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Effect of Oxygen on Formation and Activity of Nitrate Reductase in a Halophilic *Achromobacter* Species

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Formation of nitrate reductase was induced in cells of *Achromobacter* sp. when the bacteria were grown in an aerated nitrate-containing medium. Such cells did not show any nitrite reductase activity and could be used in studies concerning the influence of oxygen on the nitrate reductase activity of the living cells, the nitrite formation in a succinate-nitrate-containing medium being used as a measure of the enzyme activity. The oxygen concentration of the liquid was determined polarographically. While nitrate reduction was maximal in nitrogen atmosphere, it decreased as the oxygen content of the atmosphere increased, and stopped completely when free oxygen was registered in the suspending medium.

The influence of oxygen on the formation and activity of the nitrate- and nitrite-reducing enzymes in denitrifying bacteria has been a matter of considerable interest to soil microbiologists. It is a well-known fact that denitrification (nitrate respiration) is maximal under anaerobic conditions. Several authors, however, found that vigorous aeration was necessary for the complete suppression of the process.

Van Olden [1] found that washed cells of *Micrococcus denitrificans* showed an appreciable ability to produce nitrogen from nitrate only if they had been grown anaerobically with nitrate. He concluded that "nitrate reductase" is an "adaptive enzyme" in the sense of Karström [2].

Sacks and Barker [3] showed that exposure of *Pseudomonas denitrificans* to oxygen during growth suppressed the formation of the enzyme systems responsible for nitrite reduction much more than those responsible for the reduction of nitrate to nitrite.

Skerman and MacRae [4] demonstrated that the production of nitrate reductase in *P. denitrificans* ceased when an adequate supply of oxygen was available and that this enzyme, once formed, was not affected by the presence of oxygen.

Skerman, Lack, and Millis [5] critically discussed the works by previous authors who had studied the influence of oxygen on the denitrifying activity

of bacteria. These works had been based on the assumption that, by continuous passage of an oxygen-containing gas mixture through a bacterial suspension, equilibrium between the concentration of oxygen in the gas and the liquid phases was obtained. Skerman *et al.*[5] stressed the fact that the suspended cells are continually depleting the solution of oxygen. Therefore, the oxygen concentration of the liquid phase should be determined directly. For this purpose a polarographic method was developed. Using this method, Skerman and MacRae [6,7] showed that, with adapted cells of *P. denitrificans*, the reduction of nitrate was completely inhibited at very low oxygen concentrations in the medium.

In the present work, the influence of oxygen on the formation and activity of nitrate reductase in a strain of *Achromobacter* sp. has been studied. This bacterium is an obligate halophilic organism which was isolated from ropy herring brine and was found to produce levan in sucrose-containing media [8]. Under aerobic conditions, the growth of the bacterium is not influenced by nitrate, while, under anaerobic conditions, the growth is proportional to the concentration of nitrate if added in suboptimal amounts. Nitrate cannot be used as a nitrogen source [8]. Thus, like *Achromobacter arcticum* [9], this bacterium may be regarded as a "pure denitrifyer" in contrast to most other denitrifying bacteria, which are also capable of assimilating nitrate nitrogen.

EXPERIMENTAL

Organism. A strain of *Achromobacter* sp. which was isolated and described by Lindeberg [8].

Media. Basal medium: Tryptone (Difco) 0.5 % (w/v); yeast extract (Difco) 0.2 %; K_2HPO_4 0.05 %; $MgSO_4.7H_2O$ 0.01 %; NaCl 8 %; tap water. Stock cultures were kept on the basal medium, supplemented with 5 % of sucrose and 2 % of agar. To obtain inocula for liquid media, the bacteria were cultivated for 48 h in 0.8 % nutrient broth (Difco), supplemented with 8 % NaCl.

For shake cultures a Griffin & Tatlock microid flask shaker was used.

The optical density of the cell suspensions (when necessary diluted) was measured by means of a Beckman B spectrophotometer at 560 mμ.

Determinations of *nitrite-nitrogen* were performed according to the Griess-Ilosvay method. The extinction of the samples at 520 mμ was measured by means of a Beckman B spectrophotometer, 15 min after addition of the reagents.

The *oxygen* concentration of the suspension medium was measured polarographically according to Krog and Johansen [10,11] with a combined silver-silver chloride-platinum electrode covered with a teflon (tetrafluoroethylene plastic) membrane, 0.06 mm (0.0025 inches) thick. A Warburg flask, modified in the following way, was used for these measurements: One of the two sidearms had been removed, leaving a side-opening into the flask. The electrode was inserted through this opening to within 1 mm from the bottom of the flask. Waterproof seal was used to close the opening and to keep the electrode in place. The electrodes used were 2.5—4 mm in diameter.

The assembly used for the measurements consisted of a 1.5 V dry element, a 25 Ω resistor, and a galvanometer. The measurements were made at 0.6 V, the current at this voltage being proportional to the oxygen concentration of the medium. A calibration curve was obtained, using gas mixtures of defined composition in equilibrium with the solution used as suspension medium. The composition of these gas mixtures was controlled by means of gas chromatography *.

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Determinations of the *nitrate reductase activity* of the cells were performed using a Warburg apparatus. The flasks contained 2 ml of a 1/15 M phosphate buffer, pH 7.0, with 8 % NaCl, 20 mM sodium succinate (or, in certain series, the corresponding amounts of other compounds as indicated below), and 30 mM $NaNO_3$. The sidearm contained 0.5 ml of a suspension of the bacteria of defined optical density in 1/15 M phosphate buffer, pH 7.0, with 8 % NaCl.

The presence or absence of nitrate reducing capacity in the cells was determined as follows: The flasks were filled with nitrogen. After addition of the bacterial suspension from the sidearm, the nitrite concentration of the medium was determined every 20th min, one flask being used for each determination.

Determinations of the *nitrite reductase activity* of the bacteria were performed in the corresponding way. Instead of $NaNO_3$, the medium in the Warburg flasks contained $NaNO_2$ in concentrations corresponding to about 4 μg of NO_2—N per ml. The flasks were filled with nitrogen, and the changes of the nitrite concentration after addition of the bacteria were followed by determinations every 20th minute.

The effect of the oxygen on the nitrate reductase activity was studied in the following way: In each experiment, two Warburg flasks were filled with nitrogen, and three flasks, including the one with the electrode, with a defined mixture of nitrogen and air. After equilibration, the oxygen concentration of the liquid in the main compartment was recorded, and the bacterial suspension was then added from the sidearm. The oxygen concentration of the liquid was read at 1 min intervals and, under the experimental conditions used, reached a constant value 2—3 min after the addition of the cells. After 60 min the reaction was stopped by the addition of sulfanilic acid, and nitrite was determined.

The nitrogen used was highly purified (Norsk Hydro; < 6 ppm O_2). The Warburg experiments were performed at 25°.

RESULTS AND DISCUSSION

Ability of the bacteria to use various organic compounds as hydrogen donors in nitrate reduction. The bacterium studied is unable to oxidize carbohydrates under aerobic conditions while intermediates of the tricarboxylic acid cycle and certain amino acids are more or less readily oxidized [8].

To find out which types of organic compounds can be used by the bacterium as hydrogen donors under anaerobic conditions, when nitrate has been added as a hydrogen acceptor, a series of experiments were performed according to the method of Verhoeven [12].

The bacteria were cultivated in stationary flasks, containing the basal medium with 30 mM sodium nitrate. After two days, when heavy growth and gas formation had occurred, the cells were washed twice in 1/15 M phosphate buffer with 8 % sodium chloride, pH 7, and resuspended in a solution of the same composition to the original volume. This suspension was used for inoculation of tubes containing a melted agar medium of 42° and consisting of 1.5 % agar, 8 % sodium chloride, 30 mM sodium nitrate, and 0.5 % of the substance to be tested. The pH of the agar medium was adjusted to 7.0. The tubes were incubated at 22°.

After 4 days, gas had been formed in the tubes with ethanol, fumaric, succinic, malic, citric and pyruvic acid. No gas occurred with glycerol, carbohydrates, lactic acid, glycine, tyrosine.

As this method is not very sensitive, lactate, glycine, and tyrosine were also tested as substrates in Warburg experiments. The substances were all oxidized by the bacteria aerobically. Under anaerobic conditions, in the presence of nitrate, the same substances were used as hydrogen donors by nitrate-

reductase-containing cells (*cf.* below), and nitrite was formed. Thus, with these bacteria, the organic substances which have been found to function as substrates in normal respiration, are also used as hydrogen donors in nitrate respiration.

Influence of growth conditions on the capacity of the bacteria to reduce nitrate to nitrite. The influence of aeration and nitrate addition on the nitrate-reducing capacity of the bacteria was studied in an experiment where the bacteria were grown in the basal medium, with and without the addition of 30 mM sodium nitrate. In each series the growth was compared in stationary conical flasks (volume 300 ml, containing 250 ml of medium) and shaken flasks (volume 750 ml, containing 50 ml of medium).

In the stationary flasks without nitrate, the growth was very poor, and sufficient cell material for the following experiments was not obtained. Bacteria from the other series were collected by means of centrifugation, washed twice in 1/15 M phosphate buffer with 8 % sodium chloride, pH 7.0, and suspended in the same solution. The nitrate- and nitrite-reducing capacity of the cells was immediately tested (Figs. 1 and 2).

Under nitrogen atmosphere, the cells from the nitrate-containing, aerated cultures reduced nitrate to nitrite at a constant rate the first 80 min of the experimental time. The nitrite formed accumulated in the medium. When tested for nitrite-reducing capacity, the same type of cells showed no activity within 80 min.

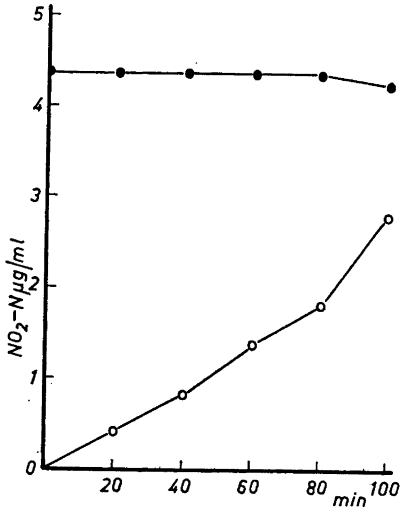

Fig. 1. Nitrate- and nitrite-reducing capacity of *Achromobacter* sp. grown in the presence of nitrate in shake cultures.
● change in nitrite concentration (nitrogen atmosphere).
○ nitrite formed from nitrate (nitrogen atmosphere).

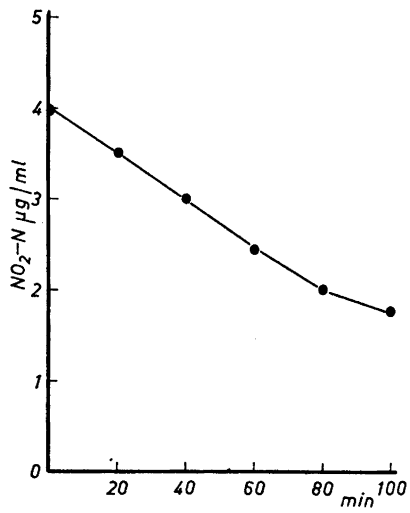

Fig. 2. Nitrite-reducing capacity of *Achromobacter* sp. grown in the presence of nitrate in stationary cultures.
● change in nitrite concentration (nitrogen atmosphere).

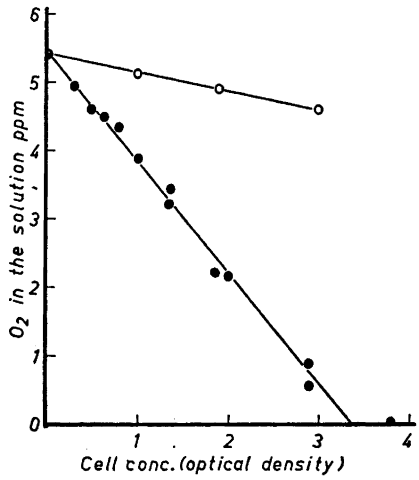

Fig. 3. The relationship between the concentrations of cells and oxygen in the liquid phase at different shaking rates.
Atmosphere: air.
○ 160 strokes per min.
● 80 strokes per min.

Cells from shaken cultures without nitrate did not show any nitrate-reducing capacity.

In the stationary nitrate-containing cultures, the bacteria had grown under intense gas formation. When the cells from these cultures were tested in the same way, however, no nitrite accumulation in the presence of nitrate could be detected. The same type of cells were tested for nitrite reducing capacity under air and under nitrogen atmosphere, respectively. In the first case, no activity could be observed, in the second case, nitrite was reduced at a constant rate. Hence, the non-occurrence of nitrite after addition of nitrate to these cells must have been due to the nitrite's being reduced at the same rate as it was formed.

Thus, when this organism has been grown in aerated cultures in the presence of nitrate, the cells are able to reduce nitrate to nitrite, while nitrite is not affected. This indicates that nitrate reductase is induced in such cultures. As long as the cultures are aerated, however, no reduction of nitrate occurs and no nitrite reductase is induced. With such cells, the nitrate-reducing capacity may be studied under varying conditions, the nitrite formation from nitrate being taken as a measure of the activity. If the measurements are performed within one hour, no nitrite reductase activity occurs.

Influence of cell concentration on the oxygen content of the suspending medium at varying shaking rates. The oxygen content of the liquid medium in the Warburg flasks was determined polarographically at various cell concentrations and at two different shaking rates, *viz.* 80 and 160 complete strokes per minute. The medium was identical with the one used in the experiments where

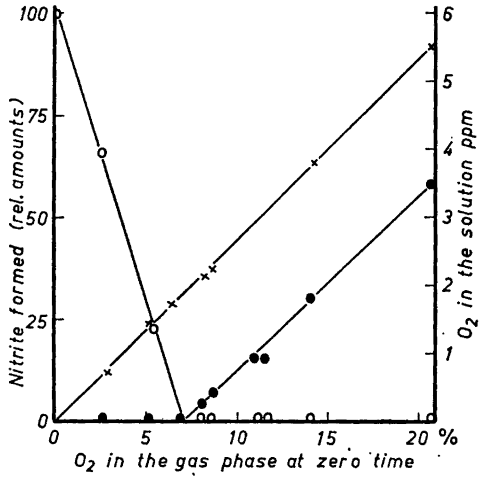

Fig. 4. Nitrate-reducing capacity of *Achromobacter* sp. at various oxygen concentrations in the liquid and gas phases.

- O nitrite formed.
- × oxygen in the cell free solution.
- ● oxygen in the solution after addition of bacteria.

nitrate reductase activity was determined, thus containing phosphate buffer, sodium chloride, succinate, and nitrate. Air was used as the gas phase. The bacteria had been grown in nitrate-containing, aerated cultures. The cell concentration varied from optical density zero to 3.8, the maximum value corresponding to about 1.6×10^9 viable counts per ml. As shown in Fig. 3, the addition of bacteria was followed by a drop in the oxygen content of the medium. The decrease was proportional to the cell concentration and was, of course, due to the difference between the rate of the oxygen uptake of the liquid and the oxygen consumption of the cells. As was to be expected, the gradient of the decrease was greatest at the lower shaking rate. Tests for nitrite were performed 60 min after the addition of the cells. In all the series giving positive oxygen values, the nitrite tests were negative.

Influence of oxygen concentration on the nitrate reductase activity of the bacteria. In a final series of experiments (Fig. 4), different concentrations of oxygen in the gas phase were used, while the cell density was kept constant (optical density 1.2, corresponding to approximately 0.5×10^9 viable counts per ml). A relatively low cell concentration was used for the purpose of keeping the oxygen consumption and the nitrite formation during the experimental time at low levels. The bacteria had been grown in nitrate-containing, aerated cultures. The composition of the atmosphere was varied from pure nitrogen, *via* different mixtures of nitrogen and air, to pure air. The lower shaking rate, 80 strokes per min, was chosen for technical reasons.

Under the experimental conditions chosen, the oxygen content of the cell-free liquid increased linearly from zero, as the oxygen pressure of the gas

phase was increased. After addition of bacterial cells, the oxygen content of the liquid was reduced by a certain amount, but was still proportional to the oxygen content of the gas phase. Nitrate reduction decreased linearly with increasing oxygen content of the gas phase, reaching the value zero near the point where oxygen was registered in the liquid by the electrode.

The results indicate that nitrate reductase-containing cells use oxygen for their respiration as long as a surplus of oxygen is present in the liquid. Only when the oxygen has been completely consumed, so that the respiratory enzymes are no longer saturated with oxygen, nitrate is used as terminal hydrogen acceptor along with oxygen. When the oxygen content of the gas phase is further decreased, the nitrate reduction increases, reaching a maximum value when oxygen is completely absent. It might be thought that, at low oxygen pressure, certain cells, preferably near to the surface of the liquid, consume the oxygen so that nitrate will be the only hydrogen acceptor available to cells more distant from the surface.

The results support the general view on the denitrification presented by Kluyver [13] and correspond with the results obtained with *Pseudomonas denitrificans* by Skerman and coworkers.

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REFERENCES

1. Van Olden, E. *Proc. Koninkl. Ned. Akad. Wetenschap.* **43** (1940) 635.
2. Karström, H. *Ergeb. Enzymforsch.* **7** (1938) 350.
3. Sacks, L. E. and Barker, H. A. *J. Bacteriol.* **58** (1949) 11.
4. Skerman, V. B. D. and MacRae, I. C. *Can. J. Microbiol.* **7** (1961) 169.
5. Skerman, V. B. D., Lack, J. and Millis, N. *Australian J. Sci. Research* **B 4** (1951) 511.
6. Skerman, V. B. D. and MacRae, I. C. *Can. J. Microbiol.* **3** (1957) 215.
7. Skerman, V. B. D. and MacRae, I. C. *Can. J. Microbiol.* **3** (1957) 505.
8. Lindeberg, G. *Proc. 2nd. Intern. Symposium on Food Microbiol.*, Cambridge 1957, p. 157.
9. Russakova, G. S. and Butkevich, V. S. *Mikrobiologiya* **10** (1941) 137.
10. Krog, J. and Johansen, K. *Rev. Sci. Instr.* **30** (1959) 108.
11. Johansen, K. and Krog, J. *Acta Physiol. Scand.* **46** (1959) 228.
12. Verhoeven, W. *Aerobic sporeforming nitrate reducing bacteria* (Diss.) Delft 1952.
13. Kluyver, A. J. *Symposium on Microbial Metabolism*, Rome 1953, p. 71.

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