Light-Mediated Nitrite Accumulation during Denitrification by *Pseudomonas* sp. Strain JR12

YORAM BARAK, YOSSEI TAL AND JAAP VAN RIJN*

Department of Animal Science, Faculty of Agricultural, Food and Environmental Quality Sciences,
The Hebrew University of Jerusalem, Rehovot 76100, Israel

Received 22 September 1997/Accepted 23 December 1997

The effect of light on the denitrifying characteristics of a nonphotosynthetic denitrifier, *Pseudomonas* sp. strain JR12, was examined. Already at low light intensities, nitrite accumulated as a result of light inhibition of nitrite but not of nitrate reduction rates. Exposure of this bacterium to light caused a photooxidation of cytochrome *c*, an intermediate electron carrier in its respiratory pathway. Photoinhibition of nitrite reduction was reversible, as nitrite reduction rates returned to preillumination levels when light-exposed cells were returned to dark conditions. Antimycin A reversed the inhibitory effect of light on nitrite reduction by preventing a reversed electron flow. Aerobic respiration by this bacterium was not affected by light.

Denitrification, in combination with nitrification, is widely applied for removal of inorganic nitrogen from nitrogen-polluted waste and drinking waters. Biological nitrogen removal by means of this combined process is often hampered by the accumulation of nitrite, an intermediate product in both nitrification and denitrification. Nitrite accumulation has received considerable attention, as this inorganic form of nitrogen is toxic to aquatic life and also to humans when it is present in drinking water (4). In denitrifiers, various environmental factors were found to underlie nitrite accumulation, among them being the type and quantity of organic substrate, oxygen, pH, nitrate availability, and temperature (23).

Light is an additional environmental factor shown to affect nitrite accumulation in nitrifying consortia (1, 2, 13, 21, 24). Less information is available on the effect of light on denitrifiers, and almost all of this limited information is restricted to photosynthetic denitrifiers. Various mechanisms were proposed to explain the light inhibition of oxygen and nitrate reduction in these latter organisms. In *Rhodospseudomonas sphaeroides*, this was explained by an energy-linked reversed reduction of NAD (14), whereas in *Rhodospseudomonas capsulata* (3, 8, 11) evidence was presented that the proton motive force generated by the photosynthetic pathway exerted a thermodynamic “back pressure” on the respiratory chain (oxygen and nitrate reduction) operating across the same membrane. Mütze (10) provided evidence for light inhibition of nitrate reduction in the nonphotosynthetic denitrifier *Paracoccus denitrificans* (formerly *Micrococcus denitrificans*). However, explanations as to the mechanism(s) underlying this inhibition were not provided.

We previously isolated a denitrifying bacterium that showed a distinct pattern of nitrite accumulation when grown on different carbon sources. Dissimilatory nitrate reduction to N_2 gas coincided with an intermediate nitrite accumulation which was high when either acetate or propionate was used and low when butyrate, valerate, or caproate was used as the carbon and electron donor (20). By means of immobilization of this *Pseudomonas* sp. isolate (strain JR12) in either alginate or

chitosan (12), we tested its nitrate removal capacity in aquariums and found that, when exposed to light, the immobilization complex produced significantly more nitrite than when incubated in the dark. The effect of light on the denitrifying capacity of this isolate is presented in this study. It is shown that, already at relatively low light intensities, nitrite reduction was significantly inhibited and returned to normal values upon switching to dark conditions. A mechanism explaining the effect of light on nitrite reduction activity is proposed. Similar findings were obtained with other denitrifying isolates as well as with a crude denitrifying consortium obtained from a fluidized-bed reactor used for nitrate removal.

MATERIALS AND METHODS

Organism. The bacterial strain examined in this study (*Pseudomonas* sp. strain JR12) was isolated from a fluidized-bed reactor used for nitrate removal in intensive fish culture systems (20). Based on metabolic properties and its fatty acid profile, this strain has been identified as *Pseudomonas stutzeri* (20). However, recent partial sequencing of the 16S rDNA classified this bacterium in RNA group I, with 99% homology to *Pseudomonas putida* and 96% homology to *P. stutzeri*. The strain was deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) under the accession number 96-563.

Culture conditions and nitrate and nitrite reduction studies. *Pseudomonas* sp. strain JR12 was cultured anaerobically at 30°C in a synthetic medium containing the following components (per liter): $Na_2HPO_4 \cdot 7H_2O$, 7.2 g; K_2HPO_4 , 1.5 g; NH_4Cl , 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; KNO_3 , 0.3 g; and a trace element solution, 2 ml (22). Either acetate or butyrate was used as a carbon source at a concentration of 5 mM. The pH of the medium was 7.2. Studies were conducted with cells harvested during the late log phase of growth (after 4 to 5 days). Cells were washed twice and resuspended in the medium described above. Although nitrate and nitrite (50 and 30 mg/liter, respectively) were added at concentrations lower than those required for complete oxidation of the volatile fatty acids, their initial concentrations were sufficient to allow determination of maximum nitrate and nitrite reduction rates (V_{max}). Experiments were conducted in a temperature-controlled (30°C) incubation vessel (300 ml), which was placed on a magnetic stirrer and fitted with nitrate, pH, and oxygen/temperature electrodes. Anaerobic conditions in the vessel were obtained by continuous flushing with prepurified nitrogen gas. High pressure within the incubation vessel prevented oxygen penetration, as verified by continuous oxygen monitoring. The experiments were initiated by the addition of one of the carbon sources. Periodically, samples were withdrawn for nitrite determinations. Changes in nitrate and pH levels were monitored every 2 to 5 min, whereas protein and ammonia concentrations in the vessel were determined in aliquots withdrawn at the beginning and the end of the experiment. A minimum of five runs were performed for each electron donor-acceptor combination. Nitrate and nitrite reduction rates (expressed per gram of protein) differed by not more than 10% between these runs. During the various experiments, the bacterial biomass (as measured by protein analysis) did not increase by more than 15%. Ammonia concentrations decreased in relation to the increase in bacterial biomass in the medium. An increase in pH (not exceeding 0.3 unit) was detected in all experiments. V_{max} values for nitrate and nitrite during each run were obtained by nonlinear regression analyses of at least 30

* Corresponding author. Mailing address: Department of Animal Science, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel. Phone: 972-8-9481302. Fax: 972-8-9465763. E-mail: van-rijn@AGRI.HUJI.AC.IL.

FIG. 1. Changes in nitrate ($\blacktriangle$) and nitrite ($\blacksquare$) concentrations upon incubation of *Pseudomonas* sp. strain JR12 (protein content, 165 mg/liter) under anoxic conditions in culture medium (see Materials and Methods) with butyrate (5 mM) as the carbon and electron donor and with nitrate as the electron acceptor in the dark and in the light ($500 \mu\text{mol}/\text{m}^2/\text{sec}$).

data points based on Michaelis-Menten kinetics using the Enzfitter software program (Elsevier-Biosoft, Amsterdam, The Netherlands). Antimycin A was solubilized in *N,N*-dimethylformamide and was added to a final concentration of $20 \mu\text{g}/\text{ml}$.

Illumination. Bacterial suspensions were illuminated with a slide projector (Zeiss Icon Perkeo, model 315 IR) equipped with a quartz iodine lamp (24 V, 150 W) with a spectrum similar to that of sunlight. Light intensity was determined with a Licor Quantum/Radiometer/Photometer (model LI-189). Different light spectra were achieved by placing green (480 to 600 nm, peak at 548 nm), blue (430 to 500 nm, peak at 473 nm), and red (600 to 700 nm, peak at 670 nm) colored glass plates in the light path between the projector and the bacterial suspension. Light intensity was controlled by varying the distance between the light source and the bacterial suspension.

Cytochrome studies. Cells, grown with butyrate as the electron and carbon donor and nitrate as the electron acceptor, were harvested in the late log phase of growth, washed with 50 mM phosphate buffer (pH 7.1), and resuspended in the same buffer at the specified concentrations. The effect of illumination on the redox state of cytochrome *c* was determined in closed 3-ml cuvettes with a Hitachi (model U-3000) double-beam spectrophotometer. Due to the lack of a suitable apparatus for illuminating the cells within the spectrophotometer, we estimated the effect of light on the redox changes of cytochrome *c* in the following way. After determination of the redox state of the bacterial suspension in the spectrophotometer, the cuvette containing the cells was taken out and illuminated for 10 min. Immediately thereafter, the cuvette was placed back into the spectrophotometer and changes in the redox state were recorded at 552 nm compared with cells fully oxidized by the addition of solid ferricyanide.

Analytical procedures. Total ammonia (NH_3 and NH_4^+) was determined as described by Scheiner (15), nitrite was measured according to the method of Strickland and Parsons (17), and nitrate was measured with a specific nitrate electrode (Radiometer, Copenhagen, Denmark) amplified with a pH meter (Radiometer; model PHM92). Protein was determined according to the method of Lowry et al. (7) with bovine serum albumin as the standard. Oxygen concen-

FIG. 2. Maximum nitrite reduction rates of *Pseudomonas* sp. strain JR12 (protein content, 135 mg/liter) incubated under anoxic conditions in culture medium (see Materials and Methods) with butyrate (5 mM) as the carbon and electron donor and with nitrite as the electron acceptor at different light intensities. Full sunlight intensity was $2,000 \mu\text{mol}/\text{m}^2/\text{s}$.

tration and temperature were measured with a model 57 temperature/oxygen probe (Yellow Springs Instruments, Yellow Springs, Ohio).

RESULTS

As found in a previous study (20), intermediate nitrite accumulation during nitrate reduction by *Pseudomonas* sp. strain JR12 was low when butyrate served as the carbon and electron donor. However, incubation of butyrate-supplemented cells in light resulted in a considerable intermediate nitrite accumulation during nitrate reduction (Fig. 1). Whereas no significant differences in the maximum nitrate reduction rates between light- and dark-incubated cells were found (264.3 ± 28.6 and $238.6 \pm 64.3 \mu\text{mol}$ of NO_3^-/g of protein/min, respectively), maximum nitrite reduction rates were significantly higher in dark-incubated cells ($333.6 \pm 14.3 \mu\text{mol}$ of NO_2^-/g of protein/min) than in light-incubated cells ($203.6 \pm 50 \mu\text{mol}$ of NO_2^-/g of protein/min). Similar results were obtained when this strain was incubated with nitrite as the sole electron acceptor in light and dark conditions or when acetate was used instead

FIG. 3. Changes in nitrite concentrations during incubation of *Pseudomonas* sp. strain JR12 (protein content, 125 mg/liter) under anoxic conditions in culture medium (see Materials and Methods) with butyrate as the carbon and electron donor under changing light ($1,500 \mu\text{mol}/\text{m}^2/\text{s}$) and dark conditions.

FIG. 4. Maximum nitrite reduction rate upon incubation of *Pseudomonas* sp. strain JR12 (protein content, 150 mg/liter) under anoxic conditions in culture medium (see Materials and Methods) with butyrate as the carbon and electron donor in the dark or under blue, green, or red light. Light intensity was 400 $\mu\text{mol}/\text{m}^2/\text{s}$. Treatments with different superscript letters are significantly different (Student's *t* test, $P < 0.05$).

of butyrate as the carbon and electron donor (data not shown).

Inhibition of nitrite reduction in this *Pseudomonas* strain was dose dependent with respect to light (Fig. 2). A 15% inhibition (as compared with reduction rates in darkness) was found at a light intensity of 5% of full sunlight intensity, and a 67% inhibition was found at full sunlight intensity (2,000 $\mu\text{mol}/\text{m}^2/\text{s}$).

Cells incubated with nitrite and butyrate were exposed to short light pulses, and nitrite disappearance from the medium was examined before and after these pulses (Fig. 3). A temporary rather than a permanent nitrite reduction inhibition by light was found, indicating that inhibition of nitrite reduction by light is reversible.

An examination of the effect of different light spectra on nitrite reduction rates (Fig. 4) revealed that inhibition of nitrite reduction was highest when cells were exposed to green light. Blue light inhibited nitrite reduction to a lesser extent,

whereas exposure of the cells to red light caused no significant inhibition compared with nitrite reduction under dark conditions.

In a previous study by our group (20), reduction of nitrite was found to be mediated by cytochrome *c* (absorption maximum, 552 nm). Our findings that green light, in particular, inhibited nitrite reduction and the fact that nitrite reduction rates but not nitrate reduction rates were affected by light led us to the conclusion that light may impair the electron transfer from cytochrome *c* to nitrite reductase. Evidence for this was obtained by an examination of the redox state of cytochrome *c* before and after light exposure of cells incubated under anoxic conditions in the presence of butyrate and in the absence of an electron acceptor. Light exposure coincided with oxidation of cytochrome *c*, the reduction state of which returned to preillumination values when the cells were placed once more in the dark (Fig. 5). Further evidence supporting the notion that light affects the electron transfer from cytochrome *c* to nitrite reductase was provided by adding antimycin A to butyrate- and nitrite-supplemented cells in the light and in the dark. Under these conditions it was found that this inhibitor reverses the inhibitory effect of light on the nitrite reduction rate (Table 1). In this bacterium, butyrate donates electrons to the respiratory chain in close vicinity to cytochrome *c*, downstream of the antimycin A block (20). Based on this knowledge, we explain the finding that antimycin A reverses the light inhibition in butyrate-supplemented cells as follows. Light brings about an oxidation of cytochrome *c*, causing a reversed, uphill electron flow away from nitrite reductase. Addition of antimycin A, a compound which blocks electron flow between cytochromes *b* and *c*, prevents such an uphill flow and, consequently, in the light and in the presence of antimycin A, nitrite reduction is not affected.

Aerobic respiration by this denitrifier was not affected by light as no differences in oxygen reduction rates were found between light- and dark-incubated cells incubated aerobically in the presence of either acetate or butyrate (Table 2).

FIG. 5. Changes in A_{552} after light exposure of *Pseudomonas* sp. strain JR12 grown on butyrate and washed and resuspended (protein content, 129 mg/liter) in phosphate buffer (pH 7.1) and butyrate (5 mM). Following dark incubation (inside the spectrophotometer), the cuvette, containing the cell suspension, was taken out of the spectrophotometer and illuminated (2,000 $\mu\text{mol}/\text{m}^2/\text{s}$) for 10 min and once more placed back in the spectrophotometer in the dark. Incubation was conducted under anoxic conditions. The absorbance was read against a reference cuvette containing ferricyanide-oxidized *Pseudomonas* sp. strain JR12.

TABLE 1. Effect of antimycin A on nitrite reduction rates during dark and light (1,500 $\mu\text{mol}/\text{m}^2/\text{s}$) incubation of butyrate-supplemented *Pseudomonas* sp. strain JR12

Illumination	Presence of antimycin A	Nitrite reduction rate (μmol of NO_2^-/g of protein/min) ^a
—	—	253.6 $\pm$ 16.8A
—	+	254.9 $\pm$ 34.5A
+	—	142.1 $\pm$ 11.6B
+	+	251.4 $\pm$ 22.8A

^a Means followed by the same letter are not significantly different by Student Newman Keuls test ($P = 0.05$), followed by analysis of variance.

DISCUSSION

In an early study by Ulken (18) on samples obtained from the Elbe river, light was found to have a profound effect on bacterial proliferation as well as on nitrification, denitrification, and thiosulfate-oxidation potentials. Based on the knowledge available then on the effect of illumination on cultures of *Nitrobacter winogradsky* (9) and *Nitrosomonas europaea* (16), she suggested (19) that photoinhibition of nitrification could be an important factor contributing to the high nitrite concentrations in the Elbe. Mütze (10) concluded that not only nitrification but also denitrification could be a source of nitrite in natural water bodies by demonstrating that, in *Micrococcus denitrificans* (presently *Paracoccus denitrificans*), oxidation of organic and inorganic (H_2) electron donors was affected by light under both aerobic and anoxic conditions. To the best of our knowledge, apart from Mütze's study, the effect of light on nonphotosynthetic denitrifiers has not been addressed and photoinhibited nitrification, not denitrification, is commonly thought to be the source of nitrite observed in light-exposed aquatic environments (5, 6, 13).

In this study we showed that light-mediated nitrite accumulation by *Pseudomonas* sp. strain JR12 was caused by photoinhibition of nitrite reduction. Photoinhibition of nitrite reduction was dose dependent and reversible. As compared with inhibition upon exposure to blue and red light, inhibition of nitrite reduction in cells illuminated with green light was the highest. Exposure of the cells to monochromatic light at 550 nm resulted in the same degree of nitrite reduction as was found when cells were exposed to green (480 to 600 nm) light (data not shown).

The sensitivity to 550-nm light and the observed light-mediated oxidation of cytochrome *c* provide evidence for the fact

that light exposure prevents electron flow from cytochrome *c* to nitrite reductase. The observation that this inhibition was relieved in the presence of antimycin A points to a reversed, uphill oxidation of cytochrome *c* by light.

Light did not affect the aerobic respiration rate in this *Pseudomonas* strain. Since we found that cytochrome *c* is affected by light, this finding points to the fact that mainly cytochrome *o* (upstream of the cytochrome *c*), not cytochrome *aa3* (downstream of cytochrome *c*), is involved in the aerobic respiratory pathway of this bacterium.

It remains to be examined to what extent light affects the denitrification patterns in other nonphotosynthetic denitrifiers. We examined the effect of light on another denitrifying isolate (*Ochrobactrum anthropi*) and on a crude denitrifying consortium obtained from a denitrifying fluidized-bed reactor and obtained results similar to those presented for *Pseudomonas* sp. strain JR12 (data not shown). It seems plausible, therefore, that light-mediated nitrite accumulation by denitrifiers is widespread. Further research will be required to determine the significance of this process in the natural habitat (e.g., habitats occupied by anoxic photosynthetic bacteria).

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TABLE 2. Maximum oxygen reduction rates of *Pseudomonas* sp. strain JR12 upon light (1,500 $\mu\text{mol}/\text{m}^2/\text{s}$) or dark incubation in the presence of acetate or butyrate

Carbon source	Illumination	Oxygen reduction rate (μmol of O_2/g of protein/min) ^a
Acetate	—	522.8 $\pm$ 27.6
	+	498.3 $\pm$ 53.0
Butyrate	—	285.6 $\pm$ 28.6
	+	300.0 $\pm$ 21.7

^a No significant differences in oxygen reduction rates were found within acetate-supplemented cells and within butyrate-supplemented cells (Student's *t* test, $P < 0.05$).

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