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Periplasmic and membrane-bound respiratory nitrate reductases in *Thiosphaera pantotropha*

The periplasmic enzyme catalyzes the first step in aerobic denitrification

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The unusual ability of *Thiosphaera pantotropha* to catalyze respiratory nitrate reduction under aerobic conditions is shown to correlate with the activity of a periplasmic nitrate reductase that is expressed under both aerobic and anaerobic growth conditions. The organism also synthesizes, but only under anaerobic conditions, a membrane-bound nitrate reductase which resembles the corresponding enzyme in *Paracoccus denitrificans* in respect of both catalytic properties and inhibition of activity in intact cells in the presence of oxygen.

Nitrate reductase; Periplasm; Nitrate transport; Oxygen control; Denitrification; *Thiosphaera pantotropha*

1. INTRODUCTION

The electron transport chains of bacteria from certain genera can utilize nitrate as a terminal electron acceptor. For some of these organisms a membrane-bound nitrate reductase has been identified, as exemplified, by *Escherichia coli* [1] and *Paracoccus denitrificans* [2]. This type of enzyme is known to comprise 3 polypeptide chains: one, an integral membrane protein, contains *b*-type haem, whilst the other two subunits, one of which contains the active site, are located at the cytoplasmic surface of the plasma membrane. The enzyme can accept electrons, via ubiquinone, from NADH and succinate dehydrogenases. It is inhibited by low concentrations of azide and utilizes chlorate as an alternative electron acceptor. The activity of this type of nitrate reductase in intact cells is inhibited in the presence of oxygen. Observations with the denitrifying organism *P. denitrificans* indicated that this inhibition was exerted indirectly via the redox state of a cellular component controlling the movement of nitrate across the cytoplasmic membrane to the active site of its reductase [3,4]. A similar mechanism for control by oxygen has subsequently been suggested for several other bacterial genera [5–7].

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Abbreviations: MV^+, reduced methylviologen; BV^+, reduced benzylviologen

The recently described organism *Thiosphaera pantotropha* is reported to reduce nitrate in the presence of oxygen as a first step of an aerobic denitrification process [8]. Aerobic respiratory reduction of nitrate by bacteria is difficult to reconcile with the general observation that such reduction is inhibited under aerobic conditions [1,3,5–9]. The present paper explains the molecular basis for aerobic respiratory nitrate reduction in *T. pantotropha*.

2. MATERIALS AND METHODS

T. pantotropha strain LMD82.5 was obtained from Dr L. Robertson (Laboratory of Microbiology, Delft University of Technology, Delft, The Netherlands). The organism was grown at 37°C on a minimal medium previously described [3], in which succinate, ammonium and nitrate are initially present as carbon source, nitrogen source and terminal electron acceptor, respectively. Anaerobic growth was in 50 ml Falcon tubes and aerobic growth was in either 1 litre or 2.5 litre conical flasks containing 100 ml or 250 ml of medium, respectively, and shaken at 200 rpm. Bacteria were harvested when the cultures reached optical densities, measured at 650 nm in 1 cm light-path cuvettes, of 0.8–1.0 for anaerobically and 0.3–0.4 for aerobically grown cells. For experiments with intact cells, bacteria were harvested by centrifugation, washed once in nitrate-free growth medium and suspended in a minimal volume of the same nitrate-free medium. Nitrate reduction by intact cells was measured using a nitrate electrode [9]. About 1 mg cell of protein were suspended in 30 ml of nitrate-free growth medium in a sealed chamber that was continually sparged with argon or compressed air for anaerobic or aerobic experiments. The chamber was maintained at a temperature of 30°C, the reactions were started by the addition of KNO_3 to give a final concentration of 100 μM. All experiments were completed within 1 h. For aerobic experiments the oxygen concentration was measured by periodically withdrawing samples, using a gastight Hamilton syringe, and determining the oxygen concentration with an oxygen electrode. In this way it was determined that the ox-

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ygen concentration was maintained at 200 ± 25 μM O_2 for the entire time-course of an aerobic experiment. The same procedure established that there was no detectable O_2 in cell suspensions in experiments carried out under argon.

Bacteria were fractionated essentially as described for type II spheroplasts in [3]. Assays using either reduced benzylviologen (BV^+), reduced methylviologen (MV^+) or NADH were performed essentially as described previously [2], by adding the electron acceptor (either potassium nitrate, 1 mM, potassium chlorate, 1 mM or dimethylsulphoxide, 1 mM) to start the reaction.

3. RESULTS AND DISCUSSION

3.1. *The localization of nitrate reductase activity in* T. pantotropha

T. pantotropha cells, grown under either anaerobic or aerobic conditions, were fractionated into cytoplasmic, membrane and periplasmic fractions. In anaerobically grown cells the BV^+-dependent nitrate reductase activity was associated predominantly, but not entirely, with the membrane fraction (Table I). By contrast, in aerobically grown cells the BV^+-dependent nitrate reductase activity was almost exclusively associated with the periplasmic fraction (Table I). The total activity of nitrate reductase was considerably lower in aerobically grown cells, but the periplasmic activity was somewhat higher. The catalytic properties of the two nitrate reductase activities from anaerobically grown cells were distinct (Table II) in the following respects: (i) the membrane, but not the periplasmic, nitrate reductase activity could be coupled to NADH dehydrogenase activity; (ii) the membrane-associated NADH- and BV^+-nitrate reductase activities were both highly sensitive to azide, but the periplasmic BV^+-nitrate reductase activity was insensitive to azide; (iii) the membrane fragments could also catalyze NADH- and BV^+-dependent chlorate reductase activity but chlorate reductase activity could not be detected in the periplasmic fraction. In principle, the chlorate reductase activity of membrane fragments could have been due to the presence of a membrane-bound dimethylsulphoxide reductase which, in *E. coli*, can also use chlorate as a substrate [10], but no dimethylsulphoxide reductase activity was detected in either cells or subcellular fractions of *T. pantotropha*. The chlorate reductase activity is therefore most probably associated with a nitrate reductase.

Table I

The subcellular location of nitrate reductase activities from anaerobically and aerobically grown *T. pantotropha*

Fraction	BV^+-nitrate oxidoreductase activity (% of total)	
	Anaerobically grown cells	Aerobically grown cells
Periplasm	14	97
Membrane	85	3
Cytoplasm	1	n.d.

Cells containing 50 mg protein were fractionated and 100% activity is equivalent to 200 μmol BV^+ reduced·min^{-1} and 50 μmol BV^+ reduced·min^{-1} for the anaerobic and aerobic grown cells, respectively. n.d., not detectable

Table II

Distinct catalytic properties of nitrate reductases in periplasm and cytoplasmic membranes of anaerobically grown *T. pantotropha*

	Activity of nitrate reductase (μmol·min^{-1})					
Substrate:	BV^+			NADH		
Electron acceptor:	NO_3^-	NO_3^- + azide	ClO_3^-	NO_3^-	NO_3^- + azide	ClO_3^-
Fraction						
Membrane	230	20	300	100	10	120
Periplasm	40	40	n.d.	n.d.	n.a.	n.d.

Subcellular fractions were prepared from one litre of anaerobically grown cells and activities given represent the total activity found in each fraction. The azide concentration when present was 20 μM. n.d., not detectable; n.a., not applicable

Catalysis of NADH- and BV^+-dependent nitrate and chlorate reduction and sensitivity to azide are all properties of the membrane-bound nitrate reductases of other nitrate-respiring bacteria [1,11]. Azide-insensitivity and the inability to reduce chlorate are characteristics of the periplasmic nitrate reductases of *Rhodobacter* species [12]. The results (Tables I and II) therefore suggest that *T. pantotropha* can express two catalytically distinct forms of respiratory nitrate reductase which resemble the membrane-bound and periplasmic enzymes previously identified in other organisms. The membrane-bound enzyme is the dominant form expressed under anaerobic conditions whilst the periplasmic enzyme is the main form found after aerobic growth.

3.2. *A comparison of nitrate reduction in aerobically and anaerobically grown cells of* T. pantotropha

The observation that a periplasmic nitrate reductase is predominantly expressed when *T. pantotropha* is grown under aerobic conditions suggested that it may be this enzyme, and not the membrane-bound nitrate reductase, that catalyzes the respiration of nitrate by intact cells under aerobic conditions. This possibility was investigated by growing *T. pantotropha* under anaerobic and aerobic conditions and measuring the capacity for nitrate reduction of each type of intact cell, using succinate as the electron donor. Under anaerobic assay conditions nitrate respiration by the anaerobically grown cells was strongly inhibited by azide (Table III). This is consistent with this activity being predominantly due to the membrane-bound nitrate reductase. Under the same conditions nitrate respiration by the aerobically grown cells was not highly sen-

Table III

The sensitivity to azide of aerobic and anaerobic nitrate reduction by cells of *T. pantotropha* grown under aerobic or anaerobic conditions

Assay conditions	Rate of nitrate reduction[a]	
	Anaerobically grown cells	Aerobically grown cells
Anaerobic	100	100
Anaerobic + azide (20 μM)	8	88
Aerobic	10	75
Aerobic + azide (20 μM)	10	75

[a] Rates are given as a percentage of the maximum rate of nitrate reduction under anaerobic conditions which was 900 nmol nitrate reduced $\cdot$ min^{-1} $\cdot$ mg cell protein^{-1} and 130 nmol nitrate reduced $\cdot$ min^{-1} $\cdot$ mg cell protein^{-1} for anaerobically and aerobically grown cells, respectively

sitive to azide (Table III), indicating the predominant presence of the periplasmic enzyme. Under aerobic assay conditions nitrate respiration by the anaerobically grown cells was inhibited by 90%. The residual air-insensitive rate of nitrate reduction was not further inhibited by azide. Nitrate respiration in cells grown aerobically was inhibited by only 25% on switching from anaerobic to aerobic conditions and the air-insensitive rate was not sensitive to azide (Table III).

These results confirm that: (i) the membrane-bound nitrate reductase is predominantly expressed during growth under anaerobic denitrifying conditions and is only operative under anaerobic conditions; (ii) the periplasmic nitrate reductase is predominantly expressed when cells are grown in an aerobic denitrifying manner and can operate under both anaerobic and aerobic conditions; and (iii) the periplasmic enzyme is present in anaerobically grown cells and accounts for their capacity for aerobic nitrate reduction.

The expression of membrane-bound and periplasmic nitrate reductases was further investigated in experiments in which the levels of the two types of nitrate reductase in intact cells were examined using two non-physiological electron donors: (i) reduced methylviologen (MV^+), which does not easily traverse the cytoplasmic membrane [13]; and (ii) BV^+, which can traverse the cytoplasmic membrane [13]. In cells grown under anaerobic conditions the MV^+-dependent nitrate reductase activity was 6-fold lower than the BV^+-dependent activity (Table IV). Thus much of the nitrate reductase expressed by anaerobically grown cells is inaccessible to MV^+ but freely accessible to BV^+, suggesting that such cells reduce nitrate predominantly at the cytoplasmic face of the cytoplasmic membrane. This is consistent with the predominant expression of a membrane-bound nitrate reductase with a molecular organization similar to that of *P. denitrificans* [2] and *E. coli* [1]. In aerobically grown cells the MV^+- and BV^+-dependent nitrate reductase activities were comparable (Table IV). This is consistent with the expression of a nitrate reductase that is freely accessible to MV^+ and BV^+, indicating a site of nitrate reduction on the periplasmic face of the cytoplasmic membrane and therefore the expression of the periplasmic nitrate reductase.

Table IV

Reduced benzylviologen is a substrate for both the periplasmic and membrane-bound nitrate reductase in intact cells of *T. pantotropha*, but reduced methylviologen is only effective with the periplasmic enzyme

Growth condition: Electron donor	Activity (nmol $\cdot$ min^{-1} $\cdot$ mg of cell protein^{-1})	
	Anaerobic	Aerobic
MV^+	30	60
BV^+	215	54

In conclusion, *T. pantotropha* has the ability to express catalytically distinct membrane-bound and periplasmic nitrate reductases. The requirement of the periplasmic enzyme for the aerobic respiratory reduction of nitrate is fully consistent with the view [3–7] that movement of nitrate across the cytoplasmic membrane to the active site of the membrane-bound reductase is inhibited under aerobic conditions.

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