Arch Microbiol (1984) 139:402–408

Archives of
Microbiology

Thermophilic *Bacilli* growing with carbon monoxide *

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Abstract. Four strains of obligately thermophilic *Bacilli* capable of growing with carbon monoxide as a sole carbon and energy source were isolated from settling ponds of a sugar factory. Most of them could be identified as strains of *Bacillus schlegelii* on the basis of cell wall composition, DNA homology, menaquinone and DNA base content. Growth with CO was very fast (t_d = 3 h) and was optimal at 65° C. No growth occurred below 50° C. As with the mesophilic carboxydotrophs, hydrogen plus carbon dioxide could also serve as autotrophic substrates. Growth of the isolates with CO depended on the presence of molybdenum in the growth medium. This suggested CO oxidase in the newly isolated *Bacilli* being a molybdenum hydroxylase similar to the enzymes from the mesophilic carboxydotrophs. Some data characterizing the CO-oxidizing activity in extracts of the thermophilic isolates are also provided.

Key words: Carboxydotrophic bacteria – *Bacillus schlegelii* – Species description – Autotrophic growth – Thermophilic bacteria – Carbon monoxide – Carbon monoxide oxidase

Carboxydotrophic bacteria can grow with CO as a sole carbon and energy source under aerobic conditions (Kim and Hegeman 1983; Meyer and Rohde 1984; Meyer and Schlegel 1983; Nozhevnikova and Yurganov 1978; Zavarzin and Nozhevnikova 1977). The overwhelming majority of them is Gram-negative and mesophilic. Mesophilic or obligately thermophilic spore-forming carboxydotrophs have not been described so far. However, there is a recent report on the isolation of a Gram-negative, moderately thermophilic CO-oxidizing bacterium. The isolate was preliminary characterized and tentatively named *Pseudomonas thermocarboxydovorans* (Bell and Colby 1983; Lyons et al. 1982). At present no explanation is available why most carboxydotrophic bacteria isolated to date can use hydrogen plus carbon dioxide for growth; but this indicates some sort of link between the metabolism of CO and H_2. This rationale offered the possibility to obtain novel strains of thermophilic carboxydotrophic bacteria by testing hydrogen bacteria maintained in culture collections for the ability to grow with CO, in addition to isolate them directly from water and soil samples. The isolations described were done with the aim to get strains of carboxydotrophic bacteria able of growing rapidly at elevated temperatures, to examine their taxonomic position and to compare their CO metabolism with that of the mesophilic *Pseudomonas carboxydovorans*.

* This paper is respectively dedicated to Professor Dr. H. G. Schlegel on the occasion of his 60th birthday

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Materials and methods

Enrichment and isolation. Strains of thermophilic carboxydotrophic *Bacilli* were isolated from the settling pond of a sugar factory at Nörten-Hardenberg, nearby Göttingen, FRG. Isolations were carried out at 65° C using a procedure based on that described by Meyer and Schlegel (1983). Thermophilic strains of *Bacillus sphaericus* (DSM 461, 462 and 463), *Bacillus schlegelii* type strain (DSM 2000), *Bacillus acidocaldarius* (DSM 446), *Bacillus stearothermophilus* (DSM 436) and *Bacillus coagulans* (DSM 1) were received from the German Culture Collection, DSM, Göttingen, FRG.

Media and growth conditions. Cells were cultivated at 65° C in a mineral medium supplemented with trace element solution TS2 under a gas atmosphere of (%, v/v) 5 CO_2, 35 CO and 60 air (Meyer and Schlegel 1983). Small cultures were incubated in desiccators, and mass cultures were done in 10-l fermentors (Braun-Biostat, Melsungen, FRG) insulated with foam rubber and kept at 65° C. For heterotrophic growth the mineral medium was supplemented with the indicated substrates. Gas mixtures were prepared by means of gas pumps (Wösthoff, Bochum, FRG) at flow rates of 0.5 l/min. Turbidity was determined at 436 nm in a Bausch and Lomb Spectronic Photometer (d = 1 cm). The biuret method was used for protein estimation (Schmidt et al. 1963). Dry weights were determined on membrane filters (0.2 µm, Sartorius, Göttingen, FRG).

Nutritional and biochemical characteristics. Utilization of organic compounds for growth was tested in liquid mineral medium supplemented with the indicated substrates (2 g/l). As a control, growth in mineral medium was also tested. Utilization of nitrogen sources (2 g/l) was examined with pyruvate (0.3%, w/v) or CO as substrates. Biochemical tests were performed as described for *Bacilli* (Smith et al. 1952; Gordon et al. 1973). Uninoculated media served as controls. All tests were done at 65° C.

Miscellaneous tests. Consumption or production of CO, O_2, N_2, CO_2, and H_2 was measured as described (Meyer and Schlegel 1978). Alternatively, gas uptake rates were measured manometrically at 50° C as described by Cypionka and Meyer (1982).

Oxidation of ^{14}CO to $^{14}CO_2$ by growing cells and assimilation of radioactivity were examined as described (Kiessling and Meyer 1982).

Biochemical features of the CO-oxidizing activity were determined in extracts as described (Cypionka et al. 1980; Meyer and Schlegel 1979).

The cell wall composition was determined as described by Schleifer and Kandler (1967a, b, 1972), and menaquinones were analysed according to Kroppenstedt (1982).

Enzyme assays. Extracts were prepared by passing cell suspensions through a French pressure cell at maximum pressure. Oxidation of CO or H_2 with methylene blue was followed at 50° C under the conditions described (Meyer and Schlegel 1979, 1980). Alcohol dehydrogenase, malate dehydrogenase and NADH oxidase were assayed according to Boehringer Informationen (1973). Oxidation of succinate with dichlorophenolindophenol was followed at 578 nm (Veeger et al. 1969). Xanthine oxidase (Roussos 1967), ribulosebisphosphate carboxylase (Bowien et al. 1976) and phosphoribulokinase (Siebert et al. 1980) were assayed as described. All enzyme assays were done at 50° C.

DNA base composition. The percentage G + C content of the DNA was determined after extraction (Marmur and Doty 1961) by the thermal denaturation method (DeLey 1970; DeLey et al. 1970). DNA from *Escherichia coli* B (52.3 mol%), obtained from Sigma Chemical Company (St. Louis, MO, USA), served as a standard. Hybridisation rates were determined from the renaturation rates of melting DNA samples (Bradley and Mordgarski 1976; DeLey et al. 1970).

Materials. Chemicals and biochemicals were obtained from usual commercial sources and were of the same purity as described (Meyer 1982).

Results

Isolation and micromorphology. Enrichments were done in Erlenmeyer-flasks by incubating 1 – 2 g of sludge with 30 ml of mineral medium. The flasks were put in desiccators containing (%, v/v) 85 CO plus 15 O_2 and kept without shaking at 65° C. After 1 week of incubation four out of twelve enrichments were positive. They were pasteurized (10 min at 80° C) and subcultured (10% inoculum). After several transfers contaminants had been diluted out and the culture had stabilized as indicated by homogeneity of the cell's shape upon microscopic examination and by indistinguishable colony morphology.

The isolates, designated OMT1, OMT2, OMT4 and OMT7 were definitely able to use CO or H_2 plus CO_2 as sole growth substrates under aerobic conditions. Growth with CO or pyruvate was optimal at 65° C and pH 7.5 – 8.0. No growth occurred at temperatures below 50° C or above 85° C. Vitamins or organic growth factors were not required. Growth was dense and uniform in liquid batch culture but was extremely poor on agar plates. Colonies were cream-colored, rough, flat and had the tendency to form protuberances. The cells were Gram-positive, straight, and rod-shaped with dimensions of 0.6 – 0.8 to 2.5 – 5.8 μm (Fig. 1). They were motile by means of up to six peritrichously inserted flagella. All isolates displayed sphaerical, terminal endospores, distinctly distending the sporangium. Electron-microscopic examination revealed regular surface patterns of subunits (Fig. 1) similar to those described for other Gram-positive thermophilic bacteria (Sleyter 1978). Ultrathin sections displayed a three-layered cell wall consisting of a densely stained central layer and lightly stained inner and outer layers (Fig. 1).

The successful isolation of CO- or H_2-oxidizing spore-forming bacteria suggested that among the aerobic thermophilic *Bacilli* kept in culture collections, some might exist able of growing with CO. Indeed, the thermophilic hydrogen bacterium *Bacillus schlegelii* could grow with CO, whereas *B. coagulans, B. stearothermophilus, B. acidocaldarius* and three strains of *B. sphaericus* failed.

Nutritional and biochemical characteristics. The substrate-utilization spectrum of the thermophilic carboxydotrophs was restricted to some few organic acids. Of 105 compounds tested, only the following could serve as growth substrates: acetate, fumarate, α-ketoglutarate, DL-malate, pyruvate, L-succinate, L-glutamic acid, L-cysteine and L-proline. Sugars, amines and aromatic compounds were not utilized. Ammonium chloride, L-asparagine, uric acid and urea were suitable nitrogen sources in autotrophic cultures; nitrate and nitrite were not used. N_2 was not fixed when the isolates were incubated in the absence of combined nitrogen at low oxygen partial pressure (0.05 – 5%, v/v).

All isolates including *B. schlegelii* shared the following features. They contained b-, c-, and a-type cytochromes, catalase, oxidase, phenylalanine desaminase, and nitrate reductase. Starch, caseine, gelatine, egg-yolk, urea, and tyrosine were not decomposed. No growth occurred in 2.5% (w/v) NaCl or at pH 5.7. The isolates were resistant to 0.01% (w/v) lysozyme. Acid or gas were not produced from carbohydrates, and indol was not formed. The isolates were negative with respect to the Voges-Proskauer reaction, utilization of citrate, fixation of N_2 or its evolution from nitrate. Strain OMT2 was different in being urease positive and phenylalanine desaminase negative.

The guanine plus cytosine contents of the DNA's of the thermophilic carboxydotrophs were determined by thermal denaturation. The following G + C contents were obtained (mol%): *B. schlegelii* (64.6), strain OMT1 (64.9), strain OMT2 (68.6), strain OMT4 (65.4), and strain OMT7 (63.9). These values exceed those of most mesophilic *Bacilli* and approach those reported for the thermophilic ones (Stenesk et al. 1968). The DNA hybridisation rates of strain OMT2 and strain OMT1 or *B. schlegelii* were 59 ± 3%, but were 100 ± 5% with all remaining combinations of strains. Plasmids were not resolved in the thermophilic carboxydotrophs with the methods of Eckard (1978) and Kado and Liu (1981).

The cell walls of all strains contained a peptidoglycan of variation A1γ (Ghysen 1968) with directly cross-linked mesodiaminopimelinic acid (m-Dpm). The strains OMT1, OMT2, OMT4 and OMT7 revealed predominantly menaquinones of the seven isoprene unit-type (92% MK-7). A high content of MK-7 is regarded typical of thermophilic *Bacilli* (Collins and Jones 1981). *B. schlegelii* contained 56% of MK-7 as well as menaquinones with six (37% MK-6), five (4% MK-5) and four (3% MK-4) isoprene units.

Growth with CO, H_2 plus CO_2, or heterotrophic substrates. *Bacillus schlegelii* type strain and the newly isolates were able to grow with CO as sole carbon and energy source at

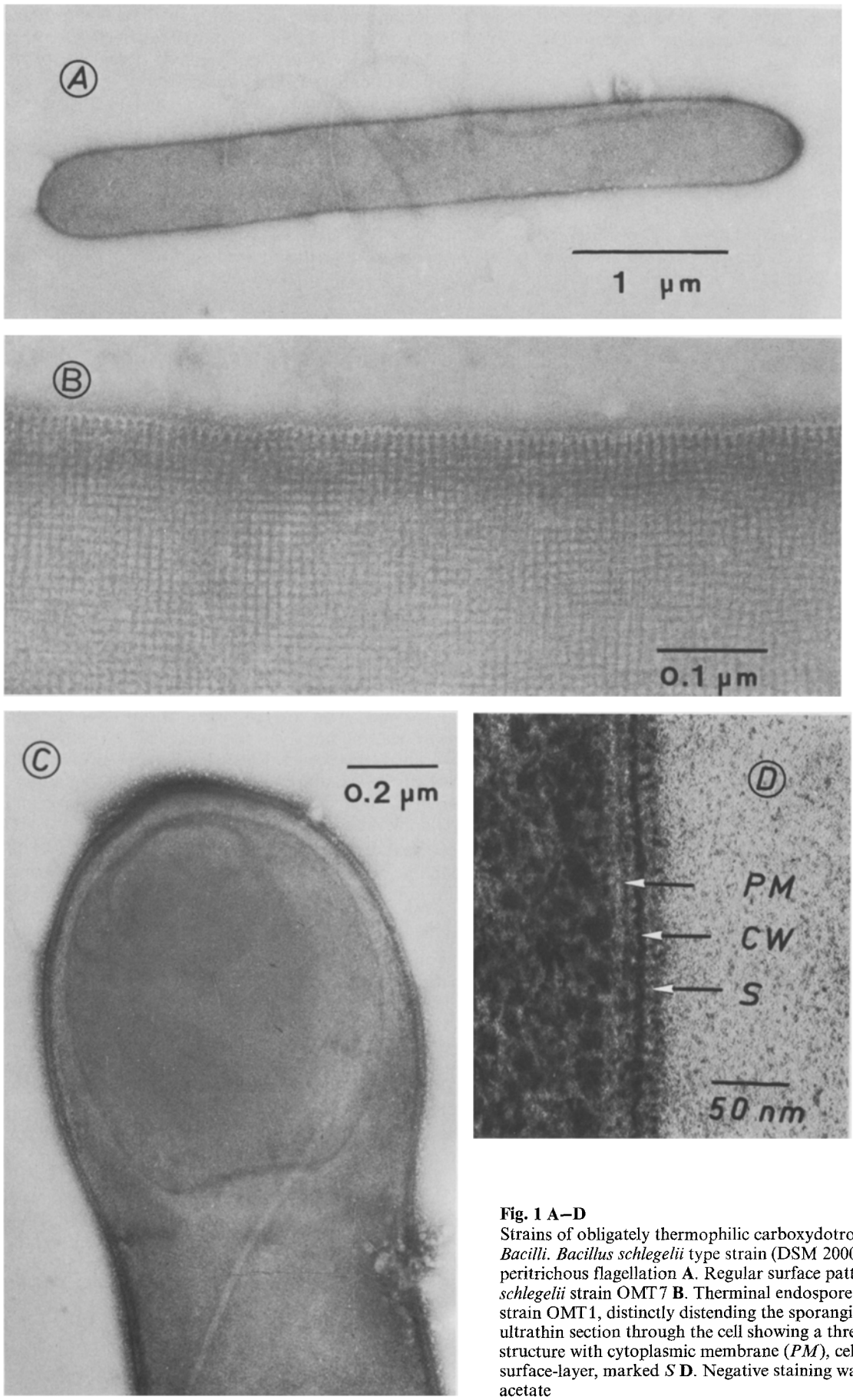

Fig. 1 A–D
Strains of obligately thermophilic carboxydotrophic *Bacilli*. *Bacillus schlegelii* type strain (DSM 2000), whole cell with peritrichous flagellation **A**. Regular surface patterns of *Bacillus schlegelii* strain OMT7 **B**. Therminal endospore of *Bacillus schlegelii* strain OMT1, distinctly distending the sporangium **C**. *Bacillus* OMT2, ultrathin section through the cell showing a three layered cell wall structure with cytoplasmic membrane (*PM*), cell wall (*CW*) and a surface-layer, marked *S* **D**. Negative staining was done with 2% uranyl acetate

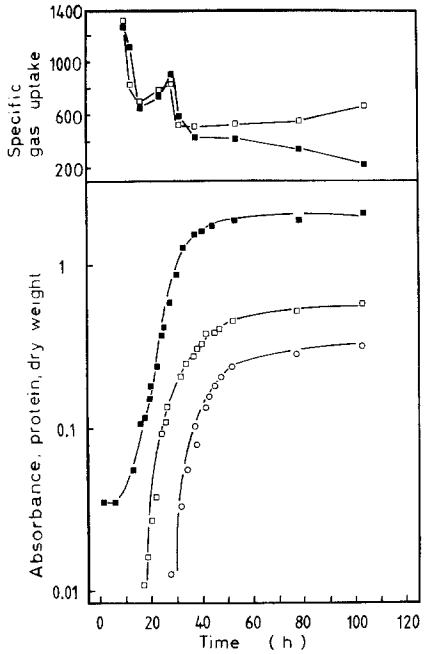

Fig. 2. Growth of *Bacillus* OMT2 with CO. *Bacillus* OMT2 was grown in a 10-l fermentor at 65° C under a gas mixture of (%, v/v) 5 CO_2, 35 CO and 60 air as described under "Materials and methods". Uptake rates of the sum of CO + O_2 (■) and the sum of H_2 + O_2 (□) were measured manometrically as detailed by Cypionka and Meyer (1982) and are given in µl gas/h · mg protein. Absorbance A_{436} (■); dry weight (□) and protein (○) in mg/ml

65° C. Growth of strain OMT2 with CO was exponential (Fig. 2) and at least five doublications were reached with all strains. The doubling times with CO (t_d in h) were: strain OMT1 (3), strain OMT2 and OMT4 (3.5), strain OMT7 (4) and *B. schlegelii* type strain (6.5). The comparatively short doubling times and high growth yields (0.12 to 0.76 g of protein/l corresponding to 0.19 to 1.10 g of dry weight/l) explain CO-autotrophic growth and disprove any growth with organic impurities which accidentally might have been present in the mineral medium or with volatile compounds present at the elevated temperatures applied. CO-oxidizing activities were high in thin suspensions and in the late exponential growth phase (Fig. 2). They amounted to [µl (CO + O_2)/h · mg protein]: OMT1 (850), OMT2 (1218), OMT4 (1210), OMT7 (958) and *B. schlegelii* type strain (1005). Pyruvate grown cells (t_d = 1.8 h) were devoid of CO-oxidizing activity, and only negligible activity was present in cells growing exponentially with H_2 plus CO_2 [12µl (CO + O_2)/h · mg protein].

All isolates grew with (%, v/v) 40 H_2, 10 CO_2 and 10 O_2 (N_2 served as balance). Doubling times of the isolates were about 7 h with H_2 plus CO_2 characterizing CO as the best autotrophic substrate. Hydrogenase was active in cells with H_2 plus CO_2 or with CO (Fig. 2). Its maximal activity amounted to [1200 µl (H_2 + O_2)/h · mg protein].

Stoichiometry of CO consumption. Uptake of CO and O_2 and formation of CO_2 were examined gas-chromatographically in a sealed culture of strain OMT2 growing with CO. The data support the equation

$$1\ O_2 + 2\ CO \rightarrow 1.85\ CO_2 + 0.15 \text{ cell carbon.}$$

As can be seen from this equation, 7.5% of the CO-born CO_2 was assimilated. The percentages of radioactive CO_2 incorporated into cell carbon ranged from 1.2% with strain OMT4 to 9.6% with strain OMT7 (Table 1).

Table 1. Oxidation and incorporation of ^{14}CO. CO autotrophically grown cells (2 ml of A_{436} = 1) were incubated in the main compartment of a Warburg vessel under a gas atmosphere of (%, v/v) 10 CO and 90 air and shaken at 50° C. The CO_2 formed was absorbed in 0.2 ml of 20% (w/v) aqueous KOH contained in the central cylinder. The reaction was started by injecting 5 to 40 µl of gaseous ^{14}CO through a septum using a gas-tight syringe. CO_2 formed and radioactivity assimilated were determined as described (Kiessling and Meyer 1982)

Strains	Percentages of ^{14}CO[a]	
	Assimilated	Oxidized to CO_2
OMT1	8.5	91.5
OMT2	2.2	97.8
OMT4	1.2	98.8
OMT7	9.6	90.4
B. schlegelii type strain	3.3	96.7

[a] The sums of radioactivity assimilated and oxidized in the different experiments varied from 67 to 533 Bq (4,000 to 32,000 dpm) and were set 100%

The specific activities of phosphoribulokinase in CO-grown strains were (nmol ribulose-5-phosphate phosphorylated/h · mg protein): strains OMT1 (322), OMT2 (604), OMT4 (612), OMT7 (649) and *B. schlegelii* (312) and of ribulosebisphosphate carboxylase (nmol of CO_2 fixed/min · mg protein): strains OMT1 (65), OMT2 (48), OMT4 (58), OMT7 (30) and *B. schlegelii* type strain (38).

Molybdenum requirement. Molybdate is required for formation of active CO oxidase in carboxydotrophic bacteria (Kalnowski 1980; Meyer and Schlegel 1983) whereas the formation of active CO dehydrogenase in *Clostridiae* depends on the presence of nickel (Diekert et al. 1979; Thauer et al. 1983). When the isolates were grown CO-autotrophically in the absence of molybdate growth slowed down and ceased after about 65 h of incubation (Fig. 3). Upon addition of 1.5 µM molybdate growth started again and same yields and doubling times as in the control were observed (Fig. 3). Molybdenum was not required with H_2 plus CO_2 or pyruvate.

Characterization of CO oxidase in extracts. After ultracentrifugation of extracts 70% (*B. schlegelii*) and 95% (the new isolates) of the CO-oxidizing activity was found in the cytoplasmic fraction. Hydrogenase was about equally distributed between the soluble and the particulate fractions. Methylene blue, toluylene blue, ferricyanide and phenazine methosulfate were readily reduced by the soluble CO- or H_2-oxidizing activities at pH 7.5 to 8.0. Low CO-oxidizing activity was also observed with pyocyanine and methylviologen. By far the highest reduction rates occurred with methylene blue or toluylene blue (CO) and phenazine methosulfate or methylene blue (H_2). They were 0.5 µmol of CO oxidized/min · mg protein and 1.0 µmol of H_2 oxidized/min · mg protein with methylene blue. NAD, NADP, FAD and FMN were not reduced by CO or H_2. Highest reduction rates of methylene blue with CO occurred at temperatures above 95° C. The activation energy was 5.18 kJ/mol (1.24 kcal/mol).

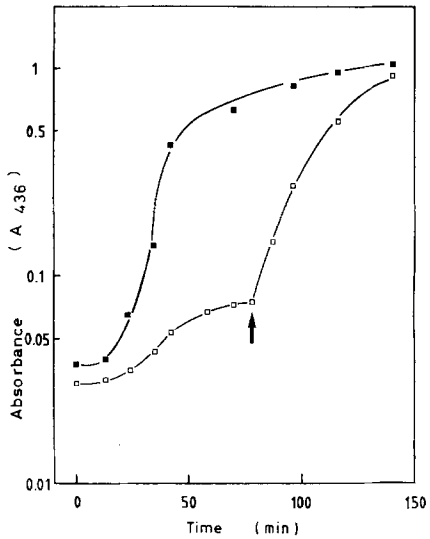

Fig. 3. Requirement of molybdenum for CO autotrophic growth. *Bacillus* OMT2 was grown at 65°C without shaking in 300-ml Erlenmeyer flasks containing 100 ml of mineral medium supplemented with 1 ml/l of trace element solution TS2 lacking molybdate (□). Samples for A_{436}-measurements were removed from the cultures as indicated. Sodium molybdate (1.5 μM) was added at the time marked by the arrow. The control culture was grown in the presence of 1.5 μM sodium molybdate (■)

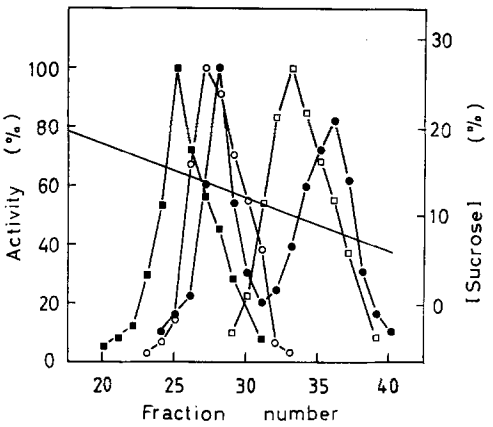

Fig. 4. Centrifugation of soluble fractions on a linear sucrose density gradient. Soluble fractions of CO-grown *Bacillus* OMT2, supplied with alcohol dehydrogenase and xanthine oxidase as molecular weight markers, were centrifuged at 200,000 × *g* for 15 h on a linear gradient of 5 to 30% sucrose in 50 mM phosphate buffer, pH 7.5. The gradient was fractionated (1 fraction = 0.25 ml) and analyzed for enzyme activities: CO oxidase (○), hydrogenase (●), alcohol dehydrogenase (□) and xanthine oxidase (■)

The M_r of the CO-oxidizing enzymes amounted to 230,000 upon centrifugation of soluble fractions on linear sucrose gradients (Fig. 4). The H_2-oxidizing activities migrated as two distinct peaks of 220,000 to 250,000 and 110,000 to 125,000 molecular weight. Only the low molecular weight band of hydrogenase was observed with the detergent-treated enzyme from *B. schlegelii* (Pinkwart et al. 1983).

Ouchterlony double immunodiffusion of purified CO oxidase from *Pseudomonas carboxydovorans* strain OM5 and soluble fractions of the thermophilic isolates with antibodies against the *P. carboxydovorans* CO oxidase revealed no precipitation lines. This indicates that the CO oxidases from *P. carboxydovorans* and from the thermophilic carboxydotrophs have no antigenic sites in common.

Discussion

Carbon monoxide metabolism of obligately thermophilic Bacilli. Bacillus schlegelii and the isolates OMT1, OMT2, OMT4 and OMT7 could use CO as sole source of carbon and energy. This is evident from their capability to grow in a simple mineral medium in the absence of any other substrate than CO, fast growth rates and high growth yields. The CO-oxidizing activities of the thermophilic strains were in the same order of magnitude as with the mesophilic carboxydotrophic bacteria (Cypionka et al. 1980; Kim and Hegeman 1981; Meyer 1982; Meyer and Schlegel 1978, 1979, 1980) and are, therefore, high enough to explain the observed growth rates. That the CO-oxidizing activity is actually used for growth is obvious from the conversion of radioactive CO to radioactive CO_2, the fixation of label into cell material and a stoichiometry of CO and O_2 uptake similar to that of the mesophilic carboxydotrophs (Meyer and Schlegel 1978, 1983). The properties shared by CO oxidases from mesophilic and thermophilic carboxydotrophic bacteria are molecular weight, reduction of methylene blue, toluylene blue and phenazine methosulfate, a pH optimum around 7.0, and the requirement of molybdenum for the formation of active enzyme. However, they displayed different temperature optima and were immunologically unrelated. The key enzymes of the Calvin cycle were found to be active in CO-grown cells of the thermophilic carboxydotrophs, and it may be suspected that CO is assimilated via the Calvin cycle subsequent to its conversion to CO_2.

Taxonomic considerations. On the basis of a positive Gram reaction, spore formation and the occurrence of thermophilic species, the assignment of the new isolates OMT1, OMT2, OMT4 and OMT7 to the genera *Desulfotomaculum, Clostridium, Sporolactobacillus* and *Sporosarcina* has to be considered. However, they cannot be included in these genera because of their strictly aerobic metabolism, the presence of cytochromes, catalase and a rod-like cell shape rather than sarcina-like aggregates. The new isolates could be assigned to the genus *Bacillus* on the basis of the descriptions given in the current edition of Bergey's Manual (Gibson and Gordon 1974) and by Gordon et al. (1973). The genus *Bacillus* comprises Gram-positive or Gram-variable, rod-shaped, straight cells with a length of 1.2 to 7.0 μm and a width of 0.3 to 2.2 μm (Gibson and Gordon 1974). Heat-resistent endospores are formed, and not more than one spore is contained in a sporangial cell. The majority of strains is aerobic and motile by means of lateral flagella. The G + C content of the DNA ranges from 32 to 62 mol% (Gibson and Gordon 1974) or from 32 to 69 mol% (F. Fahmy, D. Claus and J. Flossdorf, unpublished results) and of the thermophilic species studied by Wolf and Sharp (1981) from 32 to 69 mol%.

The carboxydotrophic isolates were Gram-positive, obligately thermophilic spore formers. Their metabolism was strictly respiratory. They contained cytochromes, oxidase and catalase and their cell wall was of the meso-diaminopimelinic acid – direct type. They were motile by

means of peritrichously inserted flagella. The G + C contents of the carboxydotrophic isolates ranged from 63.9 to 68.8 mol%. Therefore, with respect to the properties discussed, the isolates OMT1, OMT2, OMT4 and OMT7 are to be assigned to the genus *Bacillus*. *Bacillus schlegelii* has been isolated and orginally characterized as a thermophilic hydrogen bacterium (Aragno 1978; Schenk and Aragno 1979), and its ability to grow with CO has been unveiled here. This led to the idea that at least some of the strains obtained by direct isolation might be related to *B. schlegelii*. This idea was supported by very similar cell shape, a round, terminal spore distinctly distending the sporangium, peritrichous flagellation, characteristic surface patterns (Fig. 1), identical biochemical characteristics and the ability to grow chemolithoautotrophically with H_2 plus CO_2. In addition, the G + C contents of the latter strains were practically identical (63.9 to 65.4 mol%) and were clustered within a range of 1.5 mol%. Therefore, the above strains have to be considered a single species (Bradley and Mordgarski 1976; DeLey et al. 1970). This view is supported by hybridisation rates of 100% with all combinations of the DNA's from *B. schlegelii*, the strains OMT1, OMT4 and OMT7. On the basis of these considerations we propose the isolates OMT1, OMT4 and OMT7 to be named *Bacillus schlegelii* OMT1, *Bacillus schlegelii* OMT4 and *Bacillus schlegelii* OMT7.

Strain OMT2 is separated from the other isolates by a G + C content of 68.8 mol%, formation of urease, absence of phenylalanine desaminase, and by only 59% homology of its DNA with that from *B. schlegelii*. For that reason, strain OMT2 cannot be included in the species *B. schlegelii* and is proposed to be named *Bacillus* OMT2. A new species name is not suggested because only a single isolate is available thus far.

Acknowledgements. We are indebted to Drs. R. Kroppenstedt for determination of menaquinone content, M. Rohde for providing antibodies, N. Weiss and O. Kandler for advice with the examination of cell walls and D. Claus for helpful discussions.

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Received March 3, 1984/Accepted April 7, 1984