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Denitrification by *Corynebacterium nephridii*

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ABSTRACT

HART, LEWIS T. (Louisiana State University, Baton Rouge), A. D. LARSON, AND C. S. MCCLESKEY. Denitrification by *Cornyebacterium nephridii*. J. Bacteriol. **89**:1104–1108. 1965.—*Corynebacterium nephridii* was found to reduce nitrate (contrary to the original description) at a rapid rate. In the conventional 0.1% nitrate broth, neither nitrite nor nitrate was detected after 24 hr. There was no assimilation of nitrate nitrogen, and the final product of nitrate reduction was nitrous oxide. Manometric studies and growth experiments indicated that the organism is incapable of reducing nitrous oxide. *C. nephridii* is gram-negative, grows on bile salts (5%) agar, EMB Agar, and MacConkey Agar. It was proposed that this species be transferrrd to the genus *Achromobacter* and designated *Achromobacter nephridii* (Büsing, Döll, and Freytag) comb. nov.

Büsing, Döll, and Freytag (1953) and Büsing and Freytag (1954) described an organism isolated from the bladder of the medicinal leech which they designated *Corynebacterium nephridii*. Other isolations of this species have not been reported, and apparently no further investigations have been made with this bacterium.

In a comparative study of the species of the genus *Corynebacterium*, currently in progress in this laboratory, some deviations from the original description of *C. nephridii* have been noted. Büsing et al. (1953) reported this species to be gram-positive, and not forming nitrite from nitrate. We consider the organism to be gram-negative, and found (Carrier, 1963; Hart and McCleskey, 1964) that neither nitrite nor nitrate is detectable in 24-hr cultures of the bacterium grown in the conventional 0.1% nitrate broth in 16 × 150 mm tubes (Society of American Bacteriologists, 1957). The rapid and total disappearance of the nitrate, without the appearance of nitrite or gas, prompted this investigation of nitrate metabolism and other characteristics of *C. nephridii*.

MATERIALS AND METHODS

Organism and media. The culture of *C. nephridii* employed was the strain sent to the American Type Culture Collection by E. V. Morse, who had received it from Robert S. Breed. This is the original culture of Büsing, Döll, and Freytag, and the only known isolate of this species. Cultures were maintained in Trypticase (BBL) soy semisolid agar.

Sodium nitrate was added in varying amounts to Trypticase Soy Broth (BBL) or to peptone broth, as indicated in the Results section. In the aerobic tests, cultures were grown in 500-ml Erlenmeyer flasks containing 100 ml of broth and incubated at 28 C on a shaker (180 rev/min). To obtain relatively anaerobic conditions, 4 ml of medium were introduced into 13 × 100 mm screw-capped tubes and autoclaved. After sterilization, the tubes were inoculated, then aseptically filled with sterile medium, and the caps were screwed down. One of the tubes was taken for analysis at each sampling time.

Anaerobic conditions were effected with the Parker (1955) method, except that nitrogen or nitrous oxide, admitted through a sterile cotton filter, was used to flush and fill the jar.

Morphology and staining. Motility was determined by microscopic examination of hanging drop mounts of broth cultures and the use of SIM (Difco) medium. The staining procedures employed were those described in the *Manual of Microbiological Methods* (Society of American Bacteriologists, 1957). Hucker's and the Kopeloff-Beerman modifications of the Gram stain were used. Anthony's capsule stain and Burdon's fat stain were employed. Albert's stain and methylene blue were used to detect metachromatic granules.

Nitrate utilization. Nitrate reduction was determined by the sulfanilic acid alpha naphthylamine acetate method described by the Society of American Bacteriologists (1957). Ammonia nitrogen was determined by titration after distillation into 1% boric acid. Nitrite nitrogen was quantitated with the diazotization method described by the American Public Health Association (1955), and nitrate nitrogen by the method of Landman et al. (1960). Total nitrogen was measured by the micro-Kjeldahl method of Hiller, Plazin, and Van Slyke (1948).

Gas production from nitrate, nitrite, and nitrous oxide was determined by the manometric procedure described by Umbreit, Burris, and Stauffer (1959) with N_2 or N_2O as the gas phase. Sodium

azide in a final concentration of 0.004 M was used as an inhibitor of nitrate reduction.

Analysis of gas. A mass spectrometer (Consolidated Electrodynamic Corp., Pasadena, Calif.) was used to analyze the gas produced by *C. nephridii* in a nitrate medium. The apparatus employed to collect the gas for analysis is shown in Fig. 1.

RESULTS

Except for the Gram reaction and the action on nitrate, the morphological and physiological characteristics of this species were found to be as described by Büsing et al. (1953) and Büsing and Freytag (1954). We have found the organism to be gram-negative in young and old cultures. To the original description may be added the following. The organism contains fat globules (demonstrable by Burdon's method), forms colonies on MacConkey, eosin methylene blue, and brilliant green bile agar media, and does not grow in Koser's citrate medium.

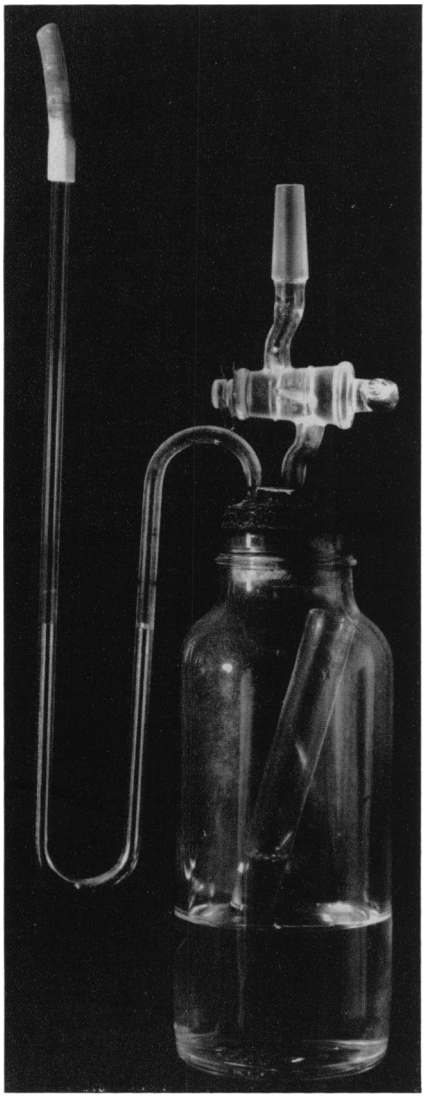

FIG. 1. *Gas-collecting apparatus, equipped with mercury manometer to indicate gas pressure and tapered joint to fit the inlet port of the mass spectrometer. Inside the culture bottle is a tube containing 20% KOH to absorb CO_2. The inoculum was introduced by syringe through a serum stopper, and the bottle was flushed with argon.*

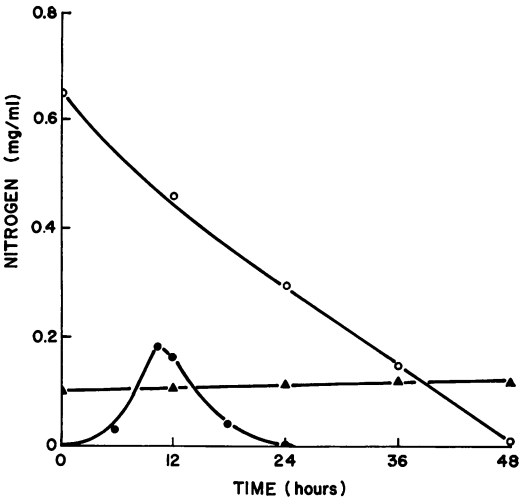

FIG. 2. *Distribution of nitrogen in cultures of Corynebacterium nephridii grown anaerobically in broth medium [Trypticase soy broth (BBL), 0.4%; sodium nitrate, 1.0%] in filled 13 × 100 mm screw-capped tubes. Symbols: ○ = nitrate nitrogen; ● = nitrite nitrogen × 100; ▲ = ammonia nitrogen.*

Utilization of nitrate. With 24-hr cultures grown in 0.1% nitrate broth in test tubes, tests for nitrite and nitrate were negative. Cultures prepared with increased sodium nitrate (0.4%) revealed a steady disappearance of nitrate, with little change in the ammonia level and a slight and transient appearance of nitrite with a peak at about 12 hr (Fig. 2). These results suggested the possibility that the organism might be assimilating the nitrate nitrogen. Under aerobic conditions, however, there was little utilization of nitrate, and nitrite persisted for days in the medium. Cultures in 1.0% nitrate broth, incubated under anaerobic conditions, were quantitatively examined at intervals for nitrate, nitrite, ammonia, and Kjeldahl nitrogen. The results are presented in Fig. 3. Kjeldahl nitrogen remained unchanged throughout the test period, whereas nitrate nitrogen steadily declined. There was little change in the ammonia nitrogen level, and nitrite nitrogen was present in only trace

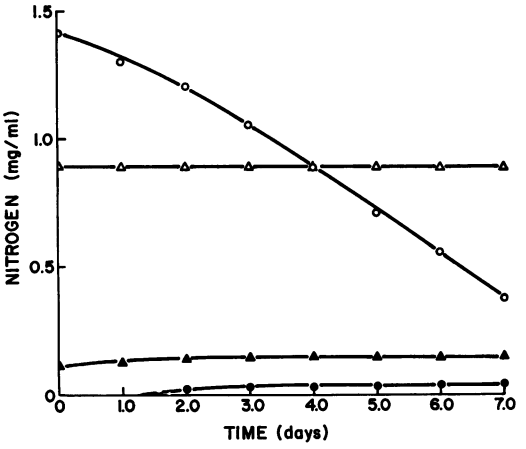

FIG. 3. *Distribution of nitrogen in cultures of Corynebacterium nephridii. Conditions as in Fig. 2 except that the concentration of $NaNO_3$ was 1.0%. Symbols:* ○ = *nitrate nitrogen;* ● = *nitrite nitrogen* × *100;* ▲ = *ammonia nitrogen;* △ = *Kjeldahl nitrogen.*

TABLE 1. *Effect of nitrate on the growth of Corynebacterium nephridii in the presence and absence of oxygen*

Medium	Aerobic	Anaerobic*
0.5% peptone	+†	−
0.5% peptone, 0.3% $NaNO_3$	+	+
0.5% peptone, 0.5% glucose	+	−
0.5% peptone, 0.5% glucose, 0.3% $NaNO_3$	+	+

* Parker (1955) method; *Clostridium sporogenes* was used as an indicator of anaerobiosis.

† Symbols: + = growth; − = no growth.

amounts. Evidence of gas was noted during pipetting of samples. Since the organism evidently was not assimilating the nitrate nitrogen, it was suspected that nitrate would serve as an electron acceptor and allow anaerobic growth. The results presented in Table 1 show that *C. nephridii* has the capacity to utilize nitrate as an electron acceptor in the absence of oxygen. Media containing no nitrate failed to support anaerobic growth.

Manometric studies. The results of manometric studies with nitrate as the electron acceptor are shown in Fig. 4. There was an immediate and continued production of gas. The addition of NaN_3 to the flask markedly inhibited gas production. The results of experiments with nitrite as the electron acceptor appear in Fig. 5. As with nitrate, gas production proceeded immediately. The reason for the nonlinearity of the curve is not known, but may be due to toxicity of nitrite.

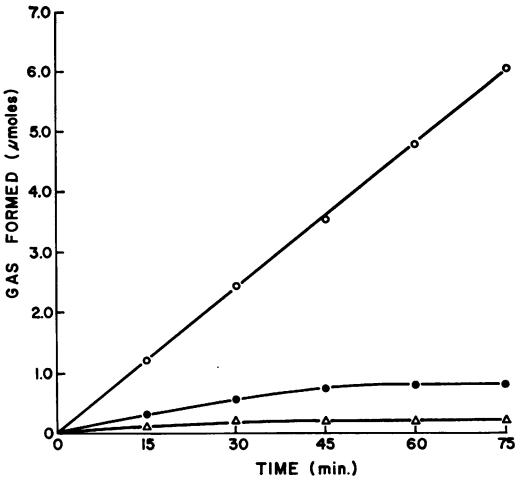

FIG. 4. *Gas production from nitrate by resting cells of Corynebacterium nephridii. Cells were grown for 48 hr in 6-oz prescription bottles filled with the same medium as in Fig. 2. The cells were washed once in distilled water. The reaction vessels contained 0.5 ml of 0.2 M potassium phosphate, 12 mg of cells, 0.2 ml of 20% KOH, 25 μmoles of $NaNO_3$, substrate, and distilled water to a volume of 3.0 ml. Gas phase, N_2; temperature, 30 C. Symbols:* △ = *endogenous;* ● = *0.004 M NaN_3 (final concentration) and 25 μmoles of glucose;* ○ = *25 μmoles of glucose.*

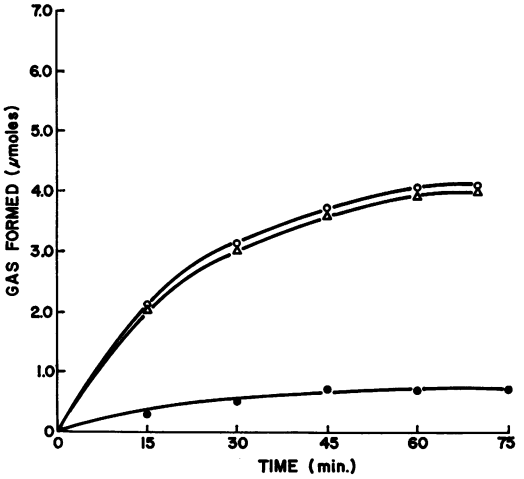

FIG. 5. *Gas production from nitrite by resting cells of Corynebacterium nephridii. The reaction vessels were prepared as described in Fig. 4 except that $NaNO_2$ was substituted for $NaNO_3$. Symbols:* ● = *endogenous;* △ = *25 μmoles of glucose plus 0.004 M sodium azide (final concentration);* ○ = *25 μmoles of glucose.*

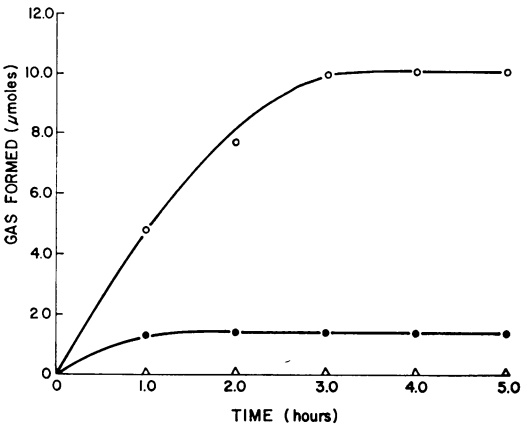

FIG. 6. *Gas production from N_2O and from nitrate under nitrous oxide by resting cells of Corynebacterium nephridii. The reaction vessels were prepared as described in Fig. 4 except that $NaNO_3$ was omitted from one series of vessels, and N_2O was the gas phase. Symbols: △ = 25 μmoles of glucose; ● = 25 μmoles of $NaNO_3$; ○ = 25 μmoles of $NaNO_3$ plus 25 μmoles of glucose.*

Gas production from nitrite was not inhibited by NaN_3. Manometric studies with N_2O as the electron acceptor (Fig. 6) showed that no gas production occurred with this material. The failure to produce gas from N_2O was not due to a toxic effect of N_2O on the reductase system, for gas was produced from nitrate in the presence of N_2O.

Anaerobic growth experiments were conducted with N_2O as the electron acceptor in peptone-glucose broth; no growth occurred in 7 days. There was no toxicity of N_2O, for good growth developed under an atmosphere of N_2O when $NaNO_3$ was supplied in the medium.

Spectrometric analysis of the gas evolved from Trypticase soy nitrate (1.0%) broth (BBL) showed it to be composed of 96.55% nitrous oxide and 3.45% nitrogen.

DISCUSSION

The failure of Büsing et al. (1953) and Büsing and Freytag (1954) to note reduction of nitrate is attributable to the very rapid utilization of nitrate by *C. nephridii* when grown under relatively anaerobic conditions, such as prevail in tubes of broth. Even when the nitrate concentration in the medium was increased to several times that recommended for the routine test procedure (Society of American Bacteriologists, 1957), only small amounts of nitrite appeared in the medium, though the nitrate rapidly disappeared. Under aerobic (shake) conditions, however, nitrite was present in detectable amounts for many days.

This organism utilizes nitrate as electron acceptor in the absence of oxygen; without nitrate in the medium, no growth occurs under anaerobic conditions. Under aerobic (shake) conditions, nitrate was consumed in small amounts, but only during the period of rapid growth, and presumably because of a deficiency of dissolved oxygen at that time. There was no assimilation of nitrate nitrogen under any conditions of growth. *C. nephridii* may, therefore, be considered as belonging to the "nitrate respiration" group of Taniguchi (1961).

Sodium azide prevented the utilization of nitrate, but not nitrite, as electron acceptor, indicating that two independent enzymes are involved in these reactions. This observation is in accord with the results obtained with other denitrifying bacteria (Delwiche, 1956).

The production of detectable gas from nitrate depended on relatively high concentrations of nitrate (1.0% or more) and the presence of a suitable electron donor such as glucose. Qualitative tests indicated that the gas was chiefly N_2O. The manometric experiments, using N O as the gas phase, showed immediate gas production from nitrate; when only N_2O was present, there was no gas production. Also, N_2O did not allow detectable growth of *C. nephridii* under anaerobic conditions, though normal growth occurred when nitrate was supplied to the organism under an atmosphere of N_2O. The evidence, therefore, indicates that *C. nephridii* reduces nitrate to N_2O via nitrite.

The steps leading to the formation of N_2O may be as suggested by Kluyver and Verhoeven (1954):

$$NO_3 \longrightarrow NO_2 \longrightarrow (HNO) \longrightarrow H_2N_2O_2 \longrightarrow N_2O + H_2O$$

C. nephridii is apparently unique among denitrifying bacteria in that it is unable to reduce N_2O. This species cannot utilize N_2O as electron acceptor for anaerobic growth, and does not form N_2 from this substance. The source of the small amount of N_2 in the gas analyzed by the mass spectrometer is not clear. The growth experiments and manometric studies indicated that the organism is unable to utilize N_2O. The necessity for high concentrations of nitrate in the medium to detect gas production may be attributed to the solubility of N_2O in water.

Taxonomy. C. nephridii possesses characteristics which are widely different from those of the type species of the genus. The organism does show some granules when stained with methylene blue or with Albert's stain, but it is gram-negative and grows on various media usually selective for

gram-negative bacteria, e.g., bile salts agar, eosin methylene blue agar, and MacConkey (Difco) Agar. Lack of uniformity in size and shape of cells is a significant characteristic of the type species of the genus *Corynebacterium;* the cells of this species are quite constant in size and shape. The wedge-shaped and club-shaped cells frequently observed in *C. diphtheriae* have not been observed in *C. nephridii.*

The characteristics of this organism appear to identify it as a member of the genus *Achromobacter* Bergey et al. It is similar to *A. parvulus* (Conn) Breed but differs from this and all other species of that genus in significant respects. The genus *Achromobacter* is so ill-defined that we hesitate to place this organism in it, but this seems preferable to leaving it as presently classified. It is proposed that this species be transferred to the genus *Achromobacter* and designated *Achromobacter nephridii* (Büsing, Döll, and Freytag) comb. nov.

ACKNOWLEDGMENTS

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