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Effects of Light and CO on the Survival of a Marine Ammonium-Oxidizing Bacterium during Energy Source Deprivation

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The chemolithotrophic ammonium-oxidizing bacterium *Nitrosomonas cryotolerans* responds uniquely to nutrient deprivation by lowering its endogenous respiration and anabolic processes to undetectable levels during starvation, thus appearing to enter a dormant state. To ascertain whether this state protects the cells from further stresses (as seen with endospore-forming bacteria), the starved cells were subjected to two known inhibitors, CO and light. It was found that long-term-starved cells were less resistant than freshly starved cells to light inhibition. Both long-term-starved cells and freshly starved cells were unaffected by CO.

Recent investigations on survival of the chemolithotrophic marine ammonium-oxidizing bacterium *Nitrosomonas cryotolerans* during energy source deprivation (11; B. H. Johnstone and R. D. Jones, Mar. Ecol. Prog. Ser., in press) have shown that this organism is possibly better adapted to survival than are heterotrophic bacteria previously studied (5, 12–14). For example, *N. cryotolerans* reduced its rate of endogenous respiration to undetectable levels after 4 weeks of starvation. No changes in cellular components such as DNA, RNA, and protein were detectable. Also, the adenylate energy charge of the organism stabilized at low levels, and a charged electron transport system was maintained. Furthermore, the response of the organism was unique in that there was neither an increase nor a decrease in cell size or total cell numbers.

Because of their lack of activity during energy source deprivation, it is possible that these bacteria are protected from other stress factors. To test this possibility, we subjected starved cells to stressing agents. Light and carbon monoxide, two relatively well studied inhibitors of ammonium oxidation, were used. Starving cells were subjected to light, which probably inhibits by photooxidizing cytochrome *c* (2), and to carbon monoxide, which competitively inhibits ammonium monooxygenase (6, 9, 24) and binds to the terminal electron acceptor, thus blocking respiration.

Light inhibition of ammonium oxidizers (6, 7, 18, 22, 27, 28; A. Vanzella, M.S. thesis, Florida International University, Miami, 1987) can occur in the photic regions of marine environments (17, 18, 25). Vanzella (M.S. thesis) observed a 37% decrease in activity under fluorescent light at intensities as low as 5 W m^{-2} (for comparison, sunlight is approximately 630 W m^{-2}).

Carbon monoxide can reach inhibitory levels in the environment through photochemical production (3, 4, 26). Inhibition can occur at concentrations as low as 2 nM (Vanzella, M.S. thesis). Conrad et al. (4) found average concentrations of 2 nM in oceanic surface waters. Very high levels of CO can be found in some environments; Conrad et al. (3) measured up to 27 nM in the surface and over 360 nM in low-O_2 waters of a eutrophic lake.

This study was initiated on the premise that cells which had entered the starvation-survival state would be more resistant to physical and chemical stresses (13), a situation analogous to the resting state of endospores. As with endospores, dormancy of vegetative cells (19–21, 23) should render the cells less susceptible to exogenous influences as a result of the lack of metabolic activity.

MATERIALS AND METHODS

Materials. All materials, unless otherwise stated, were obtained through Sigma Chemical Co., St. Louis, Mo.

Organism and culture. The ammonium-oxidizing bacterium used for this study was *N. cryotolerans* (R. D. Jones, R. Y. Morita, H.-P. Koops, and S. W. Watson, Can. J. Microbiol., in press). The bacterium was isolated from Alaskan coastal waters and is described elsewhere in more detail (10; Jones et al., in press). All cells used in this study were grown and maintained by continuous culture (dilution rate, 0.14 day^{-1}) in a 4-liter reaction vessel with the pH automatically controlled to 7.8 ± 0.05 by addition of 5% K_2CO_3. Growth was at 5°C; before inoculation, cells were adapted to growth at that temperature as described by Jones and Morita (10). The growth medium used was the same as that described by Jones and Hood (8), with the salinity adjusted to 30‰ by addition of Instant Ocean synthetic sea salts (Aquarium Systems, Inc., Mentor, Ohio). The medium was composed of inorganic salts, trace elements, and 714 μM NH_4^+ as an energy source. The culture was vigorously aerated and agitated by a magnetic stirring bar.

Starvation conditions. Three liters of cells was pelleted (5,080 × *g*, 10 min, 5°C), washed twice with starvation menstruum (SM), and resuspended in SM at a final density of approximately 1.5 × 10^7 ml^{-1}. SM consists of 30‰ Instant Ocean synthetic sea salts, 1 g of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and 1 ml of 5% K_2CO_3 solution in 1 liter of distilled H_2O. The pH of the menstruum was adjusted to 7.8 by addition of 1 N NaOH. The solution was filtered through a glass fiber prefilter (Gelman Sciences, Inc., Ann Arbor, Mich.) to remove large particles and sterilized by autoclaving. The starvation flasks were capped with neoprene stoppers to limit ammonia contamination from the air.

Effects of light and CO. After 4 weeks, the starving cells were collected and resuspended at approximately 10^6 cells ml^{-1} in sterile SM. Chemostat cells were harvested at the same time and prepared for starvation by washing, after which they also were inoculated into SM. For the light incubations, long-term-starved and freshly starved cells (200

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TABLE 1. Light and CO inhibition of long-term-starved and freshly starved cells of *N. cryotolerans*

Inhibitor	Inhibition of:			
	Long-term-starved cells		Freshly starved cells	
	% Activity[a] (mean ± SD)	% Viable cells[b]	% Activity (mean ± SD)	% Viable cells
Light	2.6 ± 1.4 (n = 9)	1.0	25 ± 16 (n = 9)	0.88
CO	107 ± 11 (n = 15)	89.0	154 ± 19 (n = 9)	86.0

[a] Rate of ammonium oxidation (micromoles per liter per hour at 10^6 cells ml^{-1}) by samples incubated in light or CO for 1 week, divided by the rate for control cultures and expressed as a percentage.

[b] Viable cells, as determined by most probable numbers for light- or CO-incubated cells, divided by viable cells for control cultures and expressed as a percentage.

ml each, 10^6 ml^{-1}) were transferred into 500-ml flasks in triplicate. The flasks were sealed with neoprene stoppers, and one flask of long-term-starved cells and one flask of freshly starved cells were covered with aluminum foil. The flasks were placed under a continuous fluorescent light source (GEF15T8CW; 6.60 W m^{-2}) at 5°C. For the CO incubations, 25 ml each of long-term-starved and freshly starved cells was transferred to 60-ml serum bottles in triplicate. The bottles were septum stoppered. The headspaces of four bottles (two containing long-term-starved cells and two containing freshly starved cells) received injections of 0.5 ml of CO (99.3% pure). The other set received no additions. The bottles were incubated at 5°C.

Cells were examined for viability and nitrite production after 1 week. Each study was performed in at least triplicate. Light intensity was measured with a LI-COR 1800 spectroradiometer.

Time course of the effect of light on starving cultures. Chemostat cultures were harvested as described above, washed, and inoculated into two 2-liter flasks with 1.5 liters of SM. The final density of cells was approximately 10^7 ml^{-1}. One flask was wrapped in foil. Cells were allowed to recover for 24 h, and then both flasks were placed under continuous light (6.60 W m^{-2}) at 5°C. At increasing time intervals, 100 ml of the cultures was withdrawn and assayed for viability and ammonium oxidation.

Ammonium oxidation. After subjection to light or CO, the cells were placed in the dark. CO was removed from the cultures by shaking the cells (125 rpm) under a fume hood after removal of the septa from the serum bottles. To measure NH_4^+ oxidation activity, 714 μM NH_4^+ [as $(NH_4)_2SO_4$, buffered at pH 7.8 with 1 M HEPES] was added. The bottles were then shaken at 125 rpm at 5°C. Cells were killed after 24 h with 0.5 ml of 1 N HCl. Nitrite production was measured by the colorimetric method of Bendschneider and Robinson (1).

Viable counts. Viability was determined by the most-probable-number method, using the chemostat medium with 1 ml of phenol red $liter^{-1}$.

RESULTS AND DISCUSSION

The CO concentration used (11 μM) caused a greater than 90% reduction of ammonium-oxidizing activity of growing *N. cryotolerans* (Vanzella, M.S. thesis). *N. cryotolerans* cells that were not growing were not affected by CO (Table 1). Viability of the cells was unaffected. For long-term-starved cells, the rate of NH_4^+ oxidation after 1 week under a CO atmosphere was 107% of the control value. For freshly starved cells, the rate of NH_4^+ oxidation was 154% of the control value. These data elucidate the competitive nature of CO inhibition (9): in the absence of a substrate, there is no deleterious effect. The increase in activity probably resulted from attempts by the cells to regenerate reducing equivalents that were lost as a result of CO oxidation (9).

If ammonium-oxidizing bacteria are able to gain energy through oxidation of CO (9), they may be able to benefit from this alternate energy source in the absence of NH_4^+ (15). However, Jones and Morita (11) found that there was no positive effect on NH_4^+ oxidation rates or survival when cells were starved in the presence of CO.

The survival of the heterotrophic bacterium Ant-300 under hydrostatic pressure is enhanced after 1 week of starvation (16). The authors explained this barotolerance as a result of decreased metabolic activity. In other words, even though the population is not increasing in biomass during starvation, it is not declining because of detrimental environmental effects. This phenomenon also appears to occur with *N. cryotolerans*; whereas CO may be deleterious to actively growing cells, cells are not affected in the absence of substrate.

Light proved to be lethal to both sets of cells, as shown by the large decrease in viable cells (Table 1). After 1 week under light, both types of cells showed a dramatic decrease in the oxidation of substrate. However, freshly starved cells possessed a higher activity (25% of the control [dark-incubated] value) than did long-term-starved cells (2.6% of the dark-incubated control value). Viable counts for both sets of cells decreased by 99%. Photoinhibition of ammonium oxidizers is greater in the absence of NH_4^+. Hooper and Terry (6) found that the presence of NH_4^+ protected *Nitrosomonas europaea* from photoinhibition. Furthermore, increasing concentrations of NH_4^+ provide incresingly greater protection from photoinhibition (6, 27; Vanzella, M.S. thesis).

Our results are in agreement with those of previous studies. Since freshly starved cells possess greater energy reserves (11), it follows that they should benefit from the protective effects of NH_4^+ oxidation. The exact mechanism for NH_4^+ protection has not been elucidated, although some mechanisms have been postulated (6).

As in previous studies (7, 27; Vanzella, M.S. thesis), it was found that in the absence of NH_4^+, photoinhibition of *N. cryotolerans* increased with increasing time of illumination (Fig. 1). The time course data show that the rate of NH_4^+ oxidation decreased rapidly when nutrient-deprived cells were illuminated. A 50% reduction in activity occurred after approximately 4.8 days of illumination, compared with a slight increase in the activity of dark-incubated controls. By 2 weeks, the illuminated cells possessed only 0.14% of their original activity. The activity of dark-incubated controls decreased by only 10% over the 2-week period.

After 3 days of 12 h of light followed by 12 h of dark, Yoshioka and Saijo (27) were able to detect a significant lag in production of NO_2^- by a *Nitrosospira* sp. after addition of NH_4^+. Vanzella (M.S. thesis) observed a lag in production of NO_2^- by *N. cryotolerans* after only 3 h under continuous light.

There was a very large decrease in the number of viable cells in illuminated cultures (Fig. 1). Viable numbers of starving cells decreased by almost 4 orders of magnitude after 2 weeks of illumination, whereas no decrease was seen for the nonilluminated starving cells. Yoshioka and Saijo (27) also observed a dramatic decrease in the numbers of viable cells during illumination in the absence of NH_4^+. In con-

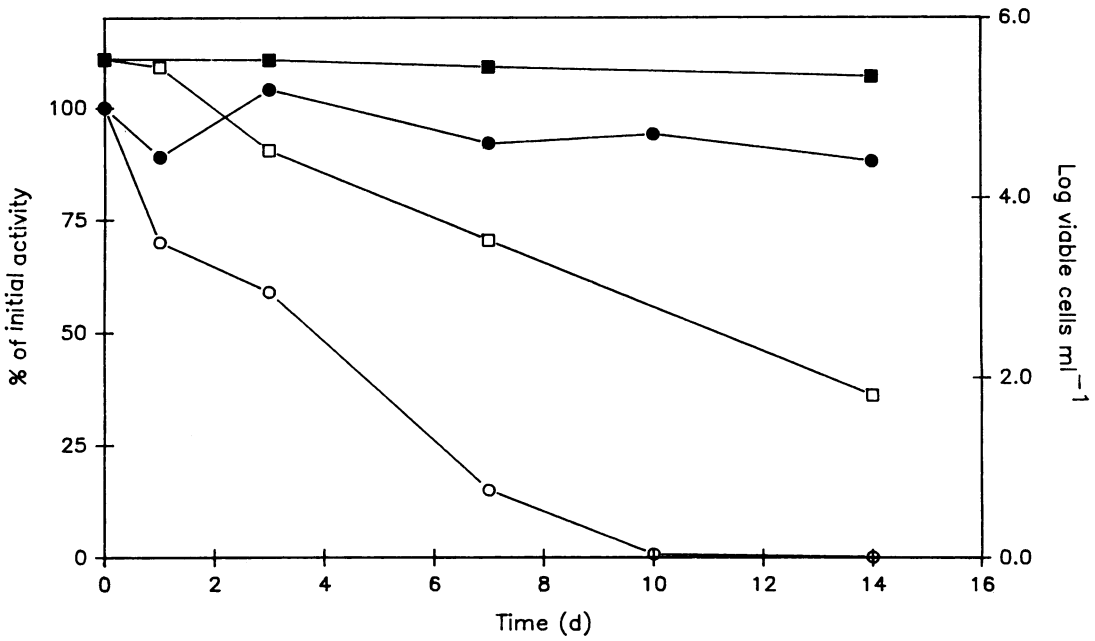

FIG. 1. Effect of light on *N. cryotolerans* undergoing starvation, showing ammonium-oxidizing activity of control (dark-incubated) (●) and light-incubated (○) cells and most probable numbers for control (■) and light-incubated (□) cells.

trast, Horrigan et al. (7) claimed that light was not lethal to ammonium-oxidizing bacteria. From these results and those of Yoshioka and Saijo (27), it appears that light inhibits substrate oxidation by ammonium oxidizers and is lethal only in the absence of substrate.

Because of a lack of metabolism, *N. cryotolerans* is not affected by CO. On the other hand, insusceptibility is not total, as evidenced by the decrease in both viability and activity of starved cells when illuminated in the absence of NH_4^+. Both long-term-starved and freshly starved cells showed a large degree of inhibition, but long-term-starved cells were much more susceptible to the effects of light. The fact that starving cells are susceptible to physical inhibitors shows that they do not enter a dormant state whereby they are protected from exogenous factors. This is a fundamental difference between an endospore and the starvation-survival state in this organism. However, other inhibitors (particularly those which inhibit key enzymes) must also be tested.

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