EFFECT OF ANAEROBIC GROWTH ON NITRATE REDUCTION BY *STAPHYLOCOCCUS EPIDERMIDIS*[1]

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ABSTRACT

JACOBS, N. J. (American Meat Institute Foundation, Chicago, Ill.), J. JOHANTGES, AND R. H. DEIBEL. Effect of anaerobic growth on nitrate reduction by *Staphylococcus epidermidis*. J. Bacteriol. **85**:782–787. 1963.—In anaerobic cultures, a strain of *Staphylococcus epidermidis* failed to reduce nitrate, although nitrate was reduced in stationary cultures grown in the presence of air. Resting suspensions of cells grown in air reduced nitrate. Although suspensions of cells grown anaerobically did not reduce nitrate, the nitrate reductase enzyme was shown to be present. When the artificial electron carrier, benzyl viologen, was added to suspensions of cells grown anaerobically, nitrate was reduced, indicating that an electron carrier required for nitrate reduction was missing. The addition of hemin to the growth medium enabled anaerobic cultures to reduce nitrate.

During an investigation on the factors affecting nitrate reduction by some strains of staphylococci, it was observed that, although these organisms produced nitrite from nitrate when grown in air, they failed to do so during growth under anaerobic conditions. This observation was of special interest, since anaerobic conditions of growth are known to enhance nitrate reduction by organisms such as *Pseudomonas denitrificans* (Sacks and Barker, 1949; Skerman and MacRae, 1957).

The findings reported in this paper indicate that inability to reduce nitrate by a *Staphylococcus epidermidis* strain under anaerobic conditions of growth results from the organism's inability to form the complete enzymatic system required for nitrate reduction. The results also give some indication of the nature of the component(s) of this system which is formed in the presence, but not in the absence, of oxygen.

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MATERIALS AND METHODS

Organism and conditions of growth. The strain (AT2) used was a typical *S. epidermidis* (Breed, Murray, and Smith, 1957) from our culture collection. The growth medium consisted of the following: Tryptone (Difco), 1 g; yeast extract (Difco), 0.5 g; NaCl, 0.5 g; K_2HPO_4, 0.5 g; glucose, 0.2 g; KNO_3, 0.3 g; and distilled water, 100 ml. Although higher concentrations of glucose would have yielded larger cell crops, the lack of pH control during growth would have interfered seriously with subsequent nitrite determination. The inoculum was grown for 1 to 2 days at 37 C in a medium similar to above but containing 0.5% of beef extract (Difco) and lacking nitrate. A 0.1 to 0.2% inoculum level was employed.

Anaerobic conditions were obtained by placing cotton-plugged Erlenmeyer flasks three-fourths filled with medium in a desiccator, evacuating three times, and replacing the atmosphere with helium after each evacuation. Static conditions of growth were obtained, except where indicated, by placing 200 ml of medium in a 2-liter Erlenmeyer flask and incubating without agitation. The flasks or desiccators were placed in a 37 C incubator for 24 hr.

For preparation of resting cells, approximately 600 ml of medium were centrifuged at 2 to 3 C. The cells were washed in 600 ml of distilled cold water (5 to 10 C), then in 40 ml of cold water, and finally resuspended in 10 to 40 ml of cold water. The static and anaerobic suspensions were adjusted to the same content according to dry weight determinations (110 C, 12 hr).

Analyses. Nitrite was determined by the method of Nason and Evans (1955), and nitrate by the method of Landmann et al. (1960). The nitrate method gave a precision of only about 5%, and, therefore, the data presented should be interpreted accordingly. (These chemical anal-

yses were conducted after appropriate dilutions were made in cold water.) If enough cells were present after dilution of samples to interfere with the colorimetric test, they were removed by centrifugation. Relative amounts of growth were determined by measuring optical densities of equally diluted cultures at 600 mμ in a Bausch and Lomb Spectronic 20 colorimeter. Nitrate reductase was assayed by a modification of the method of Taniguchi and Itagaki (1960). Experiments with resting suspensions were conducted anaerobically in Thunberg tubes. The tubes were evacuated twice and the atmosphere was replaced with helium. The tubes were then evacuated a third time and placed in a water bath (37 C) for the incubation period.

Chemicals. Benzyl viologen was purchased from Mann Research Laboratories, Inc., New York, N.Y. Hemin crystals (Armour Laboratories, Chicago, Ill.) were dissolved in 0.01 N NaOH, sterilized by filtration, and diluted in sterile water before addition to sterile culture medium.

RESULTS

Reduction of nitrate during growth under various conditions of aeration. The results indicated that nitrite was produced only when cultures were grown under limited oxygen tensions (Table 1). From another experiment, it was concluded that nitrate was quantitatively converted to nitrite.

TABLE 1. *Reduction of nitrate by growing cultures*

Growth conditions*	Nitrite formed	Growth†	Final pH
	μmoles/ml		
Deep static	21.3	0.44	6.6
Shallow static	22.1	0.51	6.6
Shaker	0.1	1.00	8.1
Anaerobic	<0.005	0.26	6.5
Uninoculated	<0.005	—	7.4

* Deep static and anaerobic cultures were in 125-ml flasks containing 100 ml of medium. Shallow static and shaker cultures were in 2-liter flasks containing 200 ml of medium. Reciprocal shaking (180 strokes per min) was used where indicated. Chemical analyses were conducted after 24 hr of incubation by diluting samples in appropriate volume of cold (10 C) water.

† Optical density after diluting with 4 volumes of water.

TABLE 2. *Nitrate reduction by resting cells*

Tube contents*	Nitrite produced	Nitrate consumed
	μmoles	*μmoles*
Static cells	1.5	1.7
Static cells + glucose	5.9	5.5
Anaerobic cells	0.05–0.07	0.1
Anaerobic cells + glucose	<0.05	0.4

* Each Thunberg tube contained 250 μmoles of phosphate buffer (pH 7.1), 9.3 μmoles of KNO_3, 4.2 mg (dry weight) of cells grown either anaerobically or under static conditions, and water to make 3.0 ml. Where indicated, 20 μmoles of glucose were added. Cells, in the sidearm, were added after anaerobiosis had been achieved. Tubes were incubated for 5 hr at 37 C.

(Approximately 19 μmoles of nitrite were formed, while approximately 18 μmoles of nitrate disappeared.) No detectable nitrite was produced when cultures were grown in a desiccator under helium or with vigorous aeration on a shaker (Table 1). Nitrate did not disappear under these conditions. These data indicate also that the difference in nitrite production between anaerobic and static cultures was not correlated with differences in total amount of growth or in the final pH of the culture. The inhibition of nitrate reduction under vigorously aerobic conditions of growth has been observed in other microorganisms (Sacks and Barker, 1949; Skerman and MacRae, 1957), and has been attributed either to an inhibition of the formation of nitrate reductase or to competiton between the two electron acceptors, oxygen and nitrate (Pichinoty and D'Ornano, 1961).

One other coagulase-negative strain and one coagulase-positive strain of *Staphylococcus* were tested under conditions identical to those described. After 24 hr of growth under static conditions, both of these strains had produced about 30 μmoles of nitrite per ml. Under anaerobic conditions, neither strain produced more than 0.1 μmole of nitrite per ml, and no appreciable amounts of nitrate had disappeared. A strain of *Escherichia coli* was also tested, but no significant differences in the amounts of nitrite produced were observed in cultures grown under anaerobic or static conditions. From these preliminary studies, it was concluded tentatively that this phenomenon may be common to all staphylococci.

Further work is in progress to test this conclusion.

Reduction of nitrate by cell suspensions from cultures grown in the presence and absence of air. To determine whether differences in ability to reduce nitrate by growing cultures reflected a difference in the enzymatic composition of cells grown with and without air, cell suspensions prepared from cultures grown under these conditions were tested for their ability to reduce nitrate. A marked difference was observed between cells grown in air and those grown anaerobically (Table 2). Cells from static cultures quantitatively converted nitrate to nitrite. No nitrite was detected in the absence of added nitrate.

Since these experiments suggested that anaerobically grown cells were deficient in some enzymatic component required for nitrate reduction, cells were tested for the presence of nitrate reductase. The assay for this enzyme, which is based on the reduction of nitrate by the reduced electron carrier, benzyl viologen, was conducted in a manner similar to that used to assay for this enzyme in *E. coli* (Taniguchi and Itagaki, 1960). Considerable amounts of nitrite were formed from nitrate in the suspensions of anaerobically grown cells with benzyl viologen (Table 3), indicating that these cells did contain the nitrate reductase enzyme. The nitrate reductase activity of the anaerobic cells was destroyed by heating the cells at 100 C for 20 min.

TABLE 3. *Test for nitrate reductase in resting cells*

Tube contents*	Nitrite formed (*μmoles*)	
	Static cells	Anaerobic cells
Nitrate, hydrosulfite, benzyl viologen	3.8	7.5
Nitrate, hydrosulfite	2.3	0.4
Nitrate, benzyl viologen	0.2	<0.1
Nitrate	0.3	<0.1
Hydrosulfite, benzyl viologen	<0.1	<0.1

* Each Thunberg tube contained 250 μmoles of phosphate buffer (pH 7.1), 6 mg of cells grown either anaerobically or under static conditions, and water to make 3.0 ml. Where indicated, 25 μmoles of KNO_3, 2 μmoles of benzyl viologen, and a few crystals of solid $Na_2S_2O_4$ were added. Immediately after the addition of hydrosulfite to the entire contents, the tubes were made anaerobic and then incubated at 37 C. After 4 hr, the tubes were opened, shaken in air until the blue color disappeared, and then analyzed.

TABLE 4. *Effect of benzyl viologen on nitrite formation by resting cells*

Tube contents*	Nitrite formed (μmoles)	
	Static cells	Anaerobic cells
Nitrate, glucose	3.5	<0.02
Nitrate, glucose, benzyl viologen	3.5	3.1
Nitrate, benzyl viologen	1.1	0.5
Nitrate	1.3	0.2
Glucose, benzyl viologen	0.04	<0.02

* Each Thunberg tube contained 250 μmoles of phosphate buffer (pH 7.1), 6 mg of either anaerobically or statically grown cells, and water to make 3 0 ml. Where indicated, 10 μmoles of KNO_3, 2 μmoles of benzyl viologen, and 20 μmoles of glucose were added. Cells in the sidearm were added after anaerobiosis had been achieved. Tubes were incubated for 3 hr at 37 C.

These results suggested that anaerobically grown cells were incapable of reducing nitrate with physiological electron donors such as glucose, not because they lacked the nitrate reductase enzyme, but due to their deficiency in some intermediary electron carrier. This possibility was supported by the results of another experiment (Table 4). It is evident that when small amounts of benzyl viologen were added to suspensions of anaerobically grown cells, these cells were as capable of reducing nitrate as were cells grown in the presence of air.

Presence of nitrate reductase in growing cultures which had not been exposed to air. The possibility was investigated that nitrate reductase was not actually present in cells grown anaerobically, but it was synthesized when the cells came in contact with oxygen during the period required to harvest and wash them. Results obtained by adding reduced benzyl viologen to a fully grown anaerobic culture within 2 to 3 min after opening the desiccator indicated that nitrate reductase could be demonstrated even when cells had not been in contact with air for any significant time (Table 5).

Nature of the missing intermediary electron carrier. Other workers (Taniguchi, Sato, and Egami, 1956; Sadana and McElroy, 1957) have suggested that cytochromes are involved in

nitrate reduction, and it has been demonstrated that anaerobiosis inhibits the formation of cytochromes in certain microorganisms (Schaeffer, 1952; Englesberg, Levy, and Gibor, 1954). Thus, it appeared possible that the electron carrier, which this *Staphylococcus* failed to synthesize under anaerobic conditions, was a cytochrome. However, preliminary spectrophotometric examinations of difference spectra (reduced minus oxidized) of cell-free extracts have been unsuccessful in elucidating any unequivocal difference in cytochrome content of cells grown under various oxygen tensions. Extracts, prepared by sonic oscillation, of cells grown with vigorous aeration exhibited absorption maxima at 553 to 560 mμ and 595 to 605 mμ in the visible region of the spectrum. Extracts from cells grown under static conditions exhibited only a weak maximum between 553 to 560 mμ. Although no maxima could be detected in extracts from anaerobic cells, it is possible that cytochromes could have been present in concentrations below the sensitivity of the technique employed. Spectrophotometric studies with anaerobic cells were further complicated in that extracts from these cells turned a dark-gray color soon after preparation.

TABLE 5. *Test for nitrate reductase in anaerobic cultures*

Contents of growth medium[a]	Addition to medium after growth[b]	Nitrite produced (μmoles/ml)[c]
With nitrate	Benzyl viologen, hydrosulfite	0.46
With nitrate (heated)[d]	Benzyl viologen, hydrosulfite	<0.003
With nitrate	Benzyl viologen	<0.003
With nitrate	Hydrosulfite	<0.003
Without nitrate	Benzyl viologen, hydrosulfite	<0.003

[a] Medium described in Materials and Methods, except that nitrite was omitted where indicated. Cultures were grown for 24 hr under anaerobic conditions in 50-ml flasks.

[b] Where indicated, 10 mg of benzyl viologen or a few crystals of hydrosulfite were added to the cultures within 2 to 3 min after the desiccator was opened. The cultures were allowed to stand for 3 min at room temperature and were vigorously shaken to oxidize the reduced benzyl viologen. Nitrite was then determined after diluting with cold water.

[c] No detectable nitrite (<0.003 μmoles per ml) was found in any of the flasks before the addition of benzyl viologen and hydrosulfite.

[d] Culture heated (120 C, 30 min) prior to addition of reagents.

TABLE 6. *Effect of hemin upon nitrate reduction by anaerobic cultures of Staphylococcus epidermidis AT2*

Medium*	Hemin concn	Nitrite produced	Growth†	Final pH
	μM	μmoles/ml		
With nitrate	0	<0.003	0.36	6.3
With nitrate	0.1	17.0	0.49	6.5
With nitrate	0.01	0.003	0.41	6.2
With nitrate	0.001	<0.003	0.44	6.6
With nitrate	0.1	<0.003	Uninoculated	7.4
Without nitrate	0.1	<0.003	0.26	6.2

* Medium was the same as described in Materials and Methods, except that nitrate was omitted or hemin added where indicated. Flasks (50 ml) containing 40 ml of medium were incubated anaerobically for 24 hr.

† Optical density after diluting with 4 volumes of water.

However, it was observed that when hemin was added to cultures grown anaerobically, nitrate was reduced (Table 6). This result supports the hypothesis that the electron carrier missing from the anaerobic cells is a cytochrome.

Effect of extended incubation on reduction of nitrate in anaerobically grown cultures. When cultures were grown anaerobically and tested after 3 days of incubation, the tests for nitrite were negative consistently and no nitrate had disappeared. However, after 5 days of anaerobic incubation, some nitrite appeared in the growth medium (as high as 8.6 μmoles per ml). The oxygen indicator (McClung and Lindberg, 1957) placed in the desiccator at the time of incubation remained in the colorless form throughout this 5-day period. The significance of this delayed reduction of nitrate has not been investigated further.

DISCUSSION

The results of this investigation indicate that, although nitrate reductase is present in anaerobically grown cells of *S. epidermidis* AT2,

this enzyme remains nonfunctional, because the organism does not possess the electron carrier(s) required for nitrate reduction when grown in the absence of air. Although the missing electron carrier(s) has not been identified, the fact that hemin allows anaerobic cultures to reduce nitrate suggests that the carrier is a heme-containing compound. This observation is consistent with recent findings that cytochromes can be involved in nitrate reduction (Taniguchi et al., 1956; Sadana and McElroy, 1957). However, we were unable to obtain unequivocal evidence regarding the cytochrome content of anaerobic cells.

The suggestion that this organism has an altered cytochrome content when grown anaerobically would agree with the findings of Ephrussi and Slonimski (1950) and Chin (1950), employing yeasts, and with those of Schaeffer (1952) and Englesberg et al. (1954), who worked with *Bacillus cereus* and *Pasteurella pestis*, respectively. However, as pointed out by Smith (1961), it is impossible to make any generalization regarding the relationship between oxygen tension during growth and cytochrome content of bacteria. Schaeffer (1952) observed that growth under various oxygen tensions had little effect on the formation of certain cytochromes in *E. coli*.

It is of interest to compare the results of our experiments, in which hemin restored nitrate reduction in anaerobic cultures, with the results of Lascelles (1956), who studied nitrate reduction in a heme-requiring *Staphylococcus* mutant. Under certain conditions, this mutant was able to grow in the absence of added hemin. Lascelles (1956) found that resting suspensions prepared from cells grown without hemin reduced nitrate only when they were supplemented with hemin. The similarity between these findings and those reported in this paper leads to the suggestion that when the *Staphylococcus* studied in this investigation is grown anaerobically, it resembles the heme-requiring *Staphylococcus* mutant in that it is unable to synthesize heme.

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