phase as the sole energy source. The CO oxidation is coupled to H_2 and CO_2 formation according to the equation $CO + H_2O \rightarrow H_2 + CO_2$. No other products of metabolism are produced. The group of CO-utilizing, H_2-producing anaerobes includes diverse bacteria. They are non-sporeforming rods differing in morphology, CO uptake rates, habitats, and maximum growth temperatures. The new carboxydotrophic thermophilic anaerobes are widely distributed in freshwater and coastal marine hydrotherms of the Kuril Islands.

Introduction

Carbon monoxide (CO) is present in volcanic gases [6, 12] and is produced in insignificant amounts by some thermophilic acetogenic [3] and methanogenic [1] bacteria. Thus, carbon monoxide should be considered as a potential substrate for extremely thermophilic anaerobic bacteria in volcanic hot environments. CO utilization by aerobic carboxydobacteria is well known [9]. A number of thermophilic acetogenic, sulfate-reducing, and methanogenic bacteria can utilize low concentrations of CO as an energy source, producing acetate, H_2S, or CH_4, respectively [15]. For these organisms CO is an unfavorable substrate because of low conversion rates and growth inhibition by high CO levels. CO oxidation is catalyzed by CO dehydrogenase, which causes the formation of reducing equivalents, but not H_2 [4]. Diekert et al. [5] reported evolution of H_2 from CO (5% in the gas phase) by resting cells of *Acetobacterium woodii*. But the stoichiometry of this reaction could not be measured accurately

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since the conversion rate was very low. Only one strain of mesophilic phototrophic bacteria, *Rhodopseudomonas gelatinosa*, was reported to be able to grow well anaerobically in the dark with CO as carbon and energy source and producing H_2 [2, 14]. Now we have isolated an obligately anaerobic, extremely thermophilic, carboxydotrophic freshwater eubacterium named *Carboxydothermus hydrogenoformans* strain Z-2901 DSM 6008 [11]. It grows in temperatures up to 78°C chemolithoautotrophically with CO as a sole carbon and energy source and thus performs a new type of chemosynthesis in thermophilic environments. Other mineral or organic compounds are not used. *C. hydrogenoformans* is characterized by high growth and CO-utilization rates with 100% CO in the gas phase. As the product of CO oxidation it forms only H_2 and CO_2 according to the equation:

$$CO + H_2O \rightarrow H_2 + CO_2 \qquad \Delta G_o = -20.0 \text{ kJ/mol}$$

The diversity and distribution of CO-utilizing, H_2-forming, obligately anaerobic, thermophilic bacteria in different terrestrial and marine hydrotherms of the Kuril Islands was studied. Sixteen freshwater and 7 marine cultures were obtained, which were able to grow at 80–85°C with CO as a sole substrate (100% in the gas phase) and to produce H_2 according to the equation. This indicates a wide distribution of bacteria of this unusual group in volcanic hot environments.

Materials and Methods

Medium and Cultivation

Cultures were grown under strictly anaerobic conditions according to the anaerobic technique of Hungate [7]. They were incubated in stoppered 125 ml flasks with 20 ml of the medium under O_2-free CO in the gas phase (100% CO, 100 kPa) and without agitation. Unless specified otherwise, the freshwater medium contained (per liter): NH_4Cl, 0.33 g; $MgCl_2 \cdot 2H_2O$, 0.33 g; $CaCl_2 \cdot 6H_2O$, 0.33 g; KH_2PO_4, 0.33 g; $NaHCO_3$, 1.0 g; $Na_2S \cdot 9H_2O$, 0.5 g; trace minerals [10], 1 ml; vitamin solution [16], 1 ml; resazurin, 1 mg; yeast extract, 0.5 g; pH 7.0 (adjusted with NaOH). For the marine cultures, 25 g/liter of natural marine salt or NaCl were added to the medium.

Light Microscopy

Cells were observed using a Zeiss Amplival phase contrast microscope with an oil immersion objective 100/1.25.

Electron Microscopy

Negative staining of whole cells was done with 2% phosphotungstic acid. Electron micrographs were obtained with a JEM-100C electron microscope.

Determination of Growth

Bacterial growth was determined by direct cell counting under a phase contrast microscope.

Analysis of Gases

CO and H_2 were analyzed using a Chrom-5 gas chromatograph (Czechoslovakia) equipped with a thermal conductivity detector and a 1.2 m glass column filled with activated coal AG-3 (USSR). The carrier gas was Ar.

Results

Collection of Samples

Samples of hot waters, muds, and thermophilic cyanobacterial mats were collected in 1988 from hydrotherms of the Kuril Islands Kunashir (Southern Kurils), Shiashkotan, and Jankich (Northern Kurils) during the RV "Professor Bogorov" cruise. They were taken anaerobically with the addition of sodium sulfide for oxygen reduction and stored in tightly stoppered bottles at 4°C.

Samples from 30 freshwater and 46 coastal marine hydrotherms served as the inoculum for enrichment of CO-utilizing thermophiles. The original temperatures of the waters and sediments were between 45 and 98°C, and pH ranged between 2.5 and 7.2. The description of sampling sites where carboxydotrophs were found is given in Table 1.

At Kunashir Island, the samples from Stolbovskii Springs, Gorjachii Pljazsh (Hot Beach), and Alechino were investigated [8].

Freshwater Stolbovskii Springs are located at the west shore of the island on the slope of Mendeleev's volcano in a deep ravine with a thick forest. The samples from these hydrotherms contain a lot of decomposing organic material from the surrounding trees and grass and from a thermophilic cyanobacterial mat formed by *Mastiglocladus* and *Phormidium.*

Gorjachii Pljazsh is located on the opposite slope of Mendeleev's volcano at the eastern shore of the island. Freshwater springs of Gorjachii Pljazsh emerge through geothermally heated soil and sand at the sea bank in a ravine covered by forest and contain leaves and grass. The marine vents are located in the littoral zone with abundant algae growth.

Alechino is situated on the southern outer slope of the Golovnina caldera at the southwest shore of Kunashir Island. There are two freshwater hot streams in the littoral zone with cyanobacterial mat development and decomposing algae. Many strongly gassed freshwater hot springs are located at a significant height on the outer border of the caldera in a large acidic solfatara with clay soil. The solfatara is surrounded by forest.

Small hot vents of Shiashkotan Island are situated in the littoral zone in the area of abundant algae development . The seawater is mixed here with a freshwater stream, flowing down the precipice from the shore. The vents are similar to the littoral hydrotherms of the Gorjachii Pljazsh.

On Yankich Island, marine samples were taken from a salt terrestrial solfatara and from hydrotherms at a depth of 23 m in the Crater Bight, which is known to have an unusually rich organic life [13].

Enrichment

To obtain enrichment cultures, medium was inoculated with 1–2 ml of the samples and incubated at 70, 75, 80, 85 or 90°C. Within one to ten days of incubation at 70, 75, 80 or 85°C, the pressure increased from 100 kPa to 160–170 kPa, and rod-shaped, non-sporeforming bacteria became visible in the enrichment cultures with 16 freshwater (53%) and 7 marine (15%) samples (Table 1). CO consumption was observed with simultaneous formation of the $H_2 + CO_2$ mixture. Other products (acetate, H_2S, CH_4) were not detected. All the cultures retained activity after several sequential transfers in the medium with 0.05 g/liter yeast extract. Because of the low yeast extract concentration, the CO-utilizing bacteria were predominant.

No growth and CO consumption occurred in enrichments incubated at 90°C. Also, there was no growth in enrichments inoculated with the samples with original pH values below 5.0 (with the exception of 2814).

Characteristics of Carboxydotrophs

The isolates could be divided into three groups according to cell morphology, rates of CO uptake and H_2 formation at 75°C, maximum growth temperature, and habitat (Table 2): 1) freshwater, rod-shaped, non-sporeforming bacteria (7 cultures) with straight or slightly curved cells, 0.4–0.5 by 1.3–2.4 μm, sometimes motile, with an average rate of CO uptake higher than 150 μmoles CO (ml culture)$^{-1}$ day^{-1} and maximum growth temperature of 80°C (Fig. 1A). *Carboxydothermus hydrogenoformans* [11] belongs to this group; 2) freshwater, rod-shaped, non-sporeforming bacteria (9 cultures) with straight nonmotile cells, 0.4 by 4.5–7.0 μm, CO-uptake rate about 20 μmoles CO (ml culture)$^{-1}$ day^{-1} and maximum growth temperature of 80–85°C (Fig. 1B); 3) marine, rod-shaped, non-sporeforming bacteria (7 cultures) with straight or slightly curved nonmotile cells, 0.4–0.5 by 5.8–9.0 μm, CO-uptake rate about 15 μmoles CO (ml culture)$^{-1}$ day^{-1} and maximum growth temperature of 80°C (Fig. 1c).

When growing at 75°C under an atmosphere of CO (100% in the gas phase, 3.9 mmoles per 20 ml of the medium) without agitation, the organisms of the first group completely consumed CO after 1–1.5 days of incubation. Organisms of the second group completely consumed CO after 10 days, and those of the third group after 20 days.

Utilization and production of gases at 75°C by the organisms of these groups are shown in Fig. 2. Under the growth conditions tested, bacteria utilized CO from the gas phase completely and formed H_2 linearly without a lag-phase. There was no inhibition of growth or CO consumption by high concentrations of CO and/or H_2. CO oxidation was coupled with H_2- and CO_2-formation in equimolar quantities according to the equation. The stoichiometric coefficient (the molar ratio of CO consumed to H_2 produced) was close to 1.0 (1.04 $\pm$

Table 1. Origin of the new CO-utilizing bacteria

Strain designation	Sample index	Sampling site	Original temperature (°C)	Original pH
Freshwater strains:				
Kunashir: Stolbovskii Springs				
Z-2901/80	2901	Mud from hot swamp (1 m below surface)	68	5.5
Z-2902	2902	Thermophilic cyanobacterial mat	65	6.0
Z-2905	2905	Sediment from hot vent	72	6.5
Z-2906	2905	Small, hot stream with leaves	82	6.5
Z-2907	2907	Sediment from water well	82	6.5
Kunashir: Gorjachii Pljazsh				
Z-2701F	2701	Water from hot vent from littoral zone	60	7.0
Z-2707	2707	Hot spring in a stream	97	6.0
Z-2711	2711	Hot soil and sand	56	6.5
Z-2712	2712	Water from a bore hole	98	6.5
Z-2713	2713	Sludge with grass from hot stream on a meadow	80	6.5
Kunashir: Alechino, littoral				
Z-2801	2801	Hot stream with sulfur and cyanobacterial mat	55	5.0
Z-2805	2805	Decomposing algae from hot stream	45	5.0
Kunashir: Alechino, solfatara				
Z-2814	2814	Mud from hot pot	95	3.0
Z-2815	2815	Sediment from hot spring	93	5.0
Z-2819	2819	Mud volcano	95	5.5
Shiashkotan: littoral				
Z-1201	1201	Hot vent with algae residues	70	6.5
Marine strains:				
Kunashir: Gorjachii Pljazsh				
Z-2701M	2701	Water from hot vent from littoral zone	60	7.0
Z-2702	2702	Small hot vent from rock from littoral zone	55	7.0
Z-2703	2703	Hot soil and sand from littoral zone	56	6.5
Z-2704	2704	Water from hot vent with algae	82	6.5
Z-2708	2708	Water and sludge from a strong hot spring from littoral zone	92	5.0
Shiashkotan: littoral				
Z-1203	1203	Tiny hot vent with algae	70	6.5
Jankich: Kraternaya Bight				
Z-1510	1510	Sediment from submarine hot vent near the seashore (depth 23 m)	75	6.5

0.05) (Table 3) for all the cultures. This clearly indicated a chemolithotrophic metabolism of the carboxydotrophic bacteria investigated.

The maximum cell yield from 3.9 mmoles of CO per 20 ml of the medium depended on the cultures. It varied from 1.-0–4.5 × 10^8 cells·ml^{-1} in the case of the first group bacteria (4.5 × 10^8 for the strain Z-2713) to 3–8 × 10^7 cells·ml^{-1} for the bacteria of the groups 2 and 3.

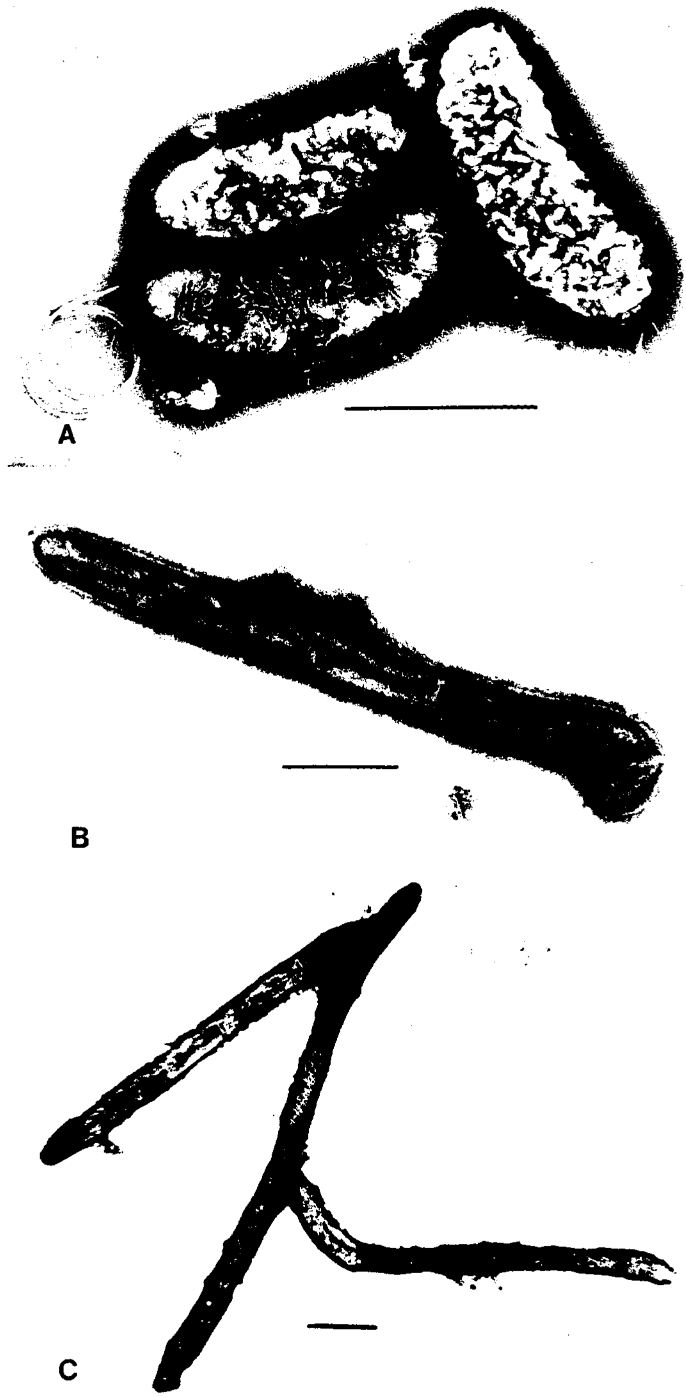

Fig. 1. Electron micrographs of (A) freshwater group 1 strain Z-2713, (B) freshwater group 2 strain Z-2819, and (C) marine group 3 strain Z-1510 (negative staining). Bar = 1 μm.

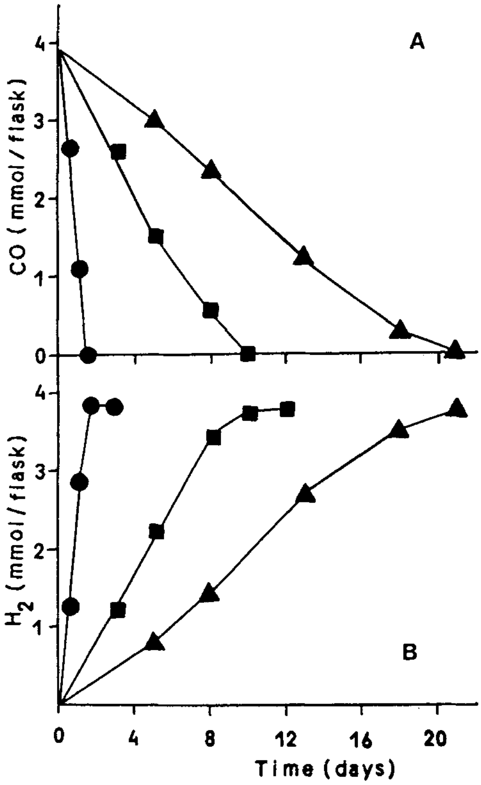

Fig. 2. CO utilization (**A**) and H_2 formation (**B**) by the strains Z-2713 (●), Z-2819 (■), and Z-1510 (▲), growing at 75°C.

Like *C. hydrogenoformans* Z-2901, the new bacteria could grow and utilize CO in transfers on completely mineral medium without yeast extract, which indicates the possibility of a chemolithoautotrophic type of metabolism.

In the presence of antibiotics penicillin, chloramphenicol, and streptomycin (100 μg per ml of the medium), there was no growth or CO utilization. Thus, the organisms investigated belong to the eubacteria.

Discussion

A new group of extremely thermophilic, obligately anaerobic, carboxydotrophic eubacteria have been formed. Like *Carboxydothermus hydrogenoformans* Z-2901 [11], which belongs to this group of organisms, they perform a novel type of chemolithotrophic metabolism. It is based on the utilization of CO as energy (and possibly carbon) source, with equimolar H_2-formation according to the equation.

New CO-utilizing, H_2-producing organisms form a diverse group and are widely distributed in freshwater and marine hot springs of the Kuril Islands. These springs usually contain allochthonous organic matter, have temperatures in the range from 45°C to 98°C, and pH values greater than 5.0. In the case of

Table 2. Characteristics of the new CO-utilizing bacteria

Strain index	Maximum growth temperature (°C)	CO-utilization rate at 75°C, μmoles (ml culture)$^{-1}$ day^{-1}
Group 1:		
Z-2901/80	80	178
Z-2902	80	185
Z-2905	80	192
Z-2906	80	155
Z-2907	80	167
Z-2713	80	187
Z-2801	80	196
Group 2:		
Z-2701F	85	16
Z-2707	80	19
Z-2711	85	22
Z-2712	80	23
Z-2805	85	16
Z-2814	85	20
Z-2815	85	23
Z-2819	85	23
Z-1201	85	21
Group 3:		
Z-2701M	80	15
Z-2702	80	13
Z-2703	80	12
Z-2704	80	15
Z-2708	80	18
Z-1203	80	15
Z-1510	80	12

sample 2814 with a pH of 3.0, the transfer of bacteria from adjacent vents cannot be excluded. High temperature of the environment is not a limiting factor, because carboxydotrophs are found in springs with temperatures at the boiling point, although they do not grow at 90°C. Among carboxydotrophic anaerobic bacteria we did not find archaebacteria, acidophiles, or thermophiles growing at 90°C.

In the hydrotherms, where carboxydotrophs are present, CO may be formed by thermal decomposition of organic matter [6]. Other possible sources of CO in these environments could be volcanic gases [8, 12] and CO production by thermophilic methanogens [1] and acetogens [3]. But no significant amounts of free CO were detected there, although trace amounts of H_2 were determined [8]. However, these results do not exclude the possible activity of anaerobic carboxydotrophic thermophiles in these volcanic environments. Furthermore, interspecies CO transfer may be occurring.

Of 76 samples investigated, 53 (70%) showed no growth of carboxydotrophic bacteria. As a rule, these did not contain organic residues and had a pH below

Table 3. CO utilization and H_2 formation at 75°C by carboxydotrophic anaerobes of different groups

Strain	Cultivation time (days)	CO utilized (mmoles)	H_2 formed (mmoles)	CO/H_2-ratio (mmole/mmole)
Z-2713	0.5	1.35	1.25	1.08
(group 1)	1.0	2.80	2.85	0.98
	1.5	3.90	3.85	1.01
Z-2819	3	1.30	1.20	1.08
(group 2)	5	2.40	2.20	1.09
	8	3.35	3.40	0.99
	10	3.90	3.75	1.04
Z-1510	5	0.90	0.80	1.13
(group 3)	8	1.55	1.40	1.11
	13	2.65	2.70	0.98
	18	3.40	3.50	0.97
	21	3.90	3.80	1.03

5.0. Other reasons for the restriction of the distribution of carboxydotrophs in hydrotherms are not known.

References

1. Conrad R, Thauer RK (1983) Carbon monoxide production by *Methanobacterium thermoautotrophicum.* FEMS Microbiol Lett 20:229–232
2. Dashkevicz MP, Uffen RL (1979) Identification of a carbon monoxide-metabolizing bacterium as a strain of *Rhodopseudomonas gelatinosa* (Molisch) van Niel. Int J System Bacteriol 29: 145–148
3. Diekert G, Hansch M, Conrad R (1984) Acetate synthesis from 2 CO_2 in acetogenic bacteria: Is carbon monoxide an intermediate? Arch Microbiol 138:224–228
4. Diekert G, Fuchs G, Thauer RK (1985) Properties and function of carbon monoxide dehydrogenase from anaerobic bacteria. In: Poole RK, Daw CS (eds) Microbial gas metabolism: Mechanistic, metabolic and biotechnological aspects. Society for General Microbiol, pp 115–129
5. Diekert G, Schrader E, Harder W (1986) Energetics of CO formation and CO oxidation in cell suspensions of *Acetobacterium woodii.* Arch Microbiol 144:386–392
6. Giggenbach WF (1980) Geothermal gas equilibria. Geochim et Cosmochim Acta 44:2021–2032
7. Hungate RE (1962) A role tube method for cultivation of strict anaerobes. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol. 3B. Academic Press, London, New York, pp 117–132
8. Markhinin EK, Stratula DS (1977) Hydrotherms of Kuril Islands. Nauka, Moscow, USSR
9. Meyer O, Schlegel HG (1983) Biology of aerobic carbon monoxide-oxidizing bacteria. Annu Rev Microbiol 37:277–310
10. Pfennig N, Lippert KD (1966) Über das Vitamin B_{12}-Bedürfnis phototropher Schwefelbakterien. Arch Microbiol 55:245–246
11. Svetlichny VA, Sokolova TG, Gerhardt M, Ringpfeil M, Kostrikina NA, Zavarzin GA (1991) *Carboxydothermus hydrogenoformans* gen. nov. and sp. nov.—A CO-utilizing thermophilic anaerobic eubacterium from hydrotherms of Kunashir Island. Syst Appl Microbiol (in press)

12. Taran YY (1988) Geochemistry of geothermal gases. Nauka, Moscow, USSR
13. Tarasov VG, Zhirmunsky AV (1989) Ecosystem studies in Kraternaya Bight, Kuril Islands. Biologiya Morya (Marine Biol) 3:4–12
14. Uffen RL (1976) Anaerobic growth of a *Rhodopseudomonas* species in the dark with carbon monoxide as sole carbon and energy source. Proc Natl Acad Sci USA 73:3298–3302
15. Uffen RL (1981) Metabolism of carbon monoxide. Enzyme and Microbial Technol 3:197–206
16. Wolin EA, Wolin MJ, Wolfe RS (1963) Formation of methane by bacterial extracts. J Biol Chem 238:2882–2886