PRODUCTION OF CARBON MONOXIDE BY BACTERIA OF THE GENERA *PROTEUS* AND *MORGANELLA*

ATSUNOBU HAYASHI,¹ HIROSHI TAUCHI, AND SEIICHI HINO²

*Botanical Institute, Faculty of Science, Hiroshima
University, Naka-ku, Hiroshima 730, Japan*

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A bacterium, isolated from human saliva, produced carbon monoxide (CO) when cultured in nutrient broth containing heme or hemoglobin. The production of CO was detected by gas chromatography, and confirmed by gas chromatography-mass spectrometry and spectrophotometry which revealed an absorption spectrum of CO-hemoglobin. The isolated bacterium was identified as a strain of *Proteus vulgaris*. Survey of 4 IFO strains of *P. vulgaris*, 1 IFO strain of *P. mirabilis* and 2 IFO strains of *Morganella morganii* (received as *P. morganii*) showed that all the 7 strains had the ability to produce CO. They produced detectable amounts of CO when cultured in a glucose-peptone medium or in the nutrient broth without the addition of heme compound, and the CO production was distinctly enhanced by the addition of heme. *Morganella morganii* IFO 3168, the most active CO producer among the strains tested, produced about 2 μ mol of CO in the absence of heme and 6 μ mol of CO in the presence of 3 μ mol of heme. The result suggests that CO production by the *Morganella* strain is different from the CO production by mammalian tissues where 1 mol of protoheme is degraded to 1 mol each of CO and biliverdin.

Small amounts of CO are produced in normal human tissues where hemoglobin is degraded to equimolar amounts of biliverdin and CO. The degradation is catalyzed by microsomal heme oxygenase (1) and the CO is produced by the oxidation of α -methene bridge carbon of the porphyrin ring (2). Some reports showed that abnormally large amounts of CO were produced in some tissues of post-mortem human bodies (3, 4). The cause of the abnormal formation is unknown and we suspected that bacterial activity might be responsible for the formation. ENGEL et al. observed CO formation by hemolytic strains of *Bacillus cereus*

¹ On leave from the Scientific Investigation Research Laboratory, Hiroshima Prefectural Police Headquarters, Hiroshima, Japan.

² To whom correspondence should be addressed.

and *Streptococcus mitis* when heme (5) or cobalamin compounds (6) were added to the culture media. No further report has appeared concerning the CO formation by these bacteria.

In this study we isolated a CO-producing bacterium from human saliva. Since we identified the bacterium to be a strain of *Proteus vulgaris*, we further examined some bacterial strains of the genera *Proteus* and *Morganella*, and found that all of the strains had the ability to produce CO.

MATERIALS AND METHODS

Organisms. A CO-producing bacterium was isolated from human saliva by the procedure described later. Beside the isolated strain, several strains of *Proteus vulgaris*, *P. mirabilis*, *Morganella morganii*, *Pseudomonas aeruginosa* and *Bacillus cereus* were obtained from the Institute for Fermentation, Osaka. In addition, *Citrobacter freundii* AHU (Faculty of Agriculture, Hokkaido University, Sapporo) 1533, *Klebsiella pneumoniae* M5a1 (7), *Escherichia coli* B, *E. coli* K12 were included among the test organisms. *Morganella morganii* IFO 3168 and IFO 3848 were received as *Proteus morganii* IFO 3168 and IFO 3848, and *Citrobacter freundii* AHU 1533 as *Escherichia freundii* AHU 1533. In this paper, these bacteria are described as *Morganella morganii* and *Citrobacter freundii*, according to the designations in Bergey's Manual of Systematic Bacteriology (8).

Culture conditions. Bacteria were precultured for 18 hr at 30°C in a nutrient broth containing meat extract (Kyokuto) 10 g, Polypeptone (Wako) 10 g and NaCl 1.5 g in 1,000 ml H₂O (pH 7.2). The nutrient broth was used in most of the main cultures, but in some experiments semisynthetic media were also used which contained Na₂HPO₄·12H₂O 24 g, KH₂PO₄ 4.5 g, (NH₄)₂SO₄ 1.0 g, MgSO₄·7H₂O 100 mg, CaCl₂ 3 mg, FeSO₄·7H₂O 0.5 mg, glucose 10 g, and Polypeptone or caseamino acids (vitamin free, Nissan) 10 g in 1,000 ml H₂O (pH 7.0). Blood-containing nutrient broth was prepared by adding sterile defibrinated horse blood (Nippon Seibutsu Zairyo Co., Ltd.) to the nutrient broth.

To test the effect of hemin on CO evolution, hemin (Sigma, Type III) was dissolved in 0.1% NaOH, and 1 ml of either sterile hemin solution or 0.1% NaOH solution was added to 9 ml of the culture medium in a 50-ml Erlenmeyer flask; the addition of NaOH did not change the pH of the media significantly. After inoculation of 0.1 ml of the preculture, the flask was sealed with a silicon stopper. In the central portion of the stopper a hole was bored and a rubber plug, used to inject gas samples by a needle into a gas chromatograph, was tightly inserted in the hole. The flasks were shaken at 30°C on a reciprocating shaker at 120 rpm with an amplitude of 5 cm.

After incubation, most of the culture flasks showed negative pressure, probably due to consumption of oxygen by bacteria. Flasks were brought to a laboratory room and after temperature equilibrium, pure helium was added to the

flask by a syringe through the rubber plug to make slightly positive pressure; the amount of added helium was generally between 1 to 5 ml and the gas volume of the flask was about 55 ml. It was necessary to take precautions against contamination of CO from laboratory sources, particularly from flames of coal gas or from alcohol lamps. A considerable amount of CO was produced after autoclaving the culture media containing both glucose and hemin, therefore we sterilized hemin solutions separately from the other components in the culture media.

Analysis of CO. In most of the present experiments, CO was analyzed by a Shimadzu gas chromatograph model GC-6AI with a thermal conductivity detector and a 2.1 m Molecular Sieve 5A (60–80 mesh) column. The column temperature was set at 100°C and helium was used as a carrier gas with a flow rate of 20 ml/min. One ml of gas sample was withdrawn from the culture flasks and injected into the gas chromatograph for analysis of CO. For quantitative analysis, various amounts of pure CO gas were injected by a microsyringe and a standard curve was drawn between the amount of CO and height of the peak on the recorder at the retention time of CO. Amounts of CO dissolved in culture solutions were not corrected, since solubility of CO in water is low.

Three other methods were used for qualitative detection of CO: For a preliminary survey of CO-producing bacteria, a Kitagawa precision CO detector tube was used, which changes color from yellow to pale blue when CO concentration is in a range of 5 to 50 ppm. The detector tube set was purchased from Komyo Rikagaku Kogyo Co., Kawasaki, Japan. Formation of CO-hemoglobin from hemoglobin was detected with a Shimadzu spectrophotometer model UV-200. Formation of CO was also confirmed by gas chromatography-mass spectrometry with a Shimadzu Auto GCMS-6020 and a Molecular Sieve 5A column (2 m), by the procedure described by KOJIMA et al. (4).

Identification of the isolated bacteria. Cells were grown on nutrient agar at 30°C for microscopic observations such as cell shape, cell size and gram stain. Gram stain was performed by the Hucker modification. For observation of flagella, cells were grown in the nutrient agar at 25°C and observed under a light microscope after staining by the Nishizawa and Sugawara method (9). The following biochemical tests were performed at 36°C. Enterotube II, Roche, purchased from the Japan Roche Co. Ltd., was used for tests of acid and gas formation from glucose, and acid formation from adonitol, lactose, arabinose, sorbitol and dulcitol. The same tube was also used for the Voges-Proskauer reaction, citrate utilization, indole production, H₂S production, urease, phenylalanine deaminase, lysine and ornithine decarboxylase. Gas formation from glucose was also examined with the Durham tube. Acid formation from sucrose, xylose, maltose and mannose were examined in semi-solid peptone water, to which 1% carbohydrate and a 0.01% bromthymol blue were added. Tests for cytochrome oxidase and catalase followed the methods described by HOLDING and COLLEE (10).

RESULTS AND DISCUSSION

Isolation of CO-producing bacteria

We were able to isolate CO-producing bacteria from human saliva after failure to isolate such bacteria from some other natural sources. About 0.1 ml of human saliva was added to 10 ml of the nutrient broth containing 10% defibrinated horse blood, and after incubation for 18 hr, gas samples from the culture flasks were analysed for CO by gas chromatography. Since the result showed the presence of CO, 0.1 ml of the culture solution was added to 10 ml of the nutrient broth containing 10 μ mol of hemin, and after cultivation for 18 hr, cells were subcultured to fresh hemin-containing broth. Final culture solutions were streaked on nutrient agar plates and colonies were isolated and transferred to nutrient agar slants.

After the isolated bacterial cells had been cultured in hemin-containing nutrient broth, gas samples from the culture flasks were analysed by gas chromatography. The chromatogram showed the CO-peak at a retention time of about 3.5 min; peaks of O₂ and N₂ appeared at about 1.0 min and 1.5 min, respectively (Fig. 1). Analysis by gas chromatography-mass spectrometry of the gas samples from culture flasks showed 3 similar peaks on the total ion current chromatogram, and the

Fig. 1. Gas chromatograms showing production of CO by isolated bacteria.

(A) Air and standard CO, (B) Gas sample from uninoculated control (nutrient broth containing 10 μ mol of hemin per 10 ml), (C) Gas sample from inoculated culture (nutrient broth without addition of hemin), (D) Gas sample from inoculated culture (nutrient broth containing 10 μ mol of hemin per 10 ml).

Table 1. Characteristics of the isolated strain.

Cell shape	Rods	Gas from glucose	—
Cell size		Acid from	
Width, μm	0.4 – 0.6	Glucose	+
Length, μm	0.8 – 2.0	Mannose	—
Gram reaction	—	Sucrose	+
Motility	+	Lactose	—
Swarming	+	Maltose	+
Flagella	Peritrichous	Arabinose	—
Oxidase test	—	Xylose	+
Catalase	+	Adonitol	—
Amino acids		Sorbitol	—
H ₂ S production	+	Dulcitol	—
Lysine decarboxylase	—	Mannitol	—
Ornithine decarboxylase	—	Citrate utilization	+
Phenylalanine deaminase	+	Voges-Proskauer test	—
Urease	+		
Indole production	+		

sample of the peak corresponding to CO showed base peak ion, m/z 28, and a small ion, m/z 29, in the mass spectrum; this feature is quite similar to that of pure CO.

The isolated bacteria also produced CO when cultured in nutrient broth containing defibrinated horse blood. The CO formation was confirmed by gas chromatographic analysis of the gas sample from culture flasks and also by spectrophotometric analysis. When the culture solutions were centrifuged to remove cells, the supernatants from inoculated cultures showed absorption maxima of CO-hemoglobin at 570 and 540 nm, while the supernatants from the uninoculated controls showed maxima of O₂-hemoglobin at 578 and 542 nm.

Identification of the isolated strain

The taxonomic characteristics of the isolated bacterium were examined and the result is shown in Table 1. The bacterium is a facultatively anaerobic gram-negative rod, and is oxidase-negative. The cells are motile with peritrichous flagella and are positive in phenylalanine deaminase and urease. These results show that the bacterium should be classified among the genera *Proteus*, *Providencia* or *Morganella*. Swarming of cells was suggested by occasional halos around the stroke colonies on the surface of agar plates. Further characteristics listed in Table 1 coincides well with those of *P. vulgaris* listed in the Bergey's Manual of Systematic Bacteriology (8), except the ability to produce gas from glucose. Our tests with both Durham tubes and semi-solid nutrient agar showed that the isolated strain is negative in gas production. Although typical *P. vulgaris* is described as positive, gas production by *P. vulgaris* is generally weak and 11 to 24% of *P. vulgaris* strains are reported to be negative (8). Therefore we concluded that the isolated organism is a strain of *P. vulgaris*.

Table 2. CO production by strains of *Proteus* and *Morganella*
(μmol CO per culture flask).

Bacteria	Culture medium			
	Nutrient broth		Glucose peptone	
	without hemin	with hemin	without hemin	with hemin
Sterile control	0.05	0.14	0.00	0.07
<i>P. vulgaris</i> (isolated strain)	0.36	2.12	0.12	1.57
<i>P. vulgaris</i> IFO 3045	0.50	0.80	0.22	1.55
<i>P. vulgaris</i> IFO 3167	0.61	0.87	0.15	1.17
<i>P. vulgaris</i> IFO 3851	0.63	2.71	0.22	1.67
<i>P. vulgaris</i> IFO 3988	0.45	0.80	0.39	2.16
<i>P. mirabilis</i> IFO 3849	0.38	1.98	0.12	1.00
<i>M. morganii</i> IFO 3168	2.03	6.10	0.93	5.99
<i>M. morganii</i> IFO 3848	0.75	2.13	0.34	3.39

Cells were grown for 24 hr at 30°C in nutrient broth or glucose-peptone medium with or without addition of 3 μmol of hemin. CO production is expressed as μmol CO per culture flask (gas volume: 55 ml).

CO production by strains of *Proteus* and *Morganella*

Strains of *P. vulgaris* IFO 3045, IFO 3167, IFO 3851, IFO 3988, *P. mirabilis* IFO 3849, *Morganella morganii* IFO 3168 and IFO 3848 were tested for their ability to produce CO. Cells were cultured in 10 ml of the nutrient broth with or without addition of 3 μmol hemin. After incubation for 24 hr, concentrations of CO in the gas phase of the culture flasks were analyzed by gas chromatography and the total amounts of CO produced in the flasks were calculated. Uninoculated controls were run in parallel. In the present experiments, the lower limit for detection of CO was about 0.02 μl per ml of the gas in the culture flask or about 0.05 μmol per total gas volume of the flask (volume: 55 ml). Table 2 shows that all the strains of *P. vulgaris*, *P. mirabilis* and *M. morganii* produced CO when cultured in the nutrient broth, and the activity of CO production was different among the strains. Production of CO was evident even when the bacteria were cultured in the nutrient broth without the addition of hemin. Since the meat extract, used for preparation of the nutrient broth, may contain some heme compounds, the experiments were repeated with the glucose-peptone medium. The results are included in Table 2. The production of CO from hemin-free glucose-peptone medium is evident with most of the strains, although the production is slight with *P. vulgaris* IFO 3167 and *P. mirabilis* IFO 3849. Results similar to those with the

Table 3. CO production by other bacteria.

Bacteria	Nutrient broth	
	without hemin	with hemin
Sterile control	0.00	0.04
<i>Pseudomonas aeruginosa</i> IFO 3445	0.04	0.08
<i>Bacillus cereus</i> IFO 3132	0.03	0.26
<i>Klebsiella pneumoniae</i> M5a1	0.03	0.08
<i>Citrobacter freundii</i> AHU 1533	0.00	0.08
<i>Escherichia coli</i> B	0.07	0.11
<i>Escherichia coli</i> K12	0.14	0.14

See the legend to Table 2.

glucose-peptone medium were obtained with the medium containing glucose and vitamin-free casamino acids.

ENGEL et al. (5) reported that hemolytic strains of *Streptococcus mitis* and *Bacillus cereus* produced CO only when cultured in the presence of heme compounds, and the amount of CO produced from 25 mg of hemoglobin (equivalent to 1.5 μ mol of heme) was 1.5 μ mol for the *B. cereus* and 0.5 μ mol for the *S. mitis* after 7 days culture. In the present experiment, strains of *Proteus* and *Morganella* produced CO without the addition of heme compounds, although CO formation was distinctly enhanced by the presence of heme compounds. The increased amount of CO production by the addition of 3 μ mol hemin to the culture of *M. morganii* IFO 3168 was about 5 μ mol. The result indicates that more than 1 mol of CO was produced from 1 mol of hemin. Since 1 mol of CO is produced when 1 mol hemin is cleaved to biliverdin by heme oxygenase, *M. morganii* IFO 3168 either produces CO by further cleavage of biliverdin molecule or produces CO by a process different from heme oxygenase. The production of CO without the addition of hemin supports the second possibility.

Table 3 shows the results of a survey for the activity of CO evolution by *Pseudomonas aeruginosa*, *Bacillus cereus* and 3 species of the enterobacteria. Low but reproducible CO evolution was detected with *E. coli* K 12 and *B. cereus* IFO 3132. The production of CO by *B. cereus* in the presence of hemin is in agreement with the report by ENGEL et al. (5).

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REFERENCES

- 1) R. TENHUNEN, H. S. MARVER, and R. SCHMID, *Proc. Natl. Acad. Sci. U.S.A.*, **61**, 748 (1968).
- 2) S. A. LANDAW, E. W. CALLAHAN, Jr., and R. SCHMID, *J. Clin. Invest.*, **49**, 914 (1970).
- 3) J. A. BEELEY and W. HARVEY, *J. Forensic Sci. Soc.*, **13**, 297 (1973).
- 4) T. KOJIMA, Y. NISHIYAMA, M. YASHIKI, and I. UNE, *Forensic Sci. Int.*, **19**, 243 (1982).

- 5) R. R. ENGEL, J. M. MATSEN, S. S. CHAPMAN, and S. SCHWARTZ, *J. Bacteriol.*, **112**, 1310 (1972).
- 6) R. R. ENGEL, S. MODLER, J. M. MATSEN, and Z. J. PETRYKA, *Biochim. Biophys. Acta*, **313**, 150 (1973).
- 7) M. C. MAHL, P. W. WILSON, M. A. FIFE, and W. H. EWING, *J. Bacteriol.*, **89**, 1482 (1965).
- 8) N. R. KRIEG and J. G. HOLT, *In Bergey's Manual of Systematic Bacteriology*, 1st ed., Vol. 1, Williams & Wilkins Co., Baltimore (1984).
- 9) Tokyo Daigaku Ikagaku Kenkyujo Gakuyukai (ed.), *Saikingaku Jissyu Teiyo*, 5th ed., Maruzen, Tokyo (1976), p. 250.
- 10) A. J. HOLDING and J. G. COLLEE, *In Methods in Microbiology*, Vol. 6A, ed. by J. R. NORRIS and D. W. RIBBONS, Academic Press, London (1971), p. 1.