A SALT-REQUIRING SULFATE-REDUCING BACTERIUM ISOLATED FROM ANTARCTICA

HIROSHI OKAZAKI* AND HIROSHI IIZUKA

*The Institute of Applied Microbiology,
University of Tokyo, Tokyo*

(Received December 27, 1971)

Determinative studies were carried out on an obligatory anaerobic, sulfate-reducing bacterium (strain No. 61) isolated from a pond water in Skarvs Nes district in the East Antarctica. This sulfate-reducing bacterium was nonsporulating, sigmoid or straight rods with a single polar flagellum, gram negative, and possessed cytochrome c_3 and desulfovibrin, characteristic of the genus *Desulfovibrio*. It could grow with lactate, malate, pyruvate, or choline when sulfate was present in the culture medium and had a stable sodium chloride requirement. These observations indicated that strain No. 61 closely resembled *Desulfovibrio desulfuricans* var. *aestuarii*.

The obligatory anaerobic sulfate-reducing bacteria utilize sulfates as terminal electron acceptors for their respiration and take part in the geo-microbiological cycle of sulfur. It is well known that representatives of the sulfate-reducing bacteria are able to grow under extraordinary conditions of temperature, salinity, and pressure. However, their growth under a low temperature has not been clearly elucidated. The presence of sulfate-reducing bacteria in the Antarctic circle has already been reported by BARGHOORN and NICHOLS (1) who studied the pyritic sediment of kettle holes in the Wright Dry Valley in the McMurdo Sound region. It is noteworthy that sulfate-reducing bacteria were found as active agents in sedimentary process under the low temperature conditions of the Antarctic environment.

Because of the extraordinary conditions under which they survive, these sulfate-reducing bacteria in the Antarctica may have physiological properties different from those of known species. Although BARGHOORN and NICHOLS (1) pointed out that the isolated sulfate-reducing bacteria belonged to the genus *Desulfovibrio*, they did not report on their purification, detailed physiological properties, or on the identification results of these bacteria.

In the investigation of geochemical cycle of sulfur in the Antarctic circle,

* Present Address: Research Laboratories, Chugai Pharmaceutical Co., Ltd., 41-8, 3 chome, Takada, Toshima-ku, Tokyo 171.

we have found two types of sulfate-reducing bacteria, which belong to the genera *Desulfovibrio* and *Desulfotomaculum*.

In the previous paper, we have reported a determinative study on a new spore-forming sulfate-reducing bacterium, *Desulfotomaculum antarcticum* (2). In the present communication, we describe the results of determinative studies on the genus *Desulfovibrio* isolated from a pond water in the Skarvs Nes district (69°30'S, 39°40'E) which is located about 60 km from the Showa base.

MATERIALS AND METHODS

The pond water was sampled by Y. Honkawa during the Eighth's Japanese Antarctic Expedition (1966–1967). The sample was stored in a frozen state until examined. For enrichment culture and isolation of sulfate-reducing bacteria, two kinds of media were employed. Postgate's medium contained in grams per liter: sodium lactate, 3.0; peptone, 2.0; yeast extract, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0; K_2HPO_4 , 0.2; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; Na-ascorbate, 0.1; NaCl, 5 or 25; pH 7.0. Starkey's medium contained in grams per liter: sodium lactate, 3.5; yeast extract, 1.0; NH_4Cl , 1.0; Na_2SO_4 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0; Mohr's salt, 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1; NaCl, 5.0 or 25.0; Na ascorbate, 0.1; Na thioglycollate, 0.1; pH 7.0.

Five ml of each sample was added into 100-ml Erlenmeyer flasks containing 20 ml of media, and incubated in a desiccator containing N_2 gas at 5°, 10°, 20° or 30° for 7 to 20 days. A pure culture was obtained by using tube culture of the Starkey's medium containing 1% agar and was checked for purity by POSTGATE's method (3). The determinative methods employed were mainly those described in POSTGATE's paper (4). Sulfate utilization by the isolated organisms was confirmed by the reaction of sulfate ions with barium ions to form a white precipitate.

Eh of the culture broth was measured with the Toa Electronics Model HN-5A pH-meter equipped with a platinum electrode. Absorption spectra of suspensions of the sulfate-reducing bacteria were measured with Shimadzu recording spectrophotometer Model MPS-50. A droplet of the cell suspension was placed on a collodion-coated copper grid, air dried, shadowed with chrome, and used for electron microscopy. The grid was examined with a JEM-7A electron microscope at an initial magnification of 3000.

RESULTS AND DISCUSSION

After incubation for 2 weeks at 20° and 30°, the enrichment culture made from sample No. 61 (pond water) showed blackening in Postgate's and Starkey's media, but not from those at 5° and 10°. Hydrogen sulfide formation and utilization of sulfate were also confirmed. However, when the enrichment cultures were performed at 5°–10° for about 50 days, the

Fig. 1. Enrichment culture made from the sample No. 61 at 10° for about 50 days.

Fig. 2. Photomicrographs of Strain No. 61.

Fig. 3. Photomicrographs of Strain No. 61.

Fig. 4. Elongated form of Strain No. 61.

Fig. 5. Electron photomicrograph of Strain No. 61.

precipitate of iron sulfide deposited in the culture broth (Fig. 1).

POSTGATE and CAMPBELL (4) reported that sulfate-reducing bacteria could be differentiated into two groups, the genus *Desulfovibrio* and *Desulfotomaculum*, on the basis of morphology and other physiological characteristics. In view of the fact that the organisms isolated in this investigation were nonsporulating, slender sigmoid rods, it was assumed that this bacterium belonged to the genus *Desulfovibrio*.

Morphological characteristics

The isolated sulfate-reducing bacterium (strain No. 61) was stained by Carbol fuchsin solution or Gram staining method after incubation on Starkey's medium at 25° and their size was measured. The organisms were slender, curved rods, 3 to 5 $\times$ 0.5 to 0.6 μ (Figs. 2 and 3), and elongated forms were frequently observed (Fig. 4). As shown in Fig. 5, electron micrographs

showed that the strain No. 61 possesses polar flagellum. The organisms were Gram negative and nonsporulating.

Physiological characteristics

Studies on utilization of carbon sources and other physiological tests were performed at 25° in Starkey's medium, because the organisms grew erratically in the medium C of BUTLIN *et al.* (5). Substrates to be tested as carbon source were added to lactate-free Starkey's medium to 0.2%. Strain No. 61 utilized lactate, malate, pyruvate, and choline. It was unable to grow on acetate, formate, propionate, tartarate, succinate, citrate, glucose, fructose, sucrose, galactose, maltose, raffinose, xylose, glycerol, and dulcitol. A reducing environments were said to be essential for the growth of sulfate-reducing bacteria. It was found that the Eh values during growth of strain No. 61 ranged from 0 to -150 mV.

No growth was observed when pyruvate was added to the sulfate-free medium. Reduction of nitrate to nitrite was not detected by the Griess-Ilosvay's reagents after incubation for 7 days in the medium containing 0.1% KNO₃. Gelatinase formation was not detected. Requirement of NaCl was examined by testing growth in a medium supplemented with various concentrations of NaCl, 0, 0.5, 2.5, 5.0, or 10.0%. This experiment showed that the strain No. 61 could not grow in the absence of NaCl and concentration of 0.5–5.0% was required for its growth. Growth was scanty in a medium containing over 10% of NaCl. Further, ability to grow with KCl was tested for the determination of chloride ion requirement. However, strain No. 61 did not grow with KCl. In this respect, strain No. 61 appears to differ from the obligatory chloride ion-requiring strain, *Desulfovibrio salexigenes* Postgate and Campbell. Temperature range of growth was examined by comparison of growth in the medium containing 25 g/liter of NaCl at 10°, 20°, 30°, 37°, or 40° for 7 to 14 days. Strain No. 61 was able to grow at 20–30°, and 30° was the maximum growth temperature. No growth was obtained at 37° and 40°. In the paper of POSTGATE and CAMPBELL (4), the temperature range for growth of five known species of *Desulfovibrio*, *D. desulfuricans*, *D. vulgaris*, *D. salexigenes*, *D. africans*, and *D. gigas*, was reported as 28–44°. Recently, STANLEY and MORITA (6) pointed out that the maximum growth temperature of psychrophilic seawater-requiring marine bacteria was affected by changes in the concentration of NaCl in the medium.

Since NaCl was found to be essential for the growth of strain No. 61, it may be considered that there is a relation between the temperature range for growth and the concentration of NaCl in this sulfate-reducing bacterium.

Respiratory pigments

The presence of cytochrome *c*₃ has been found in all the known species of the genus *Desulfovibrio*. Therefore, we tested the strain No. 61 for the cytochrome *c*₃. Intact cells of strain No. 61 were suspended in a solution of

Fig. 6. Absorption spectrum of Strain No. 61. Intact cells were suspended in bovine serum albumin and its absorbance measured by a spectrophotometer.

bovine serum albumin and absorbance of the suspension was measured (7). Reduced state of the cytochrome C_3 was prepared by the addition of a small amount of $Na_2S_2O_4$. As shown in Fig. 6, the characteristic absorption bands of cytochrome c_3 , with maxima at 419, 525, and 553 nm, were clearly confirmed. In this experiment, an absorption band in the neighborhood of 630 nm, suggesting the presence of the pigment desulfoviridin, was not seen. However, when the intact cells were treated with NaOH and exposed to UV ray of 365 nm, a red fluorescence was appeared. Therefore, it is concluded that desulfoviridin is also contained in the cells of strain No. 61. In addition to these observations, it was found that a large amount of mucous substances accumulated in aged cultures of strain No. 61. OCHYNSKY and POSTGATE (8) already reported that mucous substances, mucopolymannosides, accumulated in the cultures of *D. vulgaris* (fresh water strain) and *D. desulfuricans* (salt water strain), and that this accumulation was related to the salt habit of the strain. However, in strain No. 61, cultural conditions for the accumulation and properties of the mucous substances still remain obscure.

On the basis of these experimental facts, the taxonomical properties of strain No. 61 seemed to be similar to those of *D. desulfuricans* var. *aestuarii* Sylt 3 according to the description of Postgate, but differed in respect to the temperature range for growth and no growth in the sulfate-free medium with pyruvate as a sole carbon source. Ability to grow in the sulfate-free medium with pyruvate is a characteristic of most of the members of *D. desulfuricans*. However, Postgate and Campbell have reported some exceptions observed in this group. Accordingly, it may be reasonable to consider that the strain No. 61 belongs to *D. desulfuricans* var. *aestuarii*.

DESCRIPTION OF THE STRAIN

Desulfovibrio desulfuricans Postgate and Campbell var. *aestuarii* Stüven.

K. Stüven, *Arch. Mikrobiol.*, **35**, 152 (1960).

J. R. Postgate and L. L. Campbell, *Bacteriol. Rev.*, **30**, 732 (1966)

Strain: Skarvs Nes No. 61

Morphology: Slender, sigmoid rods, often straight rods. Nonsporulating. 3 to 10 × 0.5 to 0.6 μ . Sometimes elongated forms (15 to 20 × 0.5 to 0.6 μ) are observed. Gram negative. Shows rapid progressive motility by a single polar flagellum (Fig. 5).

Culture: Obligate anaerobe. Requiring low Eh value (0 to -150 mV) for growth. Forms black colonies in agar media containing ferrous salts. Reduces sulfates to sulfides. Accumulates a large amount of mucous substances in aged cultures.

Carbon sources: Utilizes lactate, pyruvate, malate, and choline, if sulfate is present. Does not utilize acetate, formate, propionate, tartarate, succinate, citrate, glucose, fructose, sucrose, lactose, galactose, maltose, raffinose, salicin, xylose, glycerol, or dulcitol.

Malate: Does not support growth when sulfate is absent.

Choline: Scanty growth is observed when sulfate is absent.

Pyruvate: Does not support growth when sulfate is absent.

Nitrates: Not reduced.

Gelatin: Not liquefied.

Respiratory pigment: Cell suspensions show absorption bands of cytochrome c_3 at 419, 525, and 553 nm (reduced state with $\text{Na}_2\text{S}_2\text{O}_4$).

Fluorescence: When cell suspensions are treated with NaOH and exposed to UV ray of 365 nm, red fluorescence of the chromophore of desulfovibridin is observed.

Temperature range for growth: Growth slow at 5–10°. Good growth at 20–30°. No growth at 37° or 40°.

Effect of sodium chloride: Growth at 0.5–5.0% NaCl. No growth at 0% NaCl. Poor growth above 10% NaCl. This strain shows a stable salt requirement.

Source: Isolated from the pond water collected by Y. Honkawa from

the Skarvs Nes district in the East Antarctica in 1967. Strain No. 61 was deposited in the IAM Culture Collection, The Institute of Applied Microbiology, University of Tokyo, Japan, in 1967.

REFERENCES

- 1) E.S. BARGHOORN and R.L. NICHOLS, *Science*, **134**, 190 (1961).
- 2) H. IIZUKA, H. OKAZAKI and N. SETO, *J. Gen. Appl. Microbiol.*, **15**, 11 (1969).
- 3) J. POSTGATE, *In Anreicherungskultur und Mutantenauslese*, ed. by H.G. SCHLEGEL, Gustav Fisher Verlag, Stuttgart (1965), p. 190.
- 4) J.R. POSTGATE and L.L. CAMPBELL, *Bacteriol. Rev.*, **30**, 732 (1966).
- 5) K.R. BUTLIN, M.E. ADAMS and M. THOMAS, *J. Gen. Microbiol.*, **3**, 46 (1949).
- 6) S.O. STANLEY and R.Y. MORITA, *J. Bacteriol.*, **95**, 169 (1968).
- 7) R. BARER, *Science*, **121**, 709 (1955).
- 8) F.W. OCHYNSKI and J.R. POSTGATE, *In Symposium on Marine Microbiology*. Charles C. Thomas, Springfield (1963), p. 462.