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Effect of Glucose Utilization on Nitrite Excretion by the Unicellular Cyanobacterium *Synechocystis* sp. Strain PCC 6803

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Up to 1 mM nitrite was excreted by *Synechocystis* strain 6803 cells growing under mixotrophic or photoheterotrophic conditions. This excretion is not due to a lower ratio of nitrite and nitrate reductase activities in the presence of glucose but seems to be related to a shortage of reduced ferredoxin, their electron donor, as a result of a decrease in noncyclic photosynthetic flow observed under these circumstances. Because about 60% of the reduced nitrate is excreted, the potential utilization of cyanobacteria for removal of nitrate from contaminated waters containing high concentrations of organic compounds is questioned.

Nitrate is an oxidized form of nitrogen that, at high concentrations, is a contaminant of groundwater, especially in fields treated with an excess of nitrogen fertilizer. Nitrate can be removed by several methods, e.g., physicochemical (nondegradative method) or bioreactor and microorganisms (degradative methods). In the latter case it is necessary to use denitrification enzymes or denitrifying microorganisms, which are difficult to maintain and sometimes produce undesirable air pollutants, including nitrous oxides (9, 10). Cyanobacteria are the only prokaryotes capable of reducing nitrate to ammonium by utilizing water as the primary electron donor and sunlight as the energy source. Nitrate assimilation takes place in cyanobacteria by a two-step process: (i) transport of nitrate inside the cells and (ii) its reduction to ammonium by the sequential action of ferredoxin-dependent nitrate and nitrite reductases (5). The reduction of nitrate in these organisms is a true photosynthetic process, which requires eight electrons donated by reduced ferredoxin, two for nitrate reductase and six for nitrite reductase (2, 5). The regulation of nitrate assimilation in cyanobacteria of the genera *Synechococcus* and *Anabaena*, in which the synthesis of nitrate and nitrite reductases is regulated by the nitrogen source, has been extensively studied; ammonium is a repressor in all cyanobacteria examined thus far (5–8, 11).

Nitrate assimilation has only rarely been studied in facultative heterotrophic cyanobacteria such as *Synechocystis* strain PCC 6803, a strain capable of utilizing glucose under mixotrophic and heterotrophic conditions (1, 12), probably through the pentose phosphate cycle (14) since glucose-6-phosphate dehydrogenase, the first enzyme of this pathway, has a high activity in *Synechocystis* cells in the light with or without glucose (unpublished results).

In this report we describe the excretion of nitrite by *Synechocystis* cells grown in the presence of glucose (mixotrophic growth) or with glucose plus 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (photoheterotrophic growth as defined in reference 14). This phenomenon reflects a different catalytic rate of the two reductases, most probably because of competition for available reduced ferredoxin required for reduction from nitrate to nitrite and from nitrite to ammonium.

Synechocystis sp. strain PCC 6803 was grown at 35°C under three different growth conditions. Cells were grown photoautotrophically under illumination in 1-liter Roux flasks containing 750 ml of BG11 medium (12), bubbled with a continuous stream of 5% (vol/vol) CO_2 in air. For mixotrophic and photoheterotrophic conditions, 1-liter Erlenmeyer flasks containing 500 ml of BG11 medium were supplemented with 0.11 M glucose or with 0.11 M glucose plus 5 μM DCMU, respectively. The flasks were shaken at 150 rpm in an orbital incubator illuminated with fluorescent white light from above at a light intensity of 13 W m^{-2}. All cultures were inoculated with cells grown on BG11 medium containing 40 μg of total chlorophyll and sampled every 24 h. When indicated, $NaNO_3$ was replaced by 15 mM NH_4Cl buffered with 30 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid–NaOH (TES-NaOH) buffer (pH 7.5). Nitrate reductase and nitrite reductase activities of *Synechocystis* cells were determined in situ as previously described (6, 8). Nitrate and nitrite were measured as described in references 3 and 15, respectively. Photosynthetic O_2 evolution in cells kept in 50 mM Tris-HCl buffer (pH 7.5) was determined, under saturating incandescent white light, by using a Clark-type oxygen electrode. Respiration was determined by measuring O_2 consumed in the dark, after the addition of glucose to a final concentration of 0.11 M; a Clark-type oxygen electrode was used for this purpose.

Nitrite excretion, at levels as high as 1 mM, was observed in *Synechocystis* cells growing under mixotrophic (Fig. 1B; Table 1) or photoheterotrophic (Table 1) conditions but not during photoautotrophic growth (Fig. 1A; Table 1). These results indicate deficient coordination of the two reductases when glucose is present, with nitrate reductase producing more nitrite than nitrite reductase is able to reduce. The fact that ammonium excretion was never observed implied also that the imbalance in nitrate assimilation occurred at the level of nitrite reductase, which was incapable of reducing all the nitrite produced by nitrate reductase. It is worth noting that the carbon/nitrogen ratio of *Synechocystis* cells grown with nitrate in mixotrophic cultures was 20% higher than that

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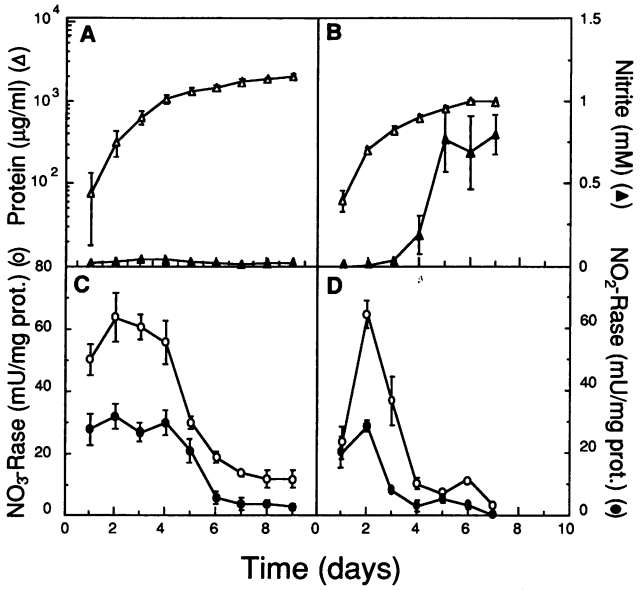

FIG. 1. Levels of nitrate and nitrite reductase activities and nitrite excretion of *Synechocystis* strain 6803 growing under photoautotrophic (A and C) and mixotrophic (B and D) conditions. Each point is the average of three separate experiments. Error bars show the standard error. Abbreviations: NO_2-Rase, nitrite reductase; NO_3-Rase, nitrate reductase.

of cells grown in photoautotrophic cultures with the same nitrogen source (Table 2). Such a difference in the C/N ratio between mixotrophic and photoautotrophic cultures was not found when cells were grown with ammonium instead of nitrate (Table 2), showing that cells grown under mixotrophic conditions had a lower rate of nitrogen assimilation when nitrate was the nitrogen source. Moreover, the comparison of growth rates of mixotrophic cultures utilizing nitrate with those of cultures utilizing ammonium (7.5- and 5.0-h doubling times, respectively) supports this fact. Therefore nitrite excretion seems to be a symptom of a hampered process of nitrogen assimilation and not the consequence of an increased nitrate reduction rate.

Nitrite excretion seems not to be due to a different nitrate/nitrite reductase ratio under mixotrophic conditions, since even higher ratios between the two activities were observed for photoautotrophically growing cells, while no excretion of nitrite was detected (Fig. 1).

Over time, nitrate reductase and nitrite reductase activities developed in a similar fashion; the specific activity was maximal at the end of the exponential growth phase (Table 1) and decreased during the stationary phase (Fig. 1C and D). This time course agrees with the behavior of enzymes that have a clear anabolic function.

TABLE 2. Carbon/nitrogen elemental ratio in *Synechocystis* strain PCC 6803 cells grown under different culture conditions[a]

Growth conditions	Carbon/nitrogen ratio[b]
Photoautotrophic	
Nitrate	4.41 ± 0.053
Ammonium	4.03 ± 0.057
Mixotrophic	
Nitrate	5.27 ± 0.007
Ammonium	4.23 ± 0.026

[a] Samples were taken 4 days (photoautotrophic) or 5 days (mixotrophic) after inoculation, washed, and dried, and the carbon/nitrogen ratio was determined with an elemental analyzer.
[b] Data are the mean of at least two independent experiments ± standard error.

To determine the rates of nitrate utilization and nitrite excretion by photoautotrophic and mixotrophic cells, *Synechocystis* cells grown under both conditions were harvested at the time when the nitrite excretion rate was maximal, washed, and resuspended on fresh medium with nitrate at a final concentration of 0.3 mM. As shown in Fig. 2, mixotrophic cells took up nitrate at a higher rate (22 ± 2.4 μmol mg of chlorophyll^{-1} h^{-1}) than did photoautotrophic cells (13 ± 1.8 μmol mg of chlorophyll^{-1} h^{-1}), but only in the first one was a significant amount of nitrite excreted, reaching a final concentration of 0.18 mM after 2 h, which corresponded to 60% of the nitrate taken up by the cells. Thus, when the cells were grown on glucose the net nitrate utilization rate was about 8 μmol · mg of chlorophyll^{-1} h^{-1}. This result supports the view that *Synechocystis* cells exhibit impaired nitrite reduction in the presence of glucose.

Glucose, in addition to being a carbon source, can lead to substantial NADPH production (14). On the other hand, glucose decreased photosystem II activity, determined by O_2 evolution, when compared with photoautotrophic conditions (Table 1). Respiration rates (O_2 uptake) were higher in glucose-grown cells than in photoautotrophically grown cells (Table 1). Similar results have been reported previously for the cyanobacterium *Anabaena variabilis* ATCC 29413 during mixotrophic growth on fructose (4). Therefore, the rate of photosynthetic reduction of ferredoxin should be lower under mixotrophic than under photoautotrophic conditions. Although NADPH, produced by glucose oxidation, can

TABLE 1. Maximum levels of nitrate and nitrite reductase, O_2 evolution and consumption, and nitrite excretion to the medium of *Synechocystis* cells under different growth conditions

Growth condition	Maximum concn (mU/mg of protein) of[a,b]:		Amt of O_2 (μmol/mg of chlorophyll/h)[b]:		Nitrite excretion (mM)[b]
	NO_3-Rase	NO_2-Rase	Evolved[c]	Consumed	
Photoautotrophic	64 ± 8	22 ± 2	232 ± 25	18 ± 2	0.005 ± 0.00
Mixotrophic	65 ± 6	29 ± 3	78 ± 10	53 ± 6	0.800 ± 0.15
Photoheterotrophic	49 ± 5	19 ± 2	ND[d]	51 ± 2	1.300 ± 0.18

[a] Both nitrate and nitrite reductase levels were determined in cells growing on nitrate as the nitrogen source. Abbreviations: NO_3-Rase, nitrate reductase; NO_2-Rase, nitrite reductase.
[b] Each value represents the mean of at least two independent experiments ± standard error.
[c] Photosynthetic oxygen evolution equals O_2 evolution in the light plus O_2 consumption in the dark.
[d] ND, not detected.

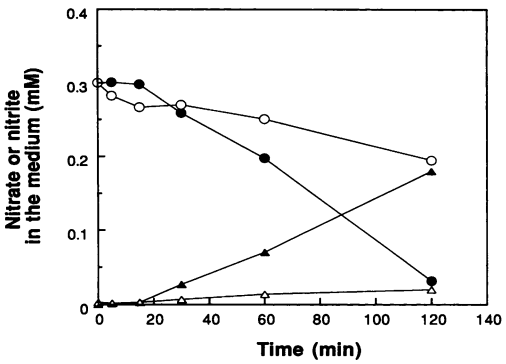

FIG. 2. Consumption of nitrate and excretion of nitrite by *Synechocystis* cells. The cell suspensions contained 4.5 μg of chlorophyll ml^{-1} (photoautotrophic growth), and 7.0 μg of chlorophyll ml^{-1} (mixotrophic growth), respectively. At zero time, 0.3 mM nitrate was added to each culture. Nitrate and nitrite (○, △, photoautotrophic; ●, ▲, mixotrophic) were determined at the indicated times.

reduce ferredoxin by the reverse reaction of ferredoxin: NADP reductase (13), a large amount of NADPH is required to overcome the difference in midpoint redox potentials (−0.42 V for reduced ferredoxin/oxidized ferredoxin versus −0.32 V for NADPH/NADP). Our results can be explained if the reduction of the ferredoxin pool by NADPH is not efficient enough to maintain nitrite reduction and, as a consequence, nitrite is excreted. In addition, the presence of DCMU, a compound which inhibits electron flow between photosystems II and I, increased nitrite excretion by about 60% in glucose cultures (Table 1), indicating that a decrease in the level of reduced ferredoxin could cause nitrite excretion under both conditions.

The fact that nitrite reductase reaction requires six reduced ferredoxins, whereas nitrate reductase requires only two (2), argues that under conditions where the available reduced ferredoxin for both reductases is limiting, nitrite reductase is unable to reduce all nitrite produced by nitrate reduction.

In conclusion, although cyanobacteria are efficient organisms at photosynthetic nitrate utilization (5), their potential use for removal of nitrate from water sources should be carefully evaluated to avoid the production of nitrite, an even more noxious contaminant than nitrate. This possibility is most likely when organic matter and other compounds which could interfere with the photosynthetic process are present in contaminated water.

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