ELSEVIER

FEMS Microbiology Letters 125 (1995) 179–184

FEMS MICROBIOLOGY LETTERS

Growth of *Nitrosomonas europaea* on hydroxylamine

Peter de Bruijn, Astrid A. van de Graaf [1], Mike S.M. Jetten, Lesley A. Robertson, J. Gijs Kuenen *

Kluyver Laboratory for Biotechnology, Department of Microbiology and Enzymology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, the Netherlands

Received 26 September 1994; revised 9 November 1994; accepted 16 November 1994

Abstract

Hydroxylamine is an intermediate in the oxidation of ammonia to nitrite, but until now it has not been possible to grow *Nitrosomonas europaea* on hydroxylamine. This study demonstrates that cells of *N. europaea* are capable of growing mixotrophically on ammonia and hydroxylamine. The molar growth yield on hydroxylamine (4.74 g mol^{-1} at a growth rate of 0.03 h^{-1}) was higher than expected. Aerobically growing cells of *N. europaea* oxidized ammonia to nitrite with little loss of inorganic nitrogen, while significant inorganic nitrogen losses occurred when cells were growing mixotrophically on ammonia and hydroxylamine. In the absence of oxygen, hydroxylamine was oxidized with nitrite as electron acceptor, while nitrous oxide was produced. Anaerobic growth of *N. europaea* on ammonium, hydroxylamine and nitrite could not be observed at growth rates of 0.03 h^{-1} and 0.01 h^{-1}.

Keywords: *Nitrosomonas europaea*; Hydroxylamine; Growth yield; Nitrous oxide; Anaerobic growth

1. Introduction

The oxidation of ammonia to nitrate by nitrifying bacteria is considered to be a strictly aerobic process, although there is evidence that autotrophic nitrifiers can survive under oxygen limitation [1,2]. Under oxygen limitation, significant inorganic nitrogen losses have been observed [3,4]. These nitrogen losses were due to the production of nitric oxide and nitrous oxide. The formation of NO and N_2O by ammonia oxidizers is attributed to reduction of nitrite by the enzyme nitrite reductase. The oxidation of hydroxylamine or hydrazine was suggested to provide the reduction equivalents [5–7]. Organic substances, such as pyruvate or formate, are also suitable electron donors for NO and N_2O production [8]. The denitrifying activity of *Nitrosomonas europaea* could not be related to growth, but it may serve as a survival mechanism in anaerobic habitats

During mixotrophic growth of *N. europaea* on ammonia and pyruvate, hydroxylamine is formed [8]. Although *N. europaea* oxidizes hydroxylamine to nitrite, this substrate does not support growth even when added continuously [9]. Utilization of hydroxylamine as growth substrate by *N. europaea* under hydroxylamine limitation has not been reported. This paper describes the growth of *N. europaea* on mixtures of hydroxylamine and ammonium under aerobic conditions in chemostat culture. During transition

* Corresponding author. Tel.: (+31-15) 785 308; Fax: (+31-15) 782 355; e-mail: J.GijsKuenen@STM.TUDelft.NL

[1] Present address: IMPULS Dutch Science and Technology Center, P.O. Box 421, 1000 AK Amsterdam, the Netherlands.

SSDI 0378-1097(94)00495-1

experiments from aerobic to anaerobic conditions, the potential for anaerobic growth of *N. europaea* was investigated. We also show that, in batch experiments under anaerobic conditions, N_2O is produced from nitrite and hydroxylamine.

During the review of the first version of this manuscript, Böttcher and Koops published a paper, in which growth of several ammonia-oxidizing bacteria on mixtures of ammonia and hydroxylamine in batch cultures was described [20].

2. Materials and methods

2.1. Organism and cultivation

Nitrosomonas europaea LMD 86.25 was obtained from the culture collection of the Department of Microbiology and Enzymology, Delft, the Netherlands. *N. europaea* was routinely maintained in batch cultures. Phenol red (0.05 mg l^{-1}) was included as a pH indicator. Sodium carbonate (7%) was used to adjust the pH to 8. The cultures were incubated at 30°C in the dark. *N. europaea* was grown in a synthetic medium containing per litre of demineralized water: KH_2PO_4, 0.2 g; $(NH_4)_2SO_4$, 2.64 g; $MgSO_4 \cdot 7H_2O$, 0.04 g and 2 ml trace element solution. The trace element solution contained per litre of demineralized water: EDTA, 50 g; $ZnSO_4 \cdot 7H_2O$, 2.2 g; $CoCl_2 \cdot 6H_2O$, 1.61 g; $MnCl_2 \cdot 4H_2O$, 5.06 g; $CuSO_4 \cdot 5H_2O$, 1.57 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.10 g; $CaCl_2 \cdot 2H_2O$, 5.54 g; $FeSO_4 \cdot 7H_2O$, 4.99 g. The medium was autoclaved at 120°C, hydroxylamine was filter-sterilized. Contamination by heterotrophs was monitored by plating onto tryptone and yeast extract agar with subsequent incubation at 30°C for 2 weeks.

2.2. Growth conditions

Continuous cultivations were performed in Applikon laboratory fermenters with a working volume of 10 l. The medium was automatically adjusted to pH 8.0 with 1 M Na_2CO_3. The aerobic cultures were continuously gassed with air and stirred at 400 rpm. During the anaerobic period of the transition experiments from aerobic to anaerobic conditions, the cultures were gassed with 5% CO_2/95% Argon. The medium vessels were kept anaerobic by sparging with Argon. The dissolved oxygen concentration was monitored with a polarographic electrode (Ingold, Urdorf, Switzerland). Ammonia-limited chemostat cultures were grown on 20 mM NH_4^+ at a dissolved oxygen concentration of 30% air saturation at 30°C and at a dilution rate of 0.03 h^{-1}. The chemostats were wrapped in black paper to exclude light.

2.3. Anaerobic batch culture experiments

Anaerobic batch culture experiments were done in the dark at 30°C in 30-ml thermostatically controlled reaction chambers which were tightly closed with butyl rubber septa after flushing with argon. The cells for these experiments were obtained by continuous centrifugation of 20 l continuous culture fluid at 10000 × *g*. The pellet was resuspended in anaerobic mineral medium. Biomass was determined after each experiment. Ammonium, nitrite and hydroxylamine concentrations were determined and rates calculated. All experiments were performed at least three times.

2.4. Analytical procedures

Nitrite was determined using the Griess-Romijn reagent [10]. Ammonia and hydroxylamine were determined colorimetrically [11,12]. Nitrous oxide was analysed with a gas chromatograph (Packard Instrument company, USA) equipped with a TCD detector and a 180-cm column of CTR packed with Porous Polymer Mixture (Chromosorb 101). The term 'ammonium' will be indicating both the protonated and unprotonated forms, since, at the pH values used in these experiments, ammonium and ammonia both would be present. Dry weight of the cell suspensions was determined by filtrating aliquots over nitrocellulose filters (pore diameter 0.45 μm, Gelman Sciences, USA). The cells were washed three times with demineralized water and dried to constant weight.

2.5. Oxygen uptake experiments

Respiration rates of cells were assayed polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments Inc., Yellow Springs, OH). Cells from ammonia-limited chemostat cultures were

assayed directly in the culture fluid. Calculations were made on the basis of an oxygen concentration of 0.24 mM in air-saturated mineral medium at 30°C.

3. Results

3.1. Chemostat cultures grown on ammonium and hydroxylamine

Growth of *N. europaea* in ammonia-limited chemostat cultures was studied at a dilution rate of 0.03 h^{-1}, being approximately 30% of its maximum growth rate [13]. The molar growth yield of *N. europaea* in these ammonia-limited chemostat cultures was 1.43 g (mol ammonia)$^{-1}$. This yield is similar to values described for other *N. europaea* strains [14]. The cells obtained from a steady state culture were capable of oxidizing hydroxylamine at a rate of 150 nmol min^{-1} (mg dry weight)$^{-1}$. Until now, it has not been possible to grow *N. europaea* on hydroxylamine, because of its toxic nature [6,9]. However, the observed capacity to oxidize hydroxylamine suggested that *N. europaea* might be able to grow mixotrophically on a mixture of ammonia and hydroxylamine, provided that both compounds were kept growth-limiting to prevent toxicity problems. Therefore hydroxylamine was included in the medium to a level that would not exceed the observed hydroxylamine oxidation capacity. In this way a mixotrophic steady state culture could be established with hydroxylamine at undetectably low concentrations. Following a similar procedure, the hydroxylamine concentration in the medium reservoir could gradually be increased without exceeding the hydroxylamine oxidation capacity of the previous steady state culture.

Addition of hydroxylamine to the reservoir medium of ammonia-limited chemostat cultures resulted in a linear increase of biomass density (Fig. 1). This indicated that *N. europaea* was able to grow on hydroxylamine, while simultaneously oxidizing ammonia. The increase in biomass was higher than growth on ammonia alone (Fig. 1) and higher than the theoretically calculated growth yield on a mixture of ammonia and hydroxylamine. During growth on ammonium and hydroxylamine, the nitrogen recovery in the form of nitrite was lower than expected

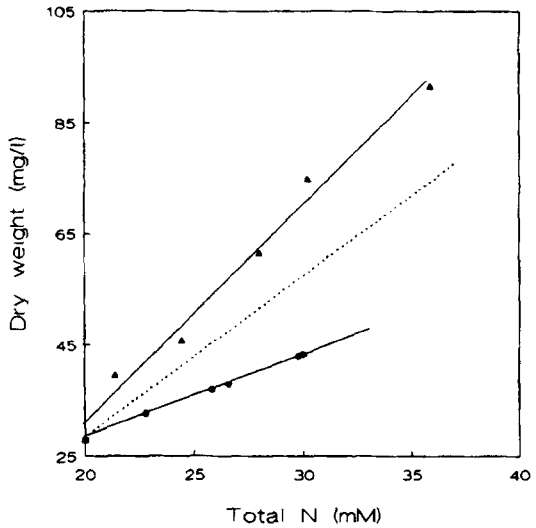

Fig. 1. Effect of increasing concentrations of hydroxylamine and ammonia in the reservoir medium on the biomass concentration in chemostat cultures of *N. europaea*. The dotted line indicates the theoretical growth yield on a mixture of hydroxylamine and ammonia on the basis of electron availability. Circles, dry weight on ammonium alone; triangles, mixotrophic growth yields on ammonium and hydroxylamine.

for biomass formation (Table 1; 0.26 mM $N_{biomass}$ for growth on 20 mM NH_4^+). This gap in the nitrogen balance could be due to formation of nitrous oxide, which was detected in the off gas. Formation of N_2O by *N. europaea* has also been described previously [4,15]. Measurements of the affinity constants (K_s) for NH_4^+ (0.2 mM) and NH_2OH (130 μM) during growth on a mixture of ammonium and hydroxylamine, or ammonia alone showed no significant difference.

Table 1
Nitrogen balances for growth of N. europaea on ammonia and hydroxylamine

$[NH_4^+]$ (mM) Medium	$[NH_2OH]$ (mM) Medium	$[NO_2^-]$ (mM) Culture fluid	N loss (%)
20.0	0	19.3	3.5
29.8	0	29.5	1.1
26.9	1.4	25.2	10.0
19.8	8.0	23.3	16.2
19.0	9.8	24.4	15.3
21.0	10.4	26.2	16.6

Growth conditions: growth rate, 0.03 h^{-1}; pH, 8.0; temperature, 30 °C.

3.2. Anaerobic activity experiments

N. europaea cells incubated under anaerobic conditions in the presence of a combination of nitrite, hydroxylamine and ammonia, converted only nitrite and hydroxylamine (Table 2). Also when cells were incubated in the presence of nitrite and hydroxylamine without ammonia, both nitrite and hydroxylamine were consumed. In both cases nitrous oxide was formed. When cells were incubated with nitrite alone nitrous oxide was also formed, while nitrite was consumed, probably by using reduction equivalents derived from storage compounds as electron donor. Incubation with hydroxylamine alone showed no consumption of hydroxylamine and no formation of nitrous oxide. This suggests that, under anaerobic conditions, *N. europaea* uses nitrite as electron acceptor and produces nitrous oxide from nitrite with hydroxylamine as electron donor. When the cells were heat-inactivated, the concentrations of nitrite, hydroxylamine and ammonia remained unchanged while there was no formation of nitrous oxide. This excludes chemical formation of nitrous oxide.

3.3. Transition experiments from aerobiosis to anaerobiosis

N. europaea was cultivated in chemostat culture at a dilution rate of 0.03 h^{-1} and at a dissolved oxygen concentration of 30% air saturation under combined ammonia and hydroxylamine limitation with a NH_4^+/NH_2OH ratio of 4. When the culture had reached steady state, the dissolved oxygen concentration was decreased to 0% by gassing the culture with 5% CO_2 and 95% argon. At the moment of the switch to anaerobic conditions, the nitrite concentration was approximately 26 mM (Table 1), thus providing ample electron acceptor for anaerobic metabolism of hydroxylamine. In Fig. 2 the density in the culture is presented as a function of time. It can be seen that the density of the culture started to decrease and that it followed the theoretical wash-out line, thus indicating that there was no significant growth. After the shift from aerobic to anaerobic conditions, ammonia and hydroxylamine directly started to accumulate into the medium. Anaerobic growth of *N. europaea* was also not observed during transition experiments at a dilution rate of 0.01 h^{-1} (not shown).

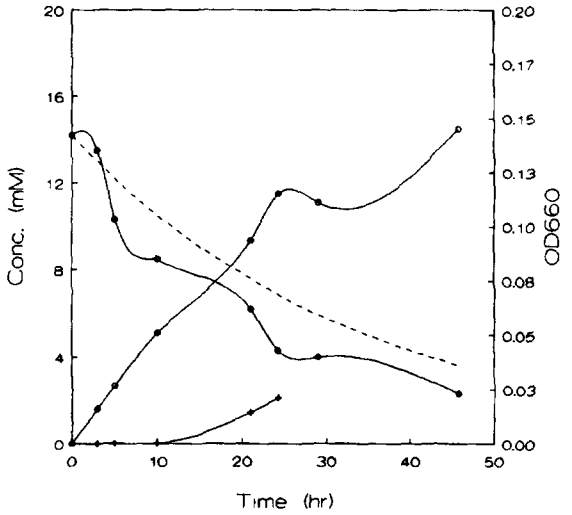

Fig. 2. Wash-out curve of *N. europaea* (●) and accumulation of NH_4^+ (○) and NH_2OH (+) during transition experiments from aerobic to anaerobic conditions at a dilution rate of 0.03 h^{-1}. The dashed line indicates the theoretical wash-out.

Table 2
Nitrous oxide production during anaerobic incubation in batch cultures of *N. europaea* cells (pH 8.0; 30°C) [a]

Addition	Increase N_2O	Decrease NO_2^-	Decrease NH_2OH
5 mM NO_2^-	1	1	0
2 mM NH_2OH	0	0	0
1 mM NH_2OH + 5 mM NO_2^-	4	5	10
5 mM NO_2^-, NH_4^+	3	6	0
2 mM NH_2OH + 5 mM NH_4^+	0	0	0
2 mM NH_2OH + 5 mM NH_4^+,NO_2^-	4	3	5
Sterilized cells	0	0	0

[a] Values are velocity in nmol min^{-1} (mg dry weight)$^{-1}$.

4. Discussion

Growth of *N. europaea* on hydroxylamine has not been reported previously. Due to the toxicity of hydroxylamine, substrate-limited growth conditions are required for utilization of this compound by *N. europaea*. It is well known that hydroxylamine is toxic at millimolar concentrations [16]. Cultures growing on ammonia and hydroxylamine could only be obtained by careful manipulation of the influent hydroxylamine concentration. When ammonia and hydroxylamine were present in the culture at growth-limiting concentrations, *N. europaea* could use hydroxylamine as a source of energy for growth. The increase in biomass concentration as a result of hydroxylamine addition was 4.74 g mol^{-1} or 1.18 g (mol redox equivalents)$^{-1}$. The molar growth yield of *N. europaea* in ammonia-limited chemostat cultures grown at the same dilution rate was 1.43 g mol^{-1} or 0.72 g (mol redox equivalents)$^{-1}$. Thus, the energetic value of the hydroxylamine redox equivalents is (1.18:0.72) × 100% = 164% of that of the redox equivalents from ammonia oxidation. Studies have shown that hydroxylamine and ammonia oxidation are coupled to proton translocation [17]. The H^+/O ratios reported for hydroxylamine are 3.9 and for ammonium ions 2.7 [17]. The energetic value of hydroxylamine redox equivalents should be (3.9:2.7) × 100% = 144% of that of the redox equivalents from ammonia oxidation, which is still 20% less than the measured values.

During growth of *N. europaea* on ammonia, a gap of 2% in the nitrogen balance was found (Table 1). This gap could be due to formation of nitrous oxide by a nitrite reductase. It is well known that ammonia-oxidizing bacteria produce small amounts of nitrous and nitric oxides in addition to nitrite. Gorreau et al. [3] found a yield of 2.5% total N as N_2O for *N. europaea*. During growth on ammonia and hydroxylamine, the gap in the nitrogen balance increased to 16% (Table 1), which is comparable to the value observed by Stüven et al. [8]. A soluble nitrite reductase has been characterized from *N. europaea* which catalyses the reduction of nitrite to N_2O and of O_2 to water. [18]. When nitrite is used as electron acceptor instead of oxygen, the limited amount of oxygen present can be used by the monooxygenase (K_m for O_2 = 15–20 μM). Oxidation of hydroxylamine to nitrite does not require molecular oxygen [19]. Cells of *N. europaea* produced N_2O by the reduction of nitrite under anaerobic conditions (Table 2). Production of N_2O increased when cells were incubated with hydroxylamine and nitrite, while hydroxylamine was consumed. This indicates that N_2O is formed by oxidation of hydroxylamine using nitrite as electron acceptor.

Although it would seem possible for *N. europaea* to grow anaerobically while oxidizing hydroxylamine, shift experiments from aerobic to anaerobic conditions at a dilution rate of 0.03 h^{-1} showed that *N. europaea* was unable to grow under these circumstances, even when the dilution rate was decreased to 0.01 h^{-1} (Fig. 2). During these experiments, ammonia and hydroxylamine accumulated into the medium. The failure to grow *N. europaea* anaerobically is probably the result of inhibition effects of hydroxylamine observed in biochemical reactions involving autotrophic CO_2 fixation [6]. It would be highly interesting to grow *N. europaea* in chemostat cultures under hydroxylamine limitation to enable accurate estimates of growth yield and maintenance substrate consumption.

Acknowledgements

This research was supported in by the Foundation of Applied Research (STW).

References

[1] Abeliovich, A. (1992) Transformations of ammonia and the environmental impact of nitrifying bacteria. Biodegradation 3, 255–264.

[2] Van de Graaf, A.A., Mulder, A., Slijkhuis, H., Robertson, L.A. and Kuenen, J.G. (1990) Anoxic ammonium oxidation: In: Proceedings of the 5th European Congress on Biotechnology (Christiansen, C., Munk, L., Viladsen, J., Eds.), pp. 388–391. Munksgaard, Copenhagen.

[3] Goreau, T.J., Kaplan, W.A., Wofsy, S.C., McElroy, M.B., Valois, F.W. and Watson, S.W. (1980) Production of NO_2^- and N_2O by nitrifying bacteria at reduced concentrations of oxygen. Appl. Environ. Microbiol. 40, 526–532.

[4] Poth, M. (1986) Dinitrogen production from nitrite by a *Nitrosomonas* isolate. Appl. Environ. Microbiol. 52, 957–959.

[5] Hooper, A.B. (1968) A nitrite-reducing enzyme from *Nitrosomonas europaea*. Preliminary characterisation with hydroxylamine as electron donor. Biochim. Biophys. Acta 126, 49–65.

[6] Poth, M. and Focht, D.D. (1985) ^{15}N Kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifier denitrification, Appl. Environ. Microbiol. 49, 1134–1141.

[7] Remde, A. and Conrad, R. (1990) Production of nitric oxide in *Nitrosomonas europaea* by reduction of nitrite. Arch. Microbiol. 154, 187–191.

[8] Stüven, R., Vollmer, M. and Bock, E. (1992) The impact of organic matter on nitric oxide formation by *Nitrosomonas europaea*. Arch. Microbiol. 158, 439–443.

[9] Watson, S.W., Bock, E., Harms, H., Koops, H.P. and Hooper, A.B. (1989) Ammonia oxidizing bacteria. In: Bergey's Manual of Systematic Bacteriology (Williams, S.T., Sharpe, M.E. and Holt, J.G., Eds.), Vol. 3, pp. 1821. Williams and Wilkins, Baltimore, MD.

[10] Griess-Romijn van Eck (1966) Physiological and chemical tests for drinking water, NEN 1056, IV-2. Nederlands Normalisatie-Instituut, Rijswijk.

[11] Frear, D.S. and Burrell, R.C. (1955) Spectrophotometric method for determining hydroxylamine reductase activity in higher plants. Anal. Chem. 27, 1664–1665.

[12] Fawcett, J.K. and Scott, J.E. (1960) A rapid and precise method for the determination of urea. J. Clin. Pathol. 13, 156–159.

[13] Loveless, J.E. and Alexander, M. (1968) The influence of metal ion concentration and pH value on the growth of a *Nitrosomonas* strain isolated from activated sludge. J. Gen. Microbiol. 52, 1.

[14] Drozd, J.W. (1980) Respiration in the ammonium-oxidizing chemoautotrophic bacteria. In: Diversity of Bacterial Respiratory Systems (Knowles, C.J., Ed.), Vol. II, pp. 87–111. CRC Press, Boca Raton, FL.

[15] Ritchie, G.A.F., and Nicholas, D.J.D. (1972) Identification of the sources of nitrous oxide produced by oxidative and reductive processes in *Nitrosomonas europaea*. Biochem. J. 126, 1181–1191.

[16] Hyman, M.R. and Wood, P.M. (1983) Methane oxidation by *Nitrosomonas europaea*. Biochem. J. 212, 31–37.

[17] Hollocher, T.C., Kumar, S. and Nicholas, D.J.D. (1982) Respiration dependent proton translocation in *Nitrosomonas europaea* and its apparent absence in *Nitrobacter agilis* during inorganic oxidations. J. Bacteriol. 149, 1013–1020.

[18] Miller, D.J. and Nicholas, D.J.D. (1985) Further characterization of the soluble cytochrome oxidase/nitrite reductase from *Nitrosomonas europaea*. J. Gen. Microbiol. 131, 2851–2854.

[19] Andersson, K.K. and Hooper, A.B. (1983) O_2 and H_2O are each the source of one O in NO_2^- produced from NH_3 by *Nitrosomonas europaea*: ^{15}N-NMR evidence. FEBS Lett. 164, 236–240.

[20] Böttcher, B. and Koops H.P. (1994) Growth of lithotrophic ammonia-oxidizing bacteria on hydroxylamine. FEMS Microbiol. Lett. 122, 263–266.