Influence of Oxygen Tension on Nitrate Reduction by a *Klebsiella* sp. Growing in Chemostat Culture

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At dissolved oxygen tensions of 15 mmHg (2 kPa) and below, nitrate-limited continuous cultures of *Klebsiella* K312 synthesized nitrate reductase (NR) and nitrite reductase (NiR) and excreted ammonia. Under anaerobic conditions over 60% of the nitrate-nitrogen utilized was excreted as ammonia. In contrast, carbon-limited cultures excreted nitrite at dissolved oxygen tensions of 15 mmHg or below and synthesized NR but not NiR. Ammonia repressed neither NR nor NiR synthesis. These observations indicate that below a critical oxygen tension of 15 mmHg *Klebsiella* K312 utilizes oxygen and nitrate as electron acceptors. This oxygen tension correlates well with the critical oxygen tension observed for a change from oxidative to fermentative metabolism in cultures of *Klebsiella aerogenes*. The product of dissimilatory nitrate reduction is ammonia in nitrate-limited cultures but principally nitrite in carbon-limited (nitrate excess) cultures.

INTRODUCTION

Nitrate respiration by bacteria grown under anaerobic conditions is a well-documented process occurring in a wide range of facultative and obligate anaerobes (Payne, 1973; Thauer *et al.*, 1977). The reduction of nitrate to nitrite is the best documented step in this system and is catalysed in most bacteria studied by a membrane-bound nitrate reductase (NR, nitrate reductase A) which is also able to reduce chlorate. Evidence obtained by measuring the molar growth yields (for the utilization of carbon sources) in anaerobic cultures in the presence and absence of nitrate indicates that nitrate reduction to nitrite is coupled to phosphorylation in a large number of organisms (Stouthamer, 1977). Such phosphorylation is usually associated with a membrane-bound NR, cytochrome *b* and quinones as electron donors for nitrate reduction (Thauer *et al.*, 1977).

The dissimilatory reduction of nitrite also occurs in a number of bacteria although the end product varies from organism to organism. Denitrifying bacteria such as *Paracoccus denitrificans*, *Thiobacillus denitrificans* and many *Pseudomonas* spp. reduce nitrite to gaseous products (Thauer *et al.*, 1977), for example the reaction $\text{NO}_2^- + 2\text{H}^+ + \text{e}^- \rightarrow \text{NO} + \text{H}_2\text{O}$ occurs in cultures of *Pseudomonas aeruginosa* (Pichinoty, 1973). Alternatively, nitrite may be reduced to ammonia according to the overall reaction $\text{NO}_2^- + 8\text{H}^+ + 6\text{e}^- \rightarrow \text{NH}_4^+ + 2\text{H}_2\text{O}$. This process, which is a potentially useful electron sink mechanism, also conserves fixed nitrogen and has been reported to occur in cultures of *Klebsiella* spp. (Hadjipetrou & Stouthamer, 1965; Dunn *et al.*, 1978), *Achromobacter fischeri* (Prakasch & Sadana, 1973), *Veillonella alcalescens* (Inderlied & Delwiche, 1973) and *Escherichia coli* (Cole, 1978). Dissimilatory nitrite reduction is catalysed by a soluble, NADH-dependent nitrite reductase (NiR) in *E. coli* (Kemp & Atkinson, 1966) and a *Klebsiella* sp. (Dunn *et al.*, 1978).

Dissimilatory nitrate reduction to ammonia is similar overall to assimilatory reduction

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when nitrate serves as culture nitrogen source. The soluble assimilatory NR from *K. aerogenes* (Van't Riet *et al.*, 1968), *Azotobacter chroococcum* (Guerrero *et al.*, 1973) and a *Pseudomonas* sp. (Brown *et al.*, 1975) are ammonia-repressible but are not influenced by the dissolved oxygen tension in the culture. In contrast, membrane-bound NR from *Pseudomonas denitrificans* and *Pseudomonas* spp. (Chang & Morris, 1962), *Klebsiella* spp. (Van't Riet *et al.*, 1968; Dunn *et al.*, 1978), *A. fischeri* (Prakasch & Sadana, 1973), *Bacillus licheniformis* (Schulp & Stouthamer, 1970), *Haemophilus parainfluenzae* (Sinclair & White, 1970), *E. coli* (Showe & de Moss, 1968) and *T. denitrificans* (Justin & Kelly, 1978) are not ammonia-repressible and are induced maximally in the absence of oxygen.

While it is well-established that nitrate dissimilation occurs maximally in anaerobic cultures, there is little detailed information on the influence of oxygen tension on this process and the synthesis of NR and NiR. Justin & Kelly (1978) reported that while NR and NiR were synthesized by *T. denitrificans* in the presence of low oxygen concentrations, nitrate reduction occurred only under anaerobic conditions.

In a previous paper we reported that a *Klebsiella* sp., strain K312, converted nitrate to ammonia anaerobically in nitrate-limited cultures and that this process was associated with NR and NiR synthesis (Dunn *et al.*, 1978). This report extends these observations to include the influence of oxygen tension on growth, NR and NiR synthesis and the products of dissimilatory nitrate reduction.

METHODS

Klebsiella sp., strain K312, was isolated by chemostat enrichment from River Tay sediments and has been described by Dunn *et al.* (1978). Population densities were estimated as viable counts by plating on to Oxoid Tryptone Soy Agar plates followed by incubation at 23 °C for 48 h. Since this organism produced extracellular polymers when nitrogen-limited, it was not possible to obtain reliable data on culture dry weights.

The defined medium of Evans *et al.* (1970) was used throughout with the concentrations of all nutrients lowered by 50%. The concentrations of the carbon (glycerol) and nitrogen sources (nitrate or ammonia) were varied as required (Table 1).

The chemostat equipment with automatic control of pH (7.5), temperature (15 °C) and dissolved oxygen tension (in a range from zero to air saturation; 1 mmHg $\approx$ 133 Pa) was as described by Brown & Rose (1969). A dilution rate of 0.1 h⁻¹ was used throughout.

For the determination of NR and NiR activities, about 500 ml culture was centrifuged at 20 000 g for 30 min at 4 °C. For NR activity determinations, the pellet was washed by resuspending in 50 mM-Tris/HCl buffer (pH 7.0) and re-centrifuging. The washed bacteria were resuspended in 8 ml Tris buffer and disrupted by two passages at 110 MPa through a French pressure cell. The homogenate was centrifuged at 30 000 g for 30 min. The pellet was discarded and the supernatant was centrifuged at 125 000 g for 1 h to give a supernatant (soluble fraction) and pellet (resuspended in 5 ml Tris buffer; particulate fraction) which were assayed for NR activity. For NiR activity determinations, the above procedure was adapted by substituting TEA buffer [50 mM-Tris/HCl, pH 8.0, containing 5 mM-EDTA and 5 mM-ascorbic acid, as described by Cole *et al.* (1974)] for Tris buffer.

NR activity was determined in soluble and particulate fractions using the method of Lowe & Evans (1964) in which benzyl viologen reduced with dithionite serves as electron donor. NiR activity was determined in soluble fractions, incubated anaerobically in Thunberg cuvettes, by the method of Cole *et al.* (1974). No NiR activity was detected in particulate fractions.

The protein content of cell extracts was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Nitrate, nitrite and ammonia in culture media were determined by methods described by Brown *et al.* (1972).

RESULTS

Previously we reported that the maximum production of ammonia from nitrate occurred anaerobically in nitrate-limited cultures (Dunn *et al.*, 1978). In the present study, growth in aerobic, nitrate-limited cultures (150 mmHg dissolved oxygen tension) was associated with only trace concentrations of nitrate remaining in the spent medium and no detectable excretion of nitrite or ammonia. These cultures contained low activities of NR but no

Table 1. *Influence of oxygen tension on the utilization of nitrate (a, b) or nitrate and ammonia (c) by chemostat cultures of Klebsiella K312 ($D = 0.1 \text{ h}^{-1}$, 15°C)*

Oxygen tension (mmHg)	Population density (ml culture) ⁻¹	Spent medium analysis (mM)			Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]		
					Nitrate reductase		Nitrite reductase
		NO ₃ ⁻	NO ₂ ⁻	NH ₄ ⁺	Soluble	Particulate	Soluble
(a) Nitrogen-limited cultures in glycerol/nitrate medium (input: 15 mM-nitrate, 15 g glycerol l ⁻¹)							
150	4.9 × 10 ⁹	0.3	—	—	12	14	ND
15	2.5 × 10 ⁹	0.3	—	2.3	98	195	113
9	2.6 × 10 ⁹	0.3	0.1	2.1	157	445	58
0.67	1.3 × 10 ⁹	0.1	0.1	4.8	741	642	448
N ₂ flow	1.3 × 10 ⁹	0.1	—	9.4	969	2058	1314
(b) Carbon-limited cultures in glycerol/nitrate medium (input: 50 mM-nitrate, 5 g glycerol l ⁻¹)							
150	2.3 × 10 ⁹	11.5	0.2	0.1	12	12	ND
15	2.5 × 10 ⁹	10.4	0.2	0.1	81	370	ND
8	2.9 × 10 ⁹	6.6	0.8	0.2	380	704	ND
5	9.9 × 10 ⁸	6.5	1.0	0.2	402	1012	ND
N ₂ flow	8.0 × 10 ⁸	4.4	5.6	0.8	771	1814	ND
(c) Carbon-limited cultures in glycerol/nitrate/ammonia medium (input: 50 mM-nitrate, 50 mM-ammonia, 5 g glycerol l ⁻¹)							
150	3.2 × 10 ⁹	21.5	—	16.1	31	43	ND
15	3.1 × 10 ⁹	20.8	0.4	16.6	50	390	ND
8	3.3 × 10 ⁹	13.5	1.9	16.9	1368	3297	ND
N ₂ flow	8.0 × 10 ⁸	3.1	9.8	20.6	1277	3782	1300

—, Not detected (< 0.01 mM). ND, Not detected [< 0.1 nmol min⁻¹ (mg protein)⁻¹].

detectable NiR (Table 1*a*). Decreasing the culture dissolved oxygen tension to 15 mmHg, however, produced a culture with a less efficient utilization of nitrate for growth with about 15% of the nitrate utilized excreted as ammonia. The activity of particulate NR was some 10 times higher than in aerobically grown cultures and NiR was synthesized in appreciable concentrations. Lowering the culture oxygen tension further, in stages to zero, led to increased amounts of ammonia excreted until under anaerobic conditions this accounted for 62% of the nitrate utilized. Only traces of nitrate and nitrite were detected at any oxygen tension. The activities of both NR and NiR increased markedly as the oxygen tension was lowered and were highest anaerobically. The culture population density during anaerobic growth was about 25% of that during aerobic growth, with intermediate counts being obtained at intermediate oxygen tensions.

In contrast to the results from nitrate-limited cultures, ammonia excretion under carbon-limitation (with nitrate present in excess) was slight even under anaerobic conditions (Table 1*b*). There was, however, a progressive increase in nitrite excretion and increase in nitrate utilization as the oxygen tension was decreased below 15 mmHg. Anaerobically, nitrite excretion accounted for 12% and ammonia excretion for 2% of the nitrate utilized. Decreased oxygen tension resulted in a similar increase in NR to that observed in nitrate-limited cultures, but no NiR was detected. The population density variations with oxygen tension were similar to those of the nitrate-limited cultures.

The effect of oxygen tension on carbon-limited cultures containing nitrate and ammonia is shown in Table 1(*c*). As in carbon-limited cultures with nitrate alone, both nitrate utilization and nitrite excretion increased as the culture oxygen tension was decreased below 15 mmHg. NR activity again increased at low oxygen tensions and NiR was synthesized anaerobically. Population density variations were again of the same order as those noted

earlier. Analysis of spent media indicated that nitrate and ammonia were assimilated simultaneously at all oxygen tensions and there was some indication that ammonia was excreted anaerobically.

DISCUSSION

The *Klebsiella* sp. used in these experiments was able to grow anaerobically with glycerol as carbon source in the absence of nitrate (or nitrite) with ammonia as nitrogen source at a dilution rate of 0.1 h^{-1} (Dunn *et al.*, 1978). This growth was marked, however, by only low population densities (5.5% of aerobically grown cells), presumably reflecting the low potential of glycerol as a fermentable carbon source. Anaerobic growth on glycerol in the presence of nitrate, however, produced a higher population density indicating the utilization of nitrate as an electron acceptor in a system coupled to phosphorylation. This dissimilatory nitrate reduction produced only 25% of the population density attained in aerobic cultures.

The adaptation of the culture to utilize nitrate as electron acceptor was apparent at 15 mmHg with the synthesis of NR and NiR (nitrogen-limited culture) and the excretion of the products of nitrate reduction. This effect was more marked at 8 to 9 mmHg and below, with increased enzyme activities, increased product excretion and a decrease in the culture population density. This suggests that oxygen and nitrate are used simultaneously as electron acceptors, evidence which contrasts with the behaviour of cultures of *T. denitrificans* mentioned in the Introduction (Justin & Kelly, 1978). The 'critical' oxygen tension at which oxygen no longer acts as principal electron acceptor correlates well with the report of Harrison (1972) for the change from oxidative to fermentative metabolism of cultures of *K. aerogenes* growing on glucose. The presence of NR and NiR in nitrate-limited cultures (with little residual nitrate) and the absence of NiR in carbon-limited (nitrate excess) cultures suggests that nitrate has some function in the control of NiR. This is not likely to be repression *per se* because NiR is synthesized anaerobically in the presence of nitrate and ammonia (Table 1c). Whatever the detailed mechanism, however, it is clear that both NR and NiR are synthesized anaerobically in the presence of nitrate and ammonia (Table 1c), that both NR and NiR are required to convert nitrate to ammonia and that ammonia does not repress the synthesis of these dissimilatory enzymes. The induction of NR and NiR at low oxygen tensions is also consistent with this dissimilatory function. The excretion of ammonia from an anaerobic, nitrate-limited culture (Table 1a) appears paradoxical and such a culture must be considered to be electron acceptor-limited.

Cole (1978) has described the excretion of ammonia by anaerobic cultures of *E. coli* growing on nitrite. In contrast to the *Klebsiella* sp., cultures of *E. coli* excreted ammonia when carbon- or nitrogen-limited. In *E. coli*, nitrite reduction to ammonia correlated well with a shift in the fermentation pattern of the organism from ethanol to acetate production indicating that nitrite was acting as an oxidizing agent. Cole (1978) further showed that the anaerobic growth yield with nitrite was similar to that with ammonia for *E. coli*, indicating that nitrite reduction was essentially an electron sink mechanism with no oxidative phosphorylation. If this were the case with the *Klebsiella* sp. it would explain why cultures preferentially reduce nitrate rather than nitrite.

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