Antonie van Leeuwenhoek **71**: 69–74, 1997.

Hydroxylamine metabolism in *Pseudomonas* PB16: involvement of a novel hydroxylamine oxidoreductase

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Key words: hydroxylamine, nitrification, aerobic denitrification, *Pseudomonas*

Summary

Pseudomonas strain PB16, a Gram-negative heterotrophic nitrifying bacterium closely related to *Pseudomonas azalaica* on the basis of 16 S rDNA analysis, was able to use hydroxylamine as an additional energy source during growth in acetate limited chemostat cultures giving an increased biomass yield. In aerobically growing cells of *Pseudomonas* PB16 only 50% of supplemented hydroxylamine could be recovered as nitrite. In addition to nitrite, N_2O could be detected in the chemostat off-gas, indicating combined heterotrophic nitrification and aerobic denitrification. The maximum specific hydroxylamine oxidizing activity observed was 450 nmol per min per mg dry weight, with a K_s of approximately 40 μM. Upon addition of hydroxylamine to the medium, *Pseudomonas* PB16 induced a soluble 132 KDa dimeric hydroxylamine oxidoreductase. The enzyme had a pH optimum of 9, and did not contain spectroscopic features typical for cytochromes, which is in contrast to hydroxylamine oxidoreductases found in autotrophic bacteria.

Introduction

A variety of heterotrophic microorganisms are capable of nitrification, provided that an organic energy source is present (Kuenen & Robertson 1994). Many of these heterotrophically nitrifying bacteria are also (aerobic) denitrifiers, and have been isolated from soil and waste water treatment systems. The role of nitrification and its coupling to simultaneous (aerobic) denitrification in these heterotrophs is subject of ongoing studies (Ferguson 1994). Heterotrophic nitrification from ammonium is not linked to the capacity for autotrophic growth (Robertson 1990). Furthermore, cultures that are actively nitrifying ammonium have been reported to have a lower than expected growth yields (Robertson et al. 1989). During growth on reduced carbon sources, dissipation of excess reducing power via heterotrophic nitrification has been suggested to be responsible for the lower yields (Robertson & Kuenen 1988). As with the autotrophic nitrifiers, hydroxylamine is an intermediate between ammonia and nitrite in the nitrifying pathway of many heterotrophs (Wehrfritz et al. 1993; van Niel et al. 1992). In contrast to ammonia, nitrification of hydroxylamine can not provide the organisms with a sink for electrons, since its oxidation produces four reducing equivalents. In principle the energy from these reducing equivalents could be used for biomass synthesis and in theory even autotrophic growth on hydroxylamine should be possible (Stouthamer 1995). However, growth experiments with *Thiosphaera pantotropha* in the presence of 2 mM hydroxylamine resulted in lower yields (Robertson et al. 1989).

Stepwise addition of hydroxylamine to continuous cultures of *Nitrosomonas europaea* enabled the cells to grow mixotrophically on ammonia and hydroxylamine (de Bruijn et al. 1995). We have chosen a similar approach to investigate the hydroxylamine metabolism of *Pseudomonas* strain PB16. This paper describes the growth of *Pseudomonas* PB16 on mixtures of acetate and increasing amounts of hydroxylamine under aerobic conditions in chemostat cultures. Inclusion of hydroxylamine in the medium, induced a high hydroxylamine oxidizing capacity in *Pseudomonas* PB16. From these cells we have purified a non-heme contain-

ing hydroxylamine oxidoreductase (HAO), markedly different from any known HAO's.

Materials and methods

Organism and cultivation

Pseudomonas strain PB16 was formerly described as *Pseudomonas denitrificans* (Robertson et al. 1990). Since *Ps. denitrificans* is no longer a valid name (ISCB 1982), the strain was analyzed via the Biolog system (Ir. Jos van de Toorn, CBS, Delft, the Netherlands) and with 16 S rDNA analysis (Dr. Fred Rainey, DSM, Braunschweig, Germany). The Biolog test did not give clear results, but indicated a distant relationship to *Ps. nitroreducens*. 16 S RNA analysis showed 99% homology to *Ps. azalaica,* but not enough physiological data are available on this species to make a sound comparison.

Continuous cultures of *Pseudomonas* PB16 were performed in Applikon laboratory fermentors with a working volume of 2 l. The medium was automatically adjusted at pH 7.0 with either 0.5 M H_2SO_4 or 0.5 M Na_2CO_3. The dissolved oxygen concentration was monitored with a polarographic electrode (Ingold, Urdorf, Switzerland), stirring rate was always higher than 700 RPM. Acetate-limited chemostat cultures were grown on 20 mM acetate at dissolved oxygen concentration of 40% air saturation at 30 °C, and a dilution rate of 0.05 h^{-1}. The synthetic medium contained per liter of demineralized water: K_2HPO_4 0.4 g; KH_2PO_4 0.15 g; $(NH_4)_2SO_4$ 1.32g; $MgSO_4.7H_2O$ 0.4 g; and 2 ml of trace element solution (de Bruijn et al. 1995).

Analytical procedures

Nitrite was determined using the Gries-Romijn reagent (Gries-Romein-Van Eck, 1966). Hydroxylamine was determined colorimetrically (Frear & Burrell, 1958). Protein concentrations were determined by the Bradford method (Bradford, 1976). The dry weight of cell suspensions was determined by filtering aliquots over nitrocellulose filters of 0.45 μm (Gelman Sciences, USA). The filters were washed three time with demineralized water and dried to constant weight. SDS/PAGE was performed using a 10% resolving gel. Protein was stained with a Biorad silver staining kit. Absorption spectra were recorded on an Aminco DW-2000 UV/Vis dual wavelength spectrophotometer (SLM instruments).

Oxygen uptake measurements

Respiration rates of cells were measured polarographically with a Clark-type oxygen electrode (Yellow Spring Instruments, Ohio USA). Cells from acetate-limited chemostat cultures were washed with 50 mM Tris-HCl pH 7.0 and concentrated in the same buffer. Calculations were made on the basis of an oxygen concentration of 0.24 mM in demineralized water at 30 °C.

Enzyme assays

Hydroxylamine oxidoreductase (HAO) activity was determined by reduction of potassium ferricyanide at 400 nm, using an extinction coefficient of 1 $mM^{-1}.cm^{-1}$. The reaction mixture contained in 1 ml: Tris-HCl pH 8.8, 50 μmol; $K_3Fe(CN)_6$, 0.05 μmol; hydroxylamine 0.2 μmol. The reaction was started by the addition of an appropriate amount of enzyme. Reported enzyme activities were always linearly proportional to the amount of protein added, and the average of three independent determinations. Kinetic parameters were determined at 25 °C, and analyzed by non-linear fitting program GRAFIT from Erithacus software (Staines UK).

The following alternative electron acceptors were tested at pH 8: dichlorophenol indophenol (DCPIP) 0.1 mM; 0.4 mM phenazine methosulfate (PMS), 0.4 mM PMS in combination with 0.2 mM methylthiazol tetrazolium bromide (MTT); 0.1 mM horse heart cytochrome C; 0.2 mM NAD(P); and 0.2 mM FAD.

Enzyme purification

Enzyme purifications except for the gel filtration were performed at 4 °C. Before disruption cells were washed once with 50 mM Tris-HCl pH 7.8 containing 5 mM $MgCl_2$ (Buffer A) and resuspended in the same buffer. The cells were disrupted in a MSE 150 W sonifier by 6 bursts of 30 seconds with intermittent cooling. Intact cells and debris were removed by centrifugating at 40,000 x g for 30 min. The clear supernatants were used as cell extract. The cell extract was loaded on a Marco Q (Biorad) column (2.5 x 40 cm) equilibrated with Buffer A. A linear gradient of 0 to 1 M NaCl in Buffer A was applied at 3 ml/min. Fractions were collected and tested for HAO activity. Active fractions were pooled, concentrated with PM30 filters

Table 1. Steady-state measurements in acetate-limited chemostat cultures of *Pseudomonas* strain PB16. The 20 mM acetate provided was completely consumed

NH_2OH (mM)	$[NO_2^-]_{out}$ (mM)	$[N_2O]_{out}$ (ppm)	q_{NH2OH} (nmol/mg dw .min)	q^{max}_{NH2OH}	Yield (mg dw/l)	Surplus Yield (g dw/mol NH_2OH)
0	0	0	<1	<1	190	0
1	0.1	2	4	14	192	2
3	0.4	25	12	54	204	4
5	1.7	46	20	113	211	4
7	3.5	134	23	450	260	10

(Amico), supplemented with 10% glycerol, frozen in liquid nitrogen and stored at -70 °C. Further purification was achieved by repetitive gel filtration on a Superdex 200 (Pharmacia) column fitted to a HPLC system equipped with a diode-array detector (Hewlett-Packard). The column was equilibrated with 0.2 M KCl in Buffer A at a flow rate of 1 ml/min. Elution of protein was followed at 280 and 416 nm. Active fractions were pooled, concentrated with PM30 filters (Amico), supplemented with 10% glycerol, frozen in liquid nitrogen and stored at -70 °C.

Results

Chemostat cultures grown on mixtures of acetate and hydroxylamine

Growth of *Pseudomonas* PB16 in acetate-limited chemostat cultures was studied at a dilution rate of 0.05 h^{-1}. The growth yield was 190 mg dry weight per liter equivalent to 9.5 g of dry weight per mol of acetate (Table 1). This yield is similar to values previously reported (Robertson *et al. 1989, 1990*). The cells obtained from a steady state were capable of oxidizing less than 1 nmol of hydroxylamine per min per mg dry weight. Despite this limited capacity to oxidize hydroxylamine, addition of 1 mM hydroxylamine to the medium resulted in complete conversion of hydroxylamine. Simultaneously the hydroxylamine oxidizing capacity increased to 14 nmol per min per mg dry weight. Thereafter the hydroxylamine concentration was gradually increased to a level that would not exceed the observed oxidation capacity. In this way steady state cultures with hydroxylamine at undetectably low concentrations could be established. The levels of hydroxylamine could gradually be increased to 7 mM. Addition of hydroxylamine to the reservoir medium resulted in an increase of biomass density

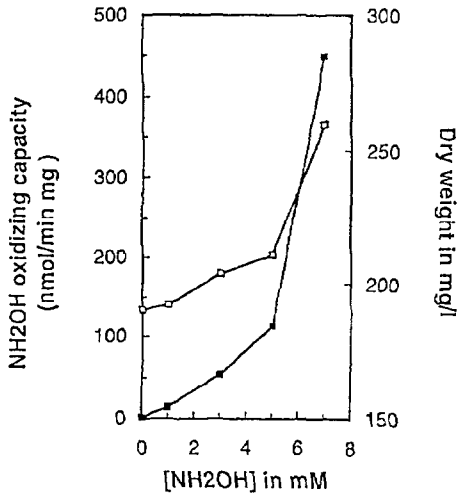

Figure 1. Effect of increasing concentrations of hydroxylamine on the biomass concentration and on hydroxylamine oxidizing capacity in acetate-limited chemostat cultures of *Pseudomonas*. Open squares, dry weight in mg/l; Closed squares, hydroxylamine oxidizing capacity in nmol/min per mg dry weigth.

(Figure 1). During mixotrophic growth on acetate and hydroxylamine, the nitrogen recovery in the form of nitrite was always lower than expected (Table 1). This gap in the nitrogen balance could be due to the formation of gaseous nitrogen compounds. Off-gas analysis showed that nitrous oxide was present (Table 1). Measurements of the kinetic parameters of cells growing mixotrophically on 20 mM acetate and 7 mM hydroxylamine showed that cells had a maximum hydroxylamine oxidizing capacity of 450 nmol NH_2OH per min per mg dry weight and a K_s of approximately 40 μM. These values come close to the numbers obtained for autotrophic bacteria (Table 2) and are 10-fold higher than those reported for other heterotrophic nitrifiers.

Table 2. Comparison hydroxylamine metabolism and hydroxylamine oxidoreductase from various bacterial sources

Organism	*Nitrosomonas europaea*	*Thiosphaera pantotropha*	*Pseudomonas* PB16	*Methylococcus capsulatus*	
q^{max}	50–1500	43	450	NR	nmol/min.mg
K_s	130	NR	12	NR	μM
Enzyme					
M_r subunit	63	20	68	39/16.4	kDa
composition	α_2-α_3	α	α_2	$\alpha\beta$	
heme/su	8	none	none	1	
heme type	P-460 heme-c			P-460	
V_{max}	28	0.13	0.45	56.4	μmol/min.mg
K_m	10	33	37	NR	μM
e-acceptor	PMS MTT Cyt_{554}	cyt C $K_3Fe(CN)_6$ Cyt_{551}	$K_3Fe(CN)_6$	PMS	
localization	periplasm	periplasm	ND	NR	
reference	Arciero 93	Wehrfritz 93	this article	Zahn 94	

NR, not reported; ND, not determined.

Purification of hydroxylamine oxidoreductase

SDS/PAGE analysis of extracts from cells grown with increasing levels of hydroxylamine showed a pronounced increase of a protein band at 68 kDa, suggesting the induction of a hydroxylamine converting enzyme. Enzyme assays of cell extracts obtained from cells grown at 7 mM hydroxylamine showed a hydroxylamine oxidoreductase (HAO) activity of approximately 100 nmol/min per mg protein when $K_3Fe(CN)_6$ was used as the electron acceptor. Five percent of the activity was retained with horse heart cytochrome c, but no activity could be found when DCPIP, PMS, PMS plus MTT, NAD(P) or FAD were provided as electron acceptor. Fractionation of the cell extract using anion exchange and repetitive gel filtration chromatography resulted in the purification of the HAO activity (Table 3). Analysis of the HAO enzyme using a calibrated Superdex 200 column indicated that the enzyme had an apparent molecular mass of 132 KDa. SDS/PAGE of the purified protein revealed the presence of a polypeptide with an molecular mass of 68 KDa (Figure 2), suggesting an α_2 dimer subunit composition. Spectral analysis of the protein showed no special features, except the protein band at 280 nm, indicating the absence of any heme groups. Analysis of the kinetic parameters of the purified protein showed a V_{max} of 450 nmol/min per mg protein and a K_m of 37 μM. The enzyme had a pH optimum around 9.

Table 3. Purification of HAO from *Pseudomonas*

Fraction	mg	Sp. Act. μmol/mg.min	Units	-fold
cell extract	136	0.11	15	1
Macro Q	22	0.21	4	2
Gel filtration I	4	0.34	1.2	3
Gel filtration II	2	0.47	0.9	5

Discussion

Until now growth of *Pseudomonas* strain PB16 on hydroxylamine has only been shown at concentrations below 2 mM (Robertson et al 1989, 1990). Cultures growing on acetate and higher concentrations of hydroxylamine could only be obtained by careful manipulation of the influent hydroxylamine concentration. When acetate and hydroxylamine were simultaneously limiting in the cultures, *Pseudomonas* PB16 could use hydroxylamine as a source of energy for growth (Table 1). The increase in biomass concentration as a result of hydroxylamine addition was maximally 10 mg/mmol of NH_2OH. This finding contrasts

Figure 2. SDS/PAGE analysis of the purified HAO enzyme of samples obtained from the second gel filtration column. The gel was stained with Biorad silver staining kit. Standard markers (Pharmacia) were phosphorylase B 94 kDa, BSA 67 kDa, ovalbumine 43 kDa, carbonic anhydrase 30 kDa, soybean trypsin inhibitor 20.1 kDa, and α-lactalbumin 14.4 kDa.

with results obtained from cells nitrifying ammonia alone (Robertson & Kuenen 1988, Wehrfritz et al. 1993). These cells generally have a reduced biomass yield, since they dissipate surplus reducing equivalents via ammonium monoxygenase. However, the oxidation of hydroxylamine was shown to be coupled to proton extrusion in several *Pseudomonas* strains (Castignetti 1990), but in batch cultures no increase in biomass yields was observed. During growth on mixtures of acetate and hydroxylamine only 50% of the hydroxylamine could be recovered as nitrite (Table 1). Off gas analysis showed that nitrous oxide was present up to 134 ppm, indicating conversion of nitrite via (aerobic) denitrification. The rate of N_2O formation was approximately 12 nmol/min. mg dry weight, which is 6-fold lower than the previously reported rates for aerobic denitrification (Robertson et al. 1989). The maximum specific hydroxylamine oxidation capacity measured was 450 nmol/min per mg dry weight. This number is comparable to the rates obtained for specialized autotrophs, and is 10-fold more than observed so far for *Alcaligenes faecalis* (33 nmol/min. mg), *Arthrobacter globiformis* (24 nmol/min.mg) and other heterotrophic nitrifiers (Table 2) (Kuenen & Robertson 1994, van Niel et al. 1992, Kurokowa et al. 1988). The affinity constant (K_s) for hydroxylamine is approximately 10-fold lower than found for *Nitrosomonas* species, indicating a very efficient conversion mechanism.

Upon disruption of the cells the hydroxylamine oxidation rate decreased 5-fold as has been reported for *Ps. putida* (Tokuyama et al. 1988), indicating a possible close membrane association or the absence of the natural electron acceptor in the enzyme assay. As has been shown for other heterotrophic nitrifiers, addition of hydroxylamine to actively nitrifying cultures stimulates total nitrification rates and induces the expression of a hydroxylamine oxidoreductase (HAO) (Wehrfritz et al. 1993, Tokuyama et al. 1988, Kurokawa et al. 1985). The 68 KDa size of the HAO subunit from *Pseudomonas* PB16 resembled the size of the HAO from the autotrophic nitrifier *Nitrosomonas europaea*, but the *Pseudomonas* enzyme did not have the spectroscopic features observed for the *N. europaea* enzyme (Arciero et al. 1993; Hooper et al. 1978). Similar to the enzyme from *Thiosphaera pantotropha*, the enzyme from *Pseudomonas* PB16 did not contain any heme prosthetic groups (Wehrfitz et al. 1993). Whether the *Pseudomonas* enzyme also contains non-heme iron has yet to be established. The HAO's of both autotrophic nitrifiers and methanotrophs contain at least one P-460 cytochrome and several c-type heme (Arciero et al. 1993, Zahn et al. 1994), which enables the enzyme to transfer the generated electrons to PMS and MTT *in vitro*. Both cytochrome c_{551} and pseudoazurin could serve as an electron acceptor for the HAO of *T. pantotropha*. Reduced cytochrome c_{551} is able to donate electrons to the denitrification enzymes, but is unable to mediate electron transfer to the quinone pool, thereby forming a possible link between heterotrophic nitrification and (aerobic) denitrification (Wehrfritz et al. 1993). A similar situation could be operative in *Pseudomonas* PB16, but its natural electron acceptor has not yet been identified. Further studies will be concentrated on the isolation of the natural electron acceptor of the HAO and on isolation of the gene encoding HAO from *Pseudomonas*.

Acknowledgements

This research was supported by the Royal Netherlands Academy of Arts and Sciences with a fellowship to M. Jetten. We thank Dr. G. de Jong for expert advice and help, B. Maaten for technical assistance, Dr. K. Othsubo for providing a reprint from Hakkokogaku

66, 401–404 and Dr. L. Robertson for critical reading of the manuscript and for stimulating discussions.

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