KOR. JOUR. MICROBIOL. March, 1985, p. 1-8 Vol. 23, No. 1

Acinetobacter Isolates Growing with Carbon Monoxide

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일산화탄소를 이용하여 성장하는 *Acinetobacter*의 분리 및 동정

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Three strains (JC1, JC2, and HY1) of aerobic carbon monoxide (CO)-utilizing *Acinetobacter* were isolated from soil through CO-enrichment culture technique. All of them were Gram-negative, non-motile, and rod-shaped but they were changed to spherical form at the end of logarithmic phase. They were resistant to penicillin and able to grow at 42°C. The guanine plus cytosine contents of the DNAs ranged from 43 to 44.5 mol%. Oxidase was not present in all cells. The colonies were smooth and whitish yellow. Heterotrophic growth occurred on several sugars, organic acids, amino acids, and alcohols. The doubling times under an atmosphere of 30% CO and 70% air at 30°C were 19 h, 25 h, and 35 h, respectively, for JC1, JC2, and HY1. JC1 was studied in more detail. The cells were grown optimally in a mineral medium (pH 6.8) under a gas mixture of 30% CO and 70% air at 30°C. Growth of the cells with CO did not depend on molybdenum. It was able to grow with 100 ppm of CO in air as a sole source of carbon and energy.

Several Gram-negative and positive carboxydobacteria are able to grow aerobically with carbon monoxide (CO) as a sole source of carbon and energy (Kim and Hegeman, 1983*a*; Krüger and Meyer, 1984; Meyer and Schlegel, 1983; Zavarzin and Nozhevnikova, 1977). The carboxydobacteria which have been studied to date, however, were isolated from only several restricted areas. Since we believed that there may be similar kinds of bacteria in Korea we carried out this work to isolate and characterize new carboxydobacteria from soils in Seoul in order to understand better the oxidation of CO by this group of bacteria.

The present work reports on the isolation and some properties of three new CO-utilizing aerobic bacteria, for which the genus name *Acinetobacter* is proposed. It also describes the effects of several environmental factors on the CO-autotrophic growth of an isolate which grows very well with CO as the sole carbon and energy source.

MATERIALS AND METHODS

Enrichment and isolation

1 g of each of soil samples from several areas in Seoul, Korea was suspended in 10 ml of liquid standard mineral base (SMB) medium (Kim and Hegeman, 1981) and the suspension was left for a couple of minutes to settle insoluble materials down. A loopful supernatant was then streaked onto solid SMB plates. The plates were incubated in a desiccator containing a gas mixture of 50% CO (99.5%, Daesung) and 50% air at 30°C. After four weeks of incubation, fast growing colonies were transferred to other SMB plates and grown for two weeks under an atmosphere of 30%

CO-70% air. This step was repeated for several times to select rapidly growing colonies. The selected colonies were then tested for homogeneity using pour-plate method after serial dilution or by growing on yeast agar phosphate medium. *Pseudomonas carboxydohydrogena* (Kim and Hegeman, 1981) and *Escherichia coli* K12 were used as controls for positive and negative CO-utilizing bacteria, respectively.

Culture conditions

Carboxydotrophic isolates were grown CO-autotrophically in SMB medium under the conditions described by Kim and Hegeman (1981). Gas mixtures were prepared using a custom-made manometer connected with a vacuum pump (Dorrel). For heterotrophic growth the mineral medium was supplemented with 0.2% sodium succinate. Turbidity was measured at 436 nm using a Hitachi 200-20 spectrophotometer.

Biochemical properties and nutritional characteristics

Several biochemical tests were carried out by the methods of Gerhardt *et al.* (1981) except for the following. Carotenoid was examined using the method of Starr and Stephen (1964). Nitrogenase was examined indirectly using C_2H_2 as described by Postgate (1972). Sensitivity to penicillin was tested using penicillin discs (10 IU, Difco). Utilization of organic compounds for heterotrophic growth was tested on solid SMB plates supplemented with 0.2% of each substrate. Several bacteria were used as controls for the above tests.

DNA base composition

DNAs were extracted by the method of Marmur and Doty (1961). The guanine and cytosine content of DNA was determined by the acid hydrolysis method and paper chromatography (Corbel *et al.*, 1979). Adenine, guanine, cytosine, and thymine (Sigma) were used as standards.

Optimal concentration of CO for cell growth

Small-size, liquid culture system was employed to determine the optimal CO concentration for the growth of the most rapidly growing isolate, JC1, at 30°C as described by Kim and Hegeman (1983*b*).

Effect of temperature on the CO-autotrophic growth:

The same small-size, liquid culture system was used to examine the effect of temperature on the growth of JC1, except that the cells were incubated under 30% CO at various temperatures.

Effect of pH on the CO-autotrophic growth

JC1 was cultivated in liquid SMB media with different pHs under a gas mixture of 30% CO-70% air at 30°C to select the best pH for CO-autotrophic growth.

Effect of molybdenum on the CO-autotrophic growth

JC1 was grown in liquid SMB medium in the presence or absence of molybdenum under 30% CO-70% air mixture at 30°C and the growth rates were compared to see if there is any effect of molybdenum on the CO-autotrophic growth of this bacterium.

Minimal CO concentration for autotrophic growth

JC1 was cultivated using the small-size, liquid culture system at 30°C under gas mixtures of low concentrations of CO with air to determine the minumum CO concentration required for autotrophic growth.

Chemicals

Chemicals of high purity were purchased from usual chemical companies as previously described (Kim and Hegeman, 1981 and 1983*b*).

RESULTS AND DISCUSSION

Isolation and morphology

After several times of incubation under CO, carboxydotrophic bacteria were stabilized as indicated by morphology and color of the colonies and by the cell shape. The three isolates, designated as JC1, JC2, and HY1, were able to grow actively with CO as a sole source of carbon and energy aerobically. JC1 and JC2 were isolated from soils sampled from a bus station and HY1 was selected from soil samples around a kitchen yard using coal briquettes as a heating source. Growth was good on agar plates as well as in liquid medium. Growth factors were not required. Colonies were whitish yellow and raised convex

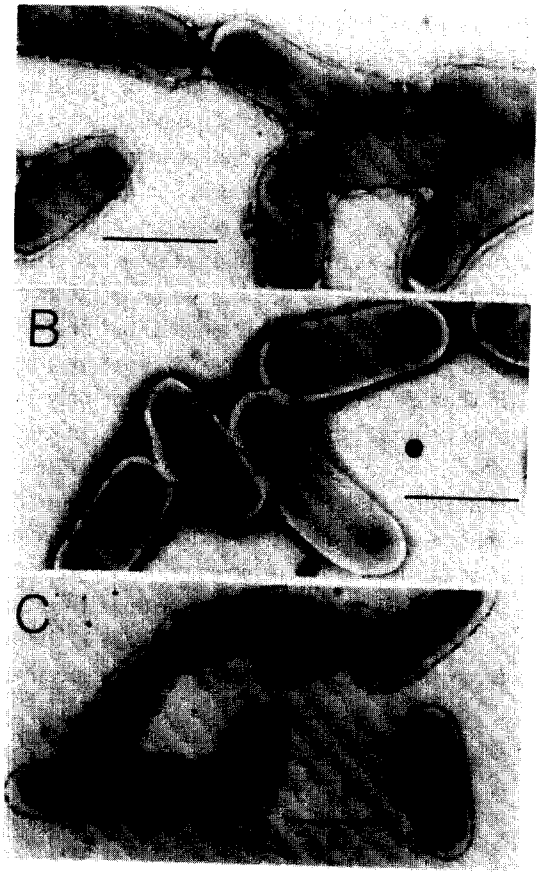

Fig. 1. Electron micrographs of isolated carboxydobacteria. Cells were harvested during exponential growth phase and were stained with phosphotungstic acid. A Hitachi H-500 electron microscope was used for observations. Symbols: JC1 (A), JC2 (B), and HY1 (C). Each bar represents 1 μm.

with smooth margin. The cells were Gram-negative, slightly curved or straight rods with dimensions of 0.6-0.7 × 1.5-2.2 μm(Fig. 1) during logarithmic phase but became near spherical form during stationary phase (Fig. 2). They were non-motile and had no flagella. Electron microscopic examination revealed that these cells reproduce by binary fission. JC1 and JC2 sometimes formed a short chain during logarithmic growth in liquid medium (Fig. 2). They also grew as large aggregates when the culture medium was agitated very slowly, implying that they may secrete slimy materials during growth with CO. The cell extracts of HY1 were nearly black when the cells were disrupted by sonic oscillation. The back color was sedimented by 20 min of centrifugation at 8,000 x g.

The isolation of the three carboxydobacteria from soils in Seoul area implies that this physiologically specific group of bacteria is not only present in the restricted areas reported to date but may be present all around the world where CO-pollution is serious.

Biochemical and nutritional properties

All isolates shared the following characteristics. They had no nitrogenase activity. Indole and H_2S were not formed and starch and gelatin were not hydrolyzed. Glucose and lactose were not fermented, implying that they get energy from respiratory metabolism. The cells were able to grow at 42°C and were resistant to 10 IU of penicillin. Catalase was present but carotenoid and poly-ß-hydroxybutyrate were not. Cells were grown anaerobically in the presence of nitrate using sodium succinate as a growth substrate. Oxidase was not present in JC1 and JC2 grown CO-autotrophically and heterotrophically and also in HY1 grown CO-autotrophically. The enzyme was detected in heterotrophically-grown cells of HY1 at the time of isolation but it disappeared after several times of transfer under CO, indicating that

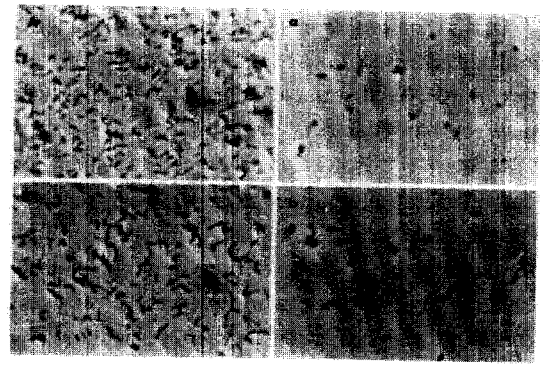

Fig. 2. Photomicrographs of JC1 and JC2. Pictures of each isolated were taken by phase contrast microscope (Nikon Labophot). Symbols: JC1 in logarithmic phase (A) and stationary phase (a); JC2 in logarithmic phase (B) and stationary phase (b). Pictures were not taken for HY1. Bar (lower right side of each micrograph) equals 1 μm.

the oxidase in HY1 is unstable.

The isolates were able to grow with various organic materials such as sugars, acids, amino acids, and alcohols. Of 31 compounds tested, the following could serve as substrates for growth in all cells: glucose, fructose, galactose, xylose, ribose, arabinose, maltose, mannose, cellobiose, propionate, lactate, succinate, acetate, L-alanine, L-cysteine, L-ornithine, L-glutamate, ethanol, isopropanol, and mannitol. In addition to the above substrates, JC1 and JC2 could use isobutanol and HY1 was able to use sucrose, lactose, L-methionine, L-tryptophane, L-asparagine, L-aspartate, and methanol. Cellulose, formate, and maleate were not utilized by all cells. Ammonium sulfate and nitrates were suitable nitrogen sources for autotrophic growth. The ability to use H_2 as an energy source was not tested.

The G+C contents were 44.5% and 43% for JC1 and HY1, respectively. The content was not determined for JC2 since the cell's morphology and nutritional and biochemical properties were indistinguishable from those of JC1, which implies that JC2 may be the same species as JC1. Plasmid was not detected from the isolates with the methods of Anderson and McKay (1983) and Birnboim and Doly (1979).

Taxonomic considerations

On the basis of Gram reaction, cell morphology, and several nutritional and biochemical properties, it may be able to consider to assign the three new carboxydotrophic isolates to the genus *Pseudomonas*. However, the cells cannot be classified in the genus because of the absence of flagella and oxidase, the ability to grow at 42°C, the spherical cell shape during stationary phase, the growth of some cells in short chains, the G+C contents, and the resistance to penicillin. The isolates could be assigned to the genus *Acinetobacter* after the description in the Bergey's manual (Lautrop, 1974) and by Sonnenwirth (1980).

The isolates are different from those well-studied carboxydobacteria such as *Pseudomonas* (Meyer and Schlegel, 1983), *Alcaligenes* (Meyer and Schlegel, 1983), *Bacillus* (Krüger and Meyer, 1984), and *Arthrobacter* (Meyer and Schlegel, 1983) in nutritional and biochemical characteristics including independence of CO-autotrophic growth on the molybdenum at least in one strain. This molybdenum independence of autotrophic growth will be discussed in another section of this paper. Together with the fact that the soluble fractions from JC1 did not have any cross-reacting material when it was subjected to react with antiserum prepared against purified CO dehydrogenase from *P. carboxydohydrogena* (data not shown), these results suggest not only the isolates but also the CO dehydrogenases of these cells may be completely new ones. It may also be deduced from the results that JC1 and JC2 are the same species while HY1 is a different one. Species names for these bacteria have not been suggested yet. Therefore, we suggest to name tentatively the JC1, JC2, and HY1 as *Acinetobacter* sp. 1, *Acinetobacter* sp. 1-1, and *Acinetobacter* sp. 2, respectively.

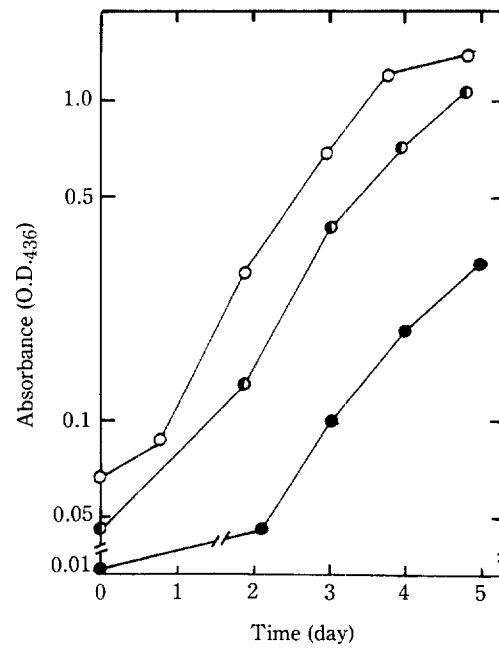

Fig. 3. Growth curve for CO-autotrophically growing cultures of isolated carboxydobacteria. Growth rates were determined by changes in turbidity under autotrophic culture conditions as described in methods. Symbols: JC1 (-○-), JC2 (-◐-), and HY1 (-●-).

Heterotrophic and autotrophic metabolism

JC1, JC2, and HY1 could grow both heterotrophically with succinate and autotrophically with CO. HY1 grew the most rapidly ($t_d = 4$ h) among the three using 0.2% succinate at 30°C. Under the same conditions JC1 and JC2 were able to grow at doubling times of 6 h and 10 h, respectively. When those cells were grown under a gas mixture of 30% CO-70% air at 30%C the doubling times were found to be 19 h, 25 h, and 35 h, respectively, for JC1, JC2, and HY1 (Fig. 3).

These results also indicate that CO is not a good substrate for the isolates as in the case of other carboxydobacteria (Kim, 1981; Zavarzin and Nozhevnikova, 1977). Under the autotrophic conditions JC1 and JC2 grew very fast and the doubling times are comparable to those of the well-known *P. carboxydohydrogena* ($t_d = 25$ h) (Kim and Hegeman, 1983*b*) and *Pseudomonas carboxydovorans* ($t_d = 20$ h) (Meyer and Schlegel, 1978). *Bacillus schlegelii* which is thermophilic has been reported to grow very fast ($t_d = 3$ h) at 65°C (Krüger and Meyer, 1984). Since JC1 was found to be the best isolate in the criterion of CO utilization, this cell was selected for further studies.

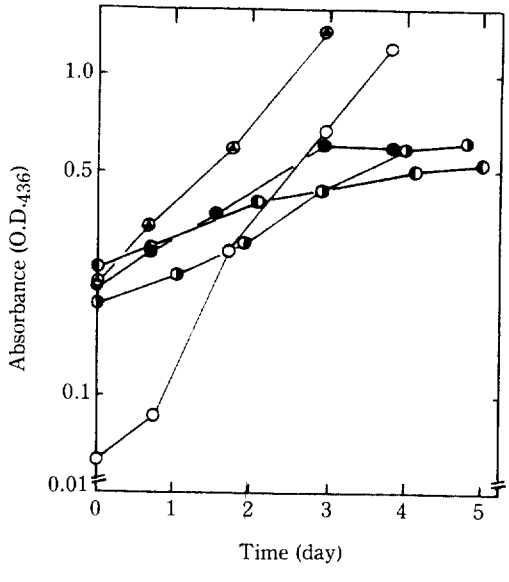

Fig. 4. Effect of CO concentration on the growth of JC1. JC1 was grown under various concentrations of CO in air to determine the optimal CO concentration for autotrophic growth as described in methods. Symbols: 5% (-◐-), 10% (-●-), 30% (-○-), 50% (-◉-), and 70% (-◑-) of CO in air.

Optimal concentration of CO for the growth of JC1

As shown in Fig. 4 the best doubling time in liquid media was obtained with 30% CO in a gas mixture with air at 30°C among those concentrations tested. The slow growth under low CO concentration may be due to the poor supply of growth substrate. When the cells were grown under 50% CO, the growth was not affected very much, implying that this bacteria is more resistant to CO than *P. carboxydohydrogena* (Kim and Hegeman, 1983b). However, the growth rate was very slow under 70% CO, indicating that CO is still inhibitory for this cell even though the cell is able to use CO as a sole growth substrate as previously noted (Kim and Hegeman, 1983*b*).

Effect of temperature and pH on the growth

When JC1 was grown under 30% CO at 20°C, 30°C and 40°C, cells were grown actively at 30°C ($t_d = 19$h) and 40°C ($t_d = 20$ h) but poorly at 20°C ($t_d = 42$ h). This implies that JC1 may be able to grow normally even at a higher temperature than 40°C at which other mesophilic carboxydobacteria are not able to grow well. This possibility needs to be tested in the future.

JC1 was grown optimally at pH 6.8 but the growth rate was not affected significantly even when the cells were cultivated at pHs 5.7 and 8.0 which accords with the result of Kim and Hegeman (1983*b*) who reported that changes in pH value do not affect the growth rate of *P. carboxydohydrogena*.

Since JC1 grows optimally in liquid SMB medium of pH 6.8 under an atmosphere of 30% CO-70% air at 30°C, subsequent cultivation of this bacterium was carried out under these conditions.

Molybdenum is not required for CO-autotrophic growth

It was found that JC1 which was cultivated CO-autotrophically without molybdenum showed about 24 h of lag period (Fig. 5). However, the cells continued to grow after the lag phase with the same doubling time as the cells grown in the

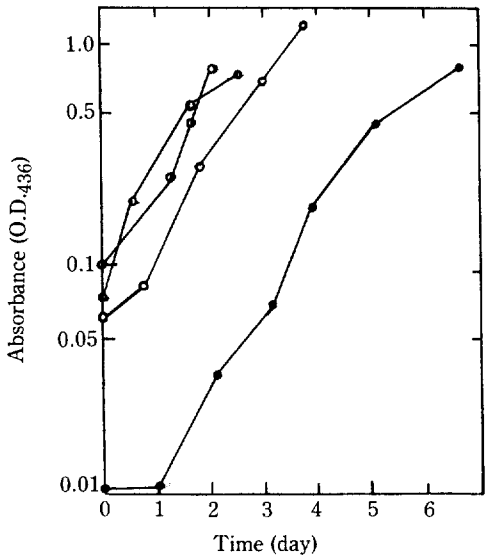

Fig. 5. Effect of molybdenum on the growth of JC1. Cells were grown in the presence or absence of molybdenum (8.3 μm) to test the effect of the mineral on the autotrophic and heterotrophic growth of the cell. Symbols: CO with molybdenum (-○-), CO without molybdenum (-●-), succinate with molybdenum (-◐-), and succinate without molybdenum (-◑-).

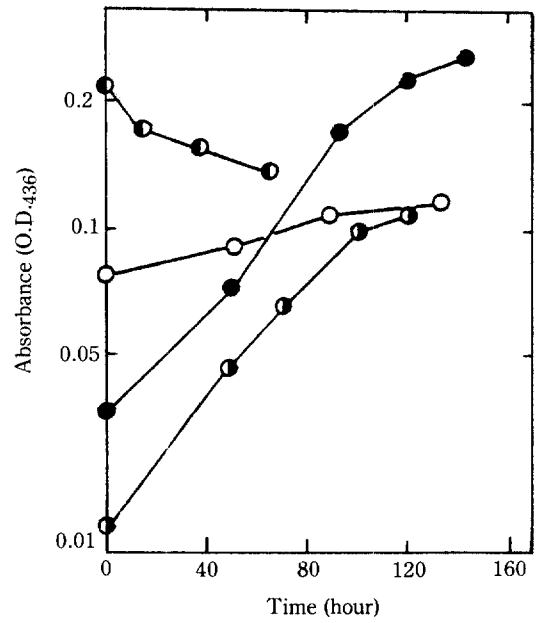

Fig. 6. Minimum concentration of CO required for autotrophic growth of JC1. Gas mixtures of low CO concentrations in air were tested to determine the minimal CO concentration for cell growth as described in methods. Symbols: laboratory air (-◐-); 0.01% CO (-○-), 0.03% CO (-◑-), and 1% CO (-●-) in air.

presence of molybdenum. The molybdenum did not affect heterotrophic growth of JC1 (Fig. 5). It has been reported that molybdenum is a cofactor of CO dehydrogenases in several carboxydobacteria (Meyer, 1982; Meyer and Rajagopalan, 1984) and that the CO-autotrophic growth of *P. carboxydovorans* (Meyer, 1982) and *Bacillus* OMT2 (Krüger and Meyer, 1984) requires the mineral, suggesting that the molybdenum may play an important role in the oxidation of CO by these bacteria. As mentioned above, JC1, however, does not require this mineral for autotrophic growth. This interesting result, together with the fact that there was no cross-reacting material in CO-grown JC1 as discussed in the previous section, the CO dehydrogenase of this cell may be a new species of CO-oxidizing enzyme. We are currently trying to purify and characterize this enzyme.

Growth with low concentration of CO

JC1 was able to grow slowly with 0.01% CO in air mixture (i.e. 100 ppm) but not with laboratory air (Fig. 6).

It has been reported that CO concentration in the atmosphere is about 0.03-0.9 ppm (Robbins *et al.*, 1968) but in urban areas the concentration can be as high as 50-100 ppm (Robinson and Robbins, 1970). The concentration may be much higher around road sides, bus stations, and garages and in the kitchens using coal briquittes as a heating or cooking source. Rainwater sometimes contains up to 200 times the concentration of CO present in the atmosphere (Swinnerton *et al.*, 1971). Therefore, this bacteria could play a role in the removal of atmospheric CO present locally at high concentrations at the places mentioned above.

적 요

일산화탄소를 이용한 enrichment 배양 방법을 통하여 흙으로 부터 일산화탄소를 이용하여 성장할 수 있는 세가지 종류(JC1, JC2, HY1)의 호기성 *Acinetobacter*들을 분리했다. 이 세균들은 모두 Gram음성 세균들로 운동성

이 없었으며, 지수성장기에는 간균의 형태를 나타내었으나 성장이 정지되었을 때에는 구균으로 변하였다. 이들은 페니실린에 대해 내성이 있었고 42℃에서도 성장을 할 수 있었다. DNA의 G+C함량은 43%~44.5%이었고, 모든 세균에서 oxidase의 활성이 나타나지 않았다. 이 세균들의 colony들은 모두 둥글고 매끄러웠으며 연한 노란색을 띄웠다. 이들은 또 여러가지 종류의 당이나 유기산, 아미노산, 알코홀 등을 이용하여 성장할 수 있었다. JC1과 JC2 및 HY1이 30%의 일산화탄소를 이용하여 30℃에서 성장할 때의 doubling time은 각각 19시간, 25시간, 그리고 35시간 이었다. JC1의 자가영양적 성장을 위한 최적조건은 pH가 6.8이었고, 온도는 30℃, 그리고 일산화탄소의 농도는 30%이었다. JC1의 자가영양적 성장에는 몰리브데늄이 필요치 않았고, 또 이 세균은 100ppm의 CO 만으로도 성장할 수 있는 것으로 밝혀졌다.

Acknowledgement:

This work was supported by a research grant from Korea Science and Engineering Foundation (1982-1984).

REFERENCES

1. Anderson, D.G., and L.L. McKay. 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl. Environ. Microbiol.* **46,** 549-552.
2. Birnboim, H.C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7,** 1513-1523.
3. Corbel, M.J., C.D. Bracewell, E.L. Thomas, and K.P.W. Gill. 1979. Techniques in the identification and classification of *Brucella* species. *In* "Identification methods for microbiologists" (Skinner F.A., and D.W. Lovelock, eds.), 2nd ed., p.70-122. Academic Press, New York.
4. Gerhardt, P., R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg, and G.B. Phillips. 1981. Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
5. Kim, Y.M. 1981. Studies on the mechanism of carbon monoxide oxidation in *Pseudomonas carboxydohydrogena.* Doctoral thesis, Indiana University, Bloomington, Indiana, U.S.A.
6. Kim, Y.M., and G.D. Hegeman. 1981. Purification and some properties of carbon monoxide dehydrogenase from *Pseudomonas carboxydohydrogena. J. Bacteriol.* **148,** 904-911.
7. Kim, Y.M., and G.D. Hegeman. 1983*a*. Oxidation of carbon monoxide by bacteria. *Intl. Rev. Cytol.* **81,** 1-32.
8. Kim, Y.M., and G.D. Hegeman. 1983*b*. Oxidation of carbon monoxide by *Pseudomonas carboxydohydrogena. Kor. J. Microbiol.* **21,** 27-35.
9. Krüger, B., and O. Meyer. 1984. Thermophilic *Bacilli* growing with carbon monoxide. *Arch. Microbiol.* **139,** 402-408.
10. Lautrop, H. 1974. Acinetobacter. *In* "Bergey's manual of determinative bacteriology" (Buchanan, R.E., and N.E. Gibbons, eds.), 8th ed., p. 436-438. The Williams and Wilkins Co., Baltimore.
11. Marmur, J., and P. Doty. 1961. Determination of the base composition of DNA from its thermal denaturation temperature. *J. Mol. Biol.* **5,** 109-118.
12. Meyer, O. 1982. Chemical and spectral properties of carbon monoxide: methylene blue oxidoreductase. *J. Biol. Chem.* **257,** 1333-1341.
13. Meyer, O., and K.V. Rajagopalan. 1984. Molybdopterin in carbon monoxide oxidase from carboxydotrophic bacteria. *J. Bacteriol.* **157,** 643-648.
14. Meyer, O., and H.G. Schlegel. Reisolation of the carbon monoxide utilizing hydrogen bacterium *Pseudomonas carboxydovorans* (Kistner) comb. nov. *Arch. Microbiol.* **118,** 35-43.
15. Meyer, O., and H.G. Schlegel. 1983. Biology of aerobic carbon monoxide-oxidizing bacteria. *Ann. Rev. Microbiol.* **37,** 227-310.
16. Postgate, J.R. 1972. The acetylene reduction test for nitrogen fixation. *In* "Methods in microbiology" (Norris, J.R., and D.W. Ribbons, eds.), Vol. 6B, p. 343-356. Academic

Press, New York.

17. Robbins, R.C., K.M. Borg, and E. Robinson. 1968. Carbon monoxide in the atmosphere. *J. Air Pollut. Control Assn.* **18,** 106-111.
18. Robinson, E., and R.C. Robbins. 1970. Gaseous atmospheric pollutants from urban and natural sources. *In* "global effects of environmental pollution" (Singer, S.F., ed.), p. 50-64. Springer-Verlag, New York.
19. Sonnenwirth, A.C. 1980. Pseudomonads and other non-fermenting bacilli. *In* "Microbiology" (Davis *et al.*, eds.), 3rd ed., p. 674-677. Harper and Row, New York.
20. Starr, M.P., and W.L. Stephens. 1964. Pigmentation and taxonomy of the genus *Xanthomonas. J. Bacteriol.* **87,** 293-302.
21. Swinnerton, J., R.A. Lamontagne, and V.J. Linnenbom. 1971. Carbon monoxide in rain water. *Science* **172,** 943-945.
22. Zavarzin, G.A., and A.N. Nozhevnikova. 1977. Aerobic carboxydobacteria. *Microbial Ecol.* **3,** 305-326.

(Received, January 15, 1985)