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Physiology and Interaction of Nitrate and Nitrite Reduction in *Staphylococcus carnosus*

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Staphylococcus carnosus **reduces nitrate to ammonia in two steps. (i) Nitrate was taken up and reduced to nitrite, and nitrite was subsequently excreted. (ii) After depletion of nitrate, the accumulated nitrite was imported and reduced to ammonia, which again accumulated in the medium. The localization, energy gain, and induction of the nitrate and nitrite reductases in *S. carnosus* were characterized. Nitrate reductase seems to be a membrane-bound enzyme involved in respiratory energy conservation, whereas nitrite reductase seems to be a cytosolic enzyme involved in NADH reoxidation. Syntheses of both enzymes are inhibited by oxygen and induced to greater or lesser degrees by nitrate or nitrite, respectively. In whole cells, nitrite reduction is inhibited by nitrate and also by high concentrations of nitrite (≥10 mM). Nitrite did not influence nitrate reduction. Two possible mechanisms for the inhibition of nitrite reduction by nitrate that are not mutually exclusive are discussed. (i) Competition for NADH nitrate reductase is expected to oxidize the bulk of the NADH because of its higher specific activity. (ii) The high rate of nitrate reduction could lead to an internal accumulation of nitrite, possibly the result of a less efficient nitrite reduction or export. So far, we have no evidence for the presence of other dissimilatory or assimilatory nitrate or nitrite reductases in *S. carnosus*.**

Nitrate can be used by many bacteria as a source of assimilable nitrogen or as a terminal electron acceptor under anoxic conditions (nitrate respiration). In the assimilatory process, which may occur aerobically or anaerobically, nitrate is ultimately reduced to ammonia (NH_3) and subsequently incorporated into biomass. This pathway is performed by many bacteria, fungi, and plants. In respiration, nitrate is used as an alternative electron acceptor when oxygen is not available. The enzymes for this pathway are found only in bacteria. Two main forms have been described so far, and in both, nitrate reduction is coupled to the generation of a proton motive force (3, 17), which is directly utilized as a source of energy or transformed into ATP by a membrane-associated ATPase. In one form, reported for *Escherichia coli* and other members of the family *Enterobacteriaceae*, the organisms reduce nitrate to nitrite, which is then excreted or further reduced to NH_3 by a dissimilatory (e.g., in *E. coli* [25]) or assimilatory nitrite reductase. The other form of nitrate respiration, denitrification, is defined as the reduction of nitrate to the gaseous oxides nitric oxide and nitrous oxide, which then may be further reduced to nitrogen (18, 27). This form, generally found in obligately respiring bacteria such as pseudomonads, is crucial to nitrogen cycling in nature (10).

Certain bacteria, such as strict aerobes, are able to perform assimilatory nitrate reduction but cannot use nitrate as the terminal electron acceptor. Other bacteria, such as *E. coli* and *Salmonella typhimurium*, do not assimilate nitrogen through nitrate reduction during aerobic growth but grow anaerobically with nitrate as the terminal electron acceptor. In contrast, bacteria such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* carry out both assimilatory and respiratory (dissimilatory) nitrate reduction.

Staphylococcus carnosus is traditionally used as a starter culture in the production of raw fermented sausages and dry-cured ham. Its main function in the curing process is to reduce nitrate to nitrite, which is necessary for the development of color and flavor. However, for toxicological reasons, the nitrite concentrations should not exceed a certain technologically interesting level. Therefore, nitrite reduction catalyzed by microorganisms is desirable. Very little information is available about the nitrate- and nitrite-reducing activity of staphylococci. Burke and Lascelles (5) partially purified a cytosolic form of the respiratory nitrate reductase of *Staphylococcus aureus*. Using a specific antiserum, they suggested that identical forms of the enzyme are present in the cytosolic and membrane fractions and are both active with artificial electron donors (4). Nitrite reduction in strains of *S. carnosus* and *Staphylococcus piscifermentans* was described by Hartmann et al. (14) and attributed to a dissimilatory nitrite reductase.

The present work focused on the physiological characterization of the nitrate- and nitrite-reducing systems of *S. carnosus*.

MATERIALS AND METHODS

Culture conditions. Cells of *S. carnosus* TM300 were grown at 37°C in modified basic medium (BM) (10 g of casein hydrolysate per liter, 5 g of yeast extract per liter, 1 g of glucose per liter, 13 g of K_2HPO_4 per liter, and 2 g of NaCl per liter; pH 7.4; 12). Aerobic cultures were incubated on a rotary shaker at 130 rpm. Anaerobic cultures were incubated in screw-cap bottles with stirring (100 rpm). The medium was supplemented with Oxyrase (20 ml/liter of medium; Oxyrase Inc. Mansfield, Ohio), an enzyme system that creates anoxic conditions in broth media at a pH range between 6.8 and 9.0. Additionally, the medium was overlaid with light mineral oil (Sigma).

Preparation of bacterial extracts. Cells were harvested in the mid-exponential growth phase, washed twice in potassium phosphate buffer (100 mM, pH 7.2), and then disrupted mechanically or by the lysostaphin-lysis method. In this method, protoplasts were prepared by (partial) degradation of the cell wall peptidoglycan by lysostaphin (280 U/liter of culture) and were subsequently lysed by a mild hyperosmotic shock. The membranes were separated from residual whole cells and cell debris by differential centrifugation and were isolated by the method of Otto et al. (24); the soluble fraction was discarded. Mechanical disruption was performed with glass beads essentially by the method of Sizemore et al. (30), except that vortexing with glass beads was performed with six cycles, with one cycle consisting of 30 s of vortexing and 5 min of rest on ice. After removal of unbroken cells by centrifugation (2,000 × *g*, 10 min, 4°C), the membrane and cytosolic fractions were separated by centrifugation (20,000 × *g*, 30 min, 4°C).

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Nitrate reductase activity. Nitrate reductase activity was assayed by the method of Lowe and Evans (20) with modifications.

(i) Intact cells. Cells were harvested in the mid-exponential growth phase and washed twice with potassium phosphate buffer (100 mM, pH 7.2). The assay mixture (total volume, 3 ml) was composed of potassium phosphate buffer (90 mM, pH 7.2), containing benzyl viologen (1 mM) as an artificial electron donor, and the reducing agent dithionite (4.7 mM). The addition of dithionite in excess facilitated handling without an anaerobic chamber. Cells were added to a final optical density at 578 nm (OD_{578}) of 0.4, and the mixture was stirred at 37°C. Subsequently, the reaction was started by the addition of sodium nitrate (10 mM). Samples were taken at various time intervals up to 2 min, and nitrate reduction was stopped by vigorously vortexing the mixture to oxidize all dithionite and benzyl viologen. After removal of the cells by centrifugation, the nitrite concentrations in the samples were measured as described below.

(ii) Bacterial extracts. The nitrate reductase assay was performed as described for intact cells, except that nitrate reduction was started by adding the bacterial extract.

Nitrite reductase activity. (i) Intact cells. Cells were harvested in the mid-exponential growth phase, washed (twice in 100 mM potassium phosphate buffer, pH 7.2) and diluted to a defined OD_{578} in 40 ml of potassium phosphate buffer (100 mM, pH 7.2) supplemented with 25 mM glucose as an electron donor. Oxyrase and mineral oil were added to generate anoxic conditions. The reaction mixture was incubated at 37°C in a screw-cap bottle with stirring (100 rpm). After 5 min of preenergization at 37°C, nitrite reduction was started by adding nitrite. At given time intervals, samples were taken, and the cells were removed by centrifugation. The concentrations of nitrite and ammonia in the supernatants were measured as described below. When nitrite reductase activities were measured under oxic conditions, Oxyrase and mineral oil were omitted from the reaction mixture, and the samples were incubated with stirring at a high rate (250 to 300 rpm).

(ii) Cytosolic extract. Nitrite reductase activity in cytosolic extract was determined essentially by the method of Coleman et al. (9). The assay was carried out in open cuvettes each containing 1 ml of 0.25 mM NADH, 1 mM NAD^+, and either 1 mM $NaNO_2$ or no nitrite in potassium phosphate buffer (100 mM, pH 7.2). The reaction was initiated by the addition of cytosolic extract. NADH-dependent nitrite reductase activity was determined from the difference between the rates of NADH oxidation in the presence and absence of nitrite.

Nitrate and nitrite reduction in resting cells. The reduction of nitrate to nitrite and subsequently to ammonia was measured in resting cells that were cultivated anaerobically in the presence of nitrite (1 mM), harvested in the mid-exponential growth phase, and washed twice in potassium phosphate buffer (100 mM, pH 7.2). The experiment was performed essentially as described for the determination of nitrite reductase activity in intact cells, except that nitrate (600 μM) instead of nitrite was added initially.

LDH activity. NAD-dependent lactate dehydrogenase (LDH) activity in the cytosolic and membrane fractions was measured at 37°C by the method of Melville et al. (22) with 0.4 mM NADH.

Analytical determinations. The nitrite concentration was determined colorimetrically by the method of Nicholas and Nason (23) as modified by Showe and DeMoss (29). The concentration of ammonia was determined, using the NADH-dependent conversion of 2-oxoglutarate to L-glutamate catalyzed by glutamate dehydrogenase (Boehringer, Mannheim, Federal Republic of Germany). The protein concentration was measured by the method of Lowry et al. (21) in the presence of sodium dodecyl sulfate (11) with bovine serum albumin as the standard.

Materials. All chemicals were reagent grade and were obtained from commercial sources.

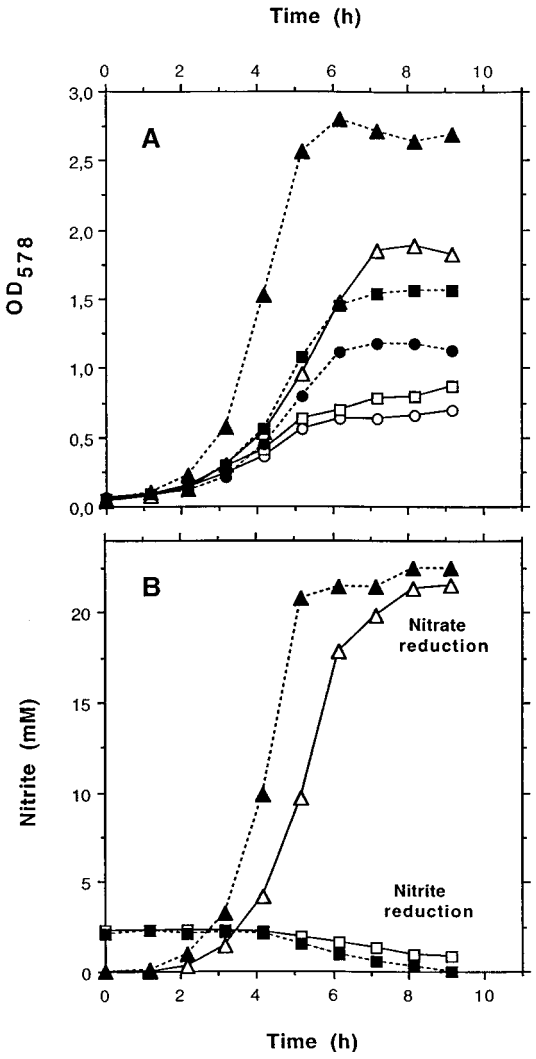

FIG. 1. Anaerobic growth of *S. carnosus* in modified basic medium with (closed symbols) or without glucose (open symbols). (A) The OD_{578} was determined without any further additions (circles) or with 25 mM nitrate (triangles) or 2 mM nitrite (squares) added to the growth medium prior to inoculation. (B) During growth, nitrate reduction (monitored as nitrite increase [triangles]) or nitrite reduction (monitored as nitrite decrease [squares]) were determined. Nitrite concentrations were determined as described in Materials and Methods.

RESULTS

Nitrate reduction. (i) Nitrate reduction during growth. During growth in the presence of nitrate, cells of *S. carnosus* reduced nitrate to nitrite, which subsequently accumulated in the growth medium. The rate of nitrite accumulation was 8- to 10-fold higher under anoxic conditions than under oxic conditions (data not shown). The presence of nitrate under anoxic conditions resulted in a higher growth yield: the final OD_{578} of 1.1 to 1.2 under anoxic conditions increased up to 2.6 to 2.7 when nitrate (25 mM) was present (Fig. 1A). When glucose was omitted from the growth medium, nitrate reduction was delayed but resulted in a similar growth stimulation (Fig. 1B).

(ii) Induction of nitrate reductase activity. For induction studies, cells were cultured in the presence or absence of oxygen and with or without nitrate (20 mM), harvested in the mid-exponential growth phase, washed twice, and used for the benzyl viologen-nitrate reductase assay. The enzyme activity of aerobically grown cells was very low and only slightly higher when nitrate had been added (Table 1). A fourfold increase in enzyme activity was observed with anaerobically grown cells. The highest activities were measured for cells grown anaerobically with nitrate (and nitrite, resulting from nitrate reduction). Upon the addition of nitrite (2 mM) during anaerobic cultivation, nitrate reductase activity was higher than without any additions but was far below the level reached with nitrate (Table 1). Nitrate reductase activities were not affected by the presence of ammonia (18 mM) in the buffer.

(iii) Location of the nitrate reductase. Cells (cultured anaerobically in the presence of nitrate) were disrupted mechanically with glass beads, and the cell fractions were used for the benzyl viologen-nitrate reductase assay. The activity in the membrane fraction (870 nmol · min^{-1} · mg of $protein^{-1}$) was only slightly higher than in the soluble fraction (600 nmol · min^{-1} · mg of $protein^{-1}$). However, disruption with glass beads might release proteins into the soluble fraction that are attached only to the membrane. Therefore, membrane vesicles

TABLE 1. Nitrate and nitrite reductase activities in resting cells[a]

Growth	Activity (nmol · min^{-1} · mg of protein^{-1})					
	Benzyl viologen-nitrate reductase			Nitrite reductase		
	–	+ NO_3^- (20 mM)	+ NO_2^- (2 mM)	–	+ NO_3^- (20 mM)	+ NO_2^- (2 mM)
Aerobic[b]	148	278	113	0	0	0
Anaerobic	617	3,220	1,522	15	79	65

[a] Cells were cultivated aerobically or anaerobically in the presence (+) or absence (−) of NO_3^- or NO_2^-. After the cells were harvested, they were washed twice in potassium phosphate buffer (100 mM, pH 7.2) and resuspended in the same buffer.

[b] For determination of the benzyl viologen-nitrate reductase activities of the aerobically grown cells, the cell densities in the assay were increased to a final OD_{578} of 1.0.

were isolated by the less destructive lysostaphin-lysis method. These membrane vesicles exhibited a very high nitrate reductase activity (3,600 nmol · min^{-1} · mg of protein^{-1}). The absence of cytosolic proteins in the preparation was proved in the presence of Triton X-100 (0.1%) with NAD-dependent LDH as the marker enzyme. At this concentration, the detergent had no influence on LDH activity in the soluble fraction (data not shown).

Nitrite reduction. (i) Nitrite reduction during growth. Whereas no nitrite reduction was detected during aerobic growth (data not shown), nitrite (2 mM) was entirely reduced during anaerobic growth. As observed with nitrate, a growth stimulation was observed when nitrite was present during anaerobic growth (Fig. 1A). Nitrite reduction was delayed when no glucose was present in the growth medium (Fig. 1B). The results indicate that nitrite can be used as a terminal electron acceptor under anoxic conditions. Despite increasing concentrations of nitrite in the growth medium (1 to 15 mM), the cells did not reduce more than 3 mM during 22 h of anaerobic cultivation. The amount of nitrite reduced and the cell yield actually decreased slightly with higher nitrite concentrations (data not shown).

(ii) Induction and end product of nitrite reduction in resting cells. Resting cells of *S. carnosus* were incubated anaerobically in nitrite-containing buffer with glucose as an electron donor. Nitrite reduction and ammonia production were monitored. As expected, no nitrite reduction was observed with aerobically grown cells, regardless of the presence of nitrite during growth (Table 1). Anaerobically grown cells reduced nitrite and converted it entirely into ammonia. After all nitrite was reduced, the ammonia concentration remained constant (data not shown). Nitrite reduction was enhanced 4.5- and 5.5-fold when cells were grown in the presence of nitrite (2 mM) and nitrate (20 mM), respectively (Table 1). The rates of nitrite reduction were not affected by the presence of ammonia (18 mM) in the buffer. However, when induced cells (grown anaerobically with nitrite) were incubated aerobically, the nitrite reductase activity was strongly inhibited (2 nmol · min^{-1} · mg of protein^{-1}).

(iii) Nitrite reductase activity in cytosolic extract. Anaerobically grown cells were either disrupted with glass beads or by the lysostaphin-lysis method, and the cell fractions were tested for nitrite-reducing activity in a photometric assay. In the presence of 1 mM nitrite, the rate of NADH oxidation by the *S. carnosus* cytosolic extract but not by the membrane preparations was clearly enhanced. This nitrite-specific cytosolic activity (350 nmol · min^{-1} · mg of protein^{-1}) was increased (504 nmol · min^{-1} · mg of protein^{-1}) when the cells had been grown anaerobically with 1 mM nitrite. Nitrite reductase activity in the cytosolic extract was completely abolished upon the addition of potassium cyanide (1 mM). Inhibition by cyanide has also been described for the NADH-dependent nitrite reductase of *E. coli* (9).

Interaction of nitrate and nitrite reduction. (i) Inhibition of nitrite reduction by nitrate. To see whether nitrate and nitrite reduction occur in parallel, the time course of nitrite and ammonia formation after the addition of nitrate was determined in resting cells that were incubated in glucose-containing buffer. In the beginning, only nitrite was formed, and no ammonia was detected (data not shown). Ammonia production started after all the nitrate was reduced. Nitrite was subsequently converted entirely into ammonia. These results indicated that nitrate inhibited nitrite reduction and were confirmed in another experiment in which anaerobically grown resting cells were incubated in nitrite- and glucose-containing buffer. After nitrite reduction had started, nitrate was added (Fig. 2). Nitrate reduction started immediately and was monitored by a rapid increase of the nitrite concentration in the buffer. Ammonia production ceased after nitrate was added and restarted only after nitrate was completely reduced (Fig. 2).

(ii) Effect of increasing concentrations of nitrate, nitrite, or ammonia on cytosolic nitrite reductase activity. To investigate nitrate inhibition of nitrite reduction, cytosolic nitrite reductase activity (nitrite concentration, 1 mM) was determined in the presence of increasing concentrations of nitrate (1, 5, 10, 20, 30, 40, and 50 mM). Surprisingly, nitrite reductase activity was unaffected by nitrate (data not shown). One could now argue that NADH can also be used as an electron donor for nitrate reduction, since benzyl viologen-nitrate reductase activity was detectable in the cytosolic fraction. However, the basic rate of NADH oxidation without nitrite remained the same whether or not nitrate was present (at concentrations of 1, 10, 30, and 50 mM). Hence, NADH is not an in vitro electron donor for nitrate reduction. This conclusion is in accordance with the results of a study on the *E. coli* nitrate reductase

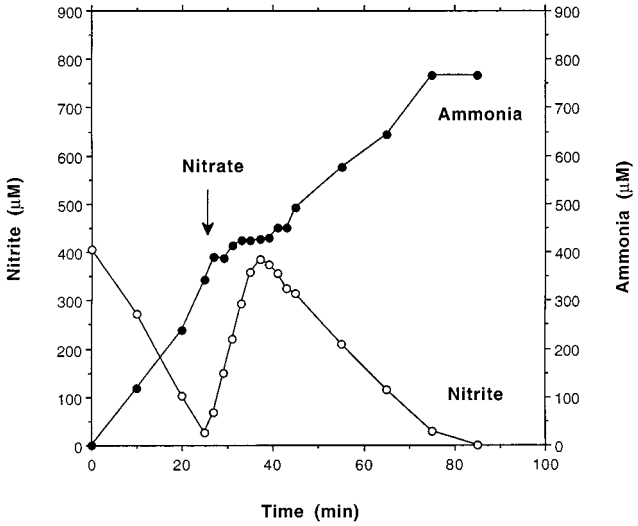

FIG. 2. Effect of nitrate on nitrite reduction in resting cells of *S. carnosus*. Cells were grown anaerobically with 1 mM nitrite and harvested in the late exponential growth phase. After the cells were washed (twice in potassium phosphate buffer, pH 7.2), they were resuspended to an OD_{578} of 2.0 in 50 ml of the same buffer supplemented with 25 mM glucose, Oxyrase, and mineral oil. Nitrite was added at zero time, and the production of nitrite and ammonia during anaerobic incubation at 37°C was monitored. After 26 min, nitrate (400 μM) was added as indicated by the arrow and incubation was continued.

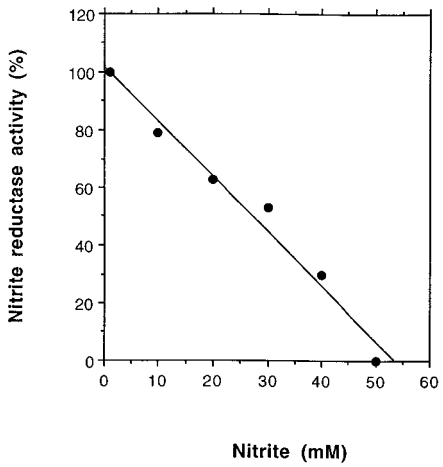

FIG. 3. Effect of increasing concentrations of nitrite on cytosolic NADH-dependent nitrite reductase activity. The cytosolic fraction, obtained after glass bead disruption of anaerobically grown cells of *S. carnosus*, was tested for NADH-dependent nitrite-reducing activity with concentrations of nitrite between 1 and 50 mM. The activity determined with 1 mM nitrite was set to be 100%. The final protein concentration in the assay was 0.176 mg/ml.

NRA (35). These results show that nitrate inhibition of nitrite reduction in resting cells is not due to a direct effect of nitrate on nitrite reductase. Since nitrate reductase activity is very high in cells of *S. carnosus*, we speculated that not nitrate but nitrite might accumulate inside the cells, causing inhibition of nitrite reductase. Indeed, when cytosolic nitrite reductase activity was determined with increasing nitrite concentrations (1, 5, 10, 20, 30, 40, and 50 mM), a slight inhibition was already observed with 10 mM of nitrite. With 50 mM nitrite, the nitrite reductase activity was completely abolished (Fig. 3). An inhibition by the end product ammonia was excluded, since the enzyme activity remained constant regardless of the presence of ammonia at various concentrations (18, 36, and 54 mM). These results demonstrate that the *S. carnosus* nitrite reductase activity is inhibited by its substrate.

(iii) Effect of Triton X-100 on nitrate and nitrite reduction in whole cells. The formation of nitrite and ammonia from nitrate was measured in resting cells (cultivated anaerobically in the presence of 1 mM nitrite) as described in the inhibition experiment, but with Triton X-100 present at a final concentration of 0.1%. In the presence of the detergent, nitrite accumulation in the medium was much slower (five- to sixfold) and a constant rate of nitrite reduction to ammonia was already detected at the beginning (data not shown). Considering that part of the nitrite resulting from nitrate reduction was immediately reduced to ammonia, the rate of nitrate reduction was still only one-third of that observed without detergent. Since Triton X-100 does not affect benzyl viologen-nitrate reductase activity in whole cells (data not shown), we speculate that it might influence nitrate transport. These results suggest that nitrite reduction in the presence of nitrate is possible under conditions in which the rate of nitrate reduction is decreased.

DISCUSSION

Under anoxic conditions, cells of *S. carnosus* rapidly reduce nitrate to nitrite, which accumulates in the external medium. Excreted nitrite was taken up only after nitrate depletion and was then further reduced to ammonia. However, nitrite reduction occurred only when the initial nitrate concentration was relatively low (below 10 mM [data not shown]). With higher concentrations, nitrite remained accumulated in the medium. The enzyme systems involved in nitrate and nitrite reduction in *S. carnosus* and their interactions were studied and compared with dissimilatory enzymes in this study.

(i) The syntheses of both enzymes were controlled by anaerobiosis and nitrate and nitrite. With nitrate, induction of nitrate reductase and of nitrite reductase was higher than with nitrite alone. Since the cells reduce nitrate, the newly produced nitrite might also contribute to the observed induction. In addition to the control of enzyme synthesis, molecular oxygen interfered with the enzymes already present in the bacteria. Both regulations ensure the most efficient use of the available terminal electron acceptors. Similar regulations have been studied intensively for other dissimilatory enzymes, such as nitrate reductase, NRA, and NADH-dependent nitrite reductase of *E. coli* (15, 32, 36).

(ii) Both nitrate and nitrite reduction resulted in higher growth yields under anoxic conditions. Dissimilatory nitrate reduction is usually coupled to the generation of a proton motive force. NADH-dependent nitrite reduction in *E. coli* is suggested to yield energy by regenerating NADH, thus allowing the formation of acetate and ATP from acetyl coenzyme A (7, 31). We assume similar mechanisms for the *S. carnosus* systems. Considering that nitrate reduction in *S. carnosus* is much faster than nitrite reduction, the energy yield per time is higher with nitrate, although the molar energy yield from nitrate or nitrite might be similar. The delay in nitrate and nitrite reduction in the absence of glucose may be due to lower intracellular levels of reduced substrates (such as NADH). These substrates can be formed only in pathways that are energetically more expendable than glycolysis.

(iii) Neither nitrate nor nitrite reductase activity was inhibited by ammonia. Inhibition by ammonia is a typical feature of assimilatory enzymes (34, 38). Whether both reductases are repressed by ammonia (or amino acids) in the growth medium cannot be tested since *S. carnosus* is only able to grow in medium supplemented with several amino acids or protein hydrolysates (unpublished results).

(iv) For nitrate and nitrite reductase in *S. carnosus*, we suggested a location in the membrane fraction and the cytosolic fraction, respectively. This is in close agreement with the location reported for other dissimilatory nitrate reductases and the NADH-dependent nitrite reductase of *E. coli* (9, 16, 31). The partial release of the benzyl viologen-nitrate reductase activity into the cytoplasm upon mechanical treatment of the cells suggests that the subunit(s) of the *S. carnosus* nitrate reductase that is catalytically active with benzyl viologen is attached to but not integrated in the membrane. This is consistent with the proposed structure of the dissimilatory nitrate reductase of other organisms (1, 2, 33).

So far, we have no evidence for the presence of other dissimilatory or assimilatory nitrate or nitrite reductases in *S. carnosus*. Previously, an *S. carnosus* Tn*917* insertion mutant was isolated that is devoid of benzyl viologen-nitrate reductase activity and does not accumulate nitrite. Preliminary sequence data indicate a defect in the nitrate reductase structural genes (26). Another Tn*917* insertion mutant was unable to reduce nitrite and had no NADH-dependent nitrite reductase activity (unpublished results). On the basis of these results, we assume that only the nitrate and nitrite reductases described here are expressed in *S. carnosus*.

Little is known about interference between nitrate and nitrite reduction. For *E. coli*, it is still controversial whether high concentrations of nitrate suppress the synthesis of nitrite reductase (6, 8, 37) or whether nitrite and nitrate (10 and 100 mM) are equally effective inducers of the enzyme (13). In vivo

inhibition of nitrite reduction by nitrate has been reported for *Pseudomonas stutzeri* (19) and *S. pisciferm entans* and *S. carnosus* (14). However, nothing is yet known about the mechanisms of nitrate inhibition. Our results confirm that in cells of *S. carnosus*, nitrite reduction was blocked until nitrate disappeared. The inhibition occurs immediately, thus genetic mechanisms are rather unlikely. On the other hand, nitrate did not inhibit nitrite reductase in the cytosolic extract, which indicates that the inhibition occurs by an indirect mechanism. Nitrate reduction is 10-fold faster than nitrite reduction in resting cells with glucose as an electron donor. Considering that NADH can be an in vivo electron donor for both reductases, nitrate reductase most likely oxidizes the bulk of the NADH because of its higher specific activity. In addition, the high rate of nitrate reduction could lead to an internal accumulation of nitrite, possibly the result of a less efficient nitrite reduction or export. The theoretical nitrite production in the absence of nitrite reduction and export was estimated to 3.25 mM nitrite produced in the cytosol per second. This calculation is based on the measured rate of nitrate reduction with glucose as the electron donor (565 nmol of nitrite formed $\cdot$ min^{-1} $\cdot$ mg of $protein^{-1}$) and an estimated internal volume of 2.9 μl/mg of protein (determined for *Lactococcus lactis* [28]). Reduction of nitrate would thus inhibit nitrite reductase after 3 s (20% inhibition at 10 mM [Fig. 3]). Interestingly, nitrite (up to 50 mM) did not inhibit nitrate reductase activity (data not shown). Both mechanisms of inhibition discussed are based on the high rate of nitrate reduction. When this rate was decreased by addition of Triton X-100, the inhibition disappeared and nitrite was reduced. More-detailed experiments concerning the discussed mechanisms or other interactions of nitrate and nitrite reduction will be performed in the future.

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