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# EVIDENCE FOR NITRIFICATION IN VIRUS INFECTED CYANOBACTERIAL CULTURES MAINTAINED WITH AMINO- ACIDS (AMINES) AS SOLE NITROGEN SOURCES

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## ABSTRACT

Cyanobacteria Phormidium uncinatum, Plectonema boryanum, Nostoc muscorum and Anacystis nidulans were grown/ incubated in combined nitrogen-free or nitrate, ammonium, glutamine or asparagines supplemented medium and infected with there respective cyanophages LPP-1, N-1, AS-1. The final cyanophage yield drastically reduced in nitrogen starved cultures but remained significantly high and roughly uniformed in combined nitrogen cultures. The nitrite and ammonia contents of Phormidium uncinatum cultures, sustained with amino acids (amines) as sole nitrogen sources, were considerably high due to inorganic nitrogen fixing and nitrate assimilating cultures failed to liberate any of the inorganic nitrogen and only nitrate was detected in ammonia supplemented cultures.

In all cases , cyanophage infection rendered significantly high ammonia and nitrite liberation activities in amino acids (amines) sustained cultures (inact and broken cells) when compared with respective healthy cultures. Nitrification like "ammonia oxidation" reaction was established and assayed in cellular and cell-free preparations of infected and uninfected cyanobacteria. These data suggest a potent role of ammonia and amino acids (amines) oxidation in supporting phage multiplication via generating additional cellular reductants.

**Key words:** Cyanobacteria, ammonia oxidase, combined nitrogen sources nitrification.

## INTRODUCTION

Infection of a cyanobacterial (blue green algae) cell with cyanophage is well known to result in a dramatic alteration in the physiology of the host. Substantial modification in host nucleic acid and protein synthesis or break down[9,27,28], photosynthetic $CO_2$ fixation[8,19], nitrite metabolism[5,7], oxidative pentose pathway and respiration[6,12], after cyanophage infection are well documented. Multiplication of cyanophage is generally accepted as being dependent on light through host photosynthesis. Oxygenic photosynthesis of host cyanobacteria has been claimed to be the mechanism for mainly providing energy (ATP) for cyanophage development[21]. Besides energy, a constant supply of reductants (NADPH) is also a fundamental requirement for phage DNA synthesis. Virus infected syanobacterium cells have been suggested to devise a mechanism to channel most of its carbon assimilate through glucose-6-phosphate dehydrogenes mediated reaction for generating NADPH[6]. There has been a report of cyanophage LPP-1 mediated blockage of *Plectonema* $CO_2$ fixation[19] in order to deviate most of the photo synthetically generated NADPH for phage multiplication.

Here, we provide evidence for oxidative deamination reaction of amino acids (amines) and subsequent ammonia oxidation to nitrite by certain cyanobacterial species. These physiological processes are quite analogous to nitrification, originally detected in some chemoautotrophic bacteria but periodically reported in other bacteria and fungi[18], green algae and higher plants[13]. This is the first report of its kind in cyanobacteria. The activities in virus infected cyanobacteria

substantially increased with varying degree when compared with respective healthy cultures. Such oxidation reactions appear to supply additional reductants during phage multiplication.

## MATERIALS AND METHODS

### *Cyanobacteria and Cyanophages*

The axenic cultures of *Phormidium uncinatum* (IU 1462/7), *Plectonema boryanum* (IU 590) and *Anacystis nidulans* (IU 625) as well as their respective phages LPP-1 and AS-1 were obtained through the courtesy of Dr. R.S. Safferman, National Environmental Centre, Cincinnati, Ohio, USA. Cyanobacterium *Nostoc muscorum* (ATCC 7120) and its cyanophage N-1 were kindly provided by Dr. D.V. Amla, National Botanical Research Institute, Lucknow (India). Cyanobacterial cultures were routinely maintained and grown in modified Chu-10 basal medium (14) supplemented with 5mM $KNO_3$ ($NO_{3-}$ - medium). Cultures were grown in air-conditioned culture room at 28°C and illuminated with cool white fluorescent light (light intensity 40-50µmol m-2 s-1 photon fluence rate). Where ever required the basal Chu-10 medium ($N_2$ medium) was enriched with asparagines (10mM), glutamine (10mM) or $NH_4Cl$ (5 mM) and buffered to pH 8-8.5 with 25 mM HEPS/ NaOH buffer and referred to as asparagine, glutamine or $NH_4^+$ medium. Host cyanobacteria were subcultered at every 8-12 days interval by aseptically transferring aliquots of 5 ml exponentially growing cultures to 200 ml fresh media. Subsequently, these cultures were manually shaken twice in a day to keep exponential condition for experiments. Cyanobacterial cells were counted by employing Feinoptic Haemocytometer. An average of three independent readings was taken into consideration.

Cyanophages were obtained for the experiments by infecting batch cultures of host cyanobacteria ($5\times10^7$ cells $ml^{-1}$) with the respective viruses at a multiplicity of infection of 0.1. After lyses, the lysates were centrifuged for 10 min at 10,000 × g to separate the cell debris. The virus concentration was determined throughout the experiments by using the plaque assay techniques[20]. Aliquots of 0.5 ml diluted lysate were thoroughly mixed with 2 ml of thick liquid cyanobacterial host suspension and 2.5 ml of melted nutrient agar medium with 1% agar and plated on agar plates having a base of 0.8% agar containing medium (30 ml in 100 mm × 17 mm petri dishes). The inverted plates were counted for the resulting plaque counts after four days of incubation. The clonal populations of virus were raised from a single plaque isolate and lysates were always filtered through a sintered glass filter (2.5 cm GF/C; Whatman, England) before being used in experiments. Bacterial contamination in the cultural/ lysates was checked at every stage.

For infection experiments, exponentially growing cultures of the host cyanobacteria in late log phase were adjusted to a cell density of approximately $5\times10^7$ cells $ml^{-1}$ and infected with respective cyanophages at an initial multiplicity of infection of 5. One-step cyanophage growth was carried out[19] with the initial multiplicity of infection value set at 0.1.

### *Nitrogen Incubation Experiments*

Exponentially growing cyanobacterial cultures in late log phase were first centrifuged and washed with sterile $N_2$ medium and subsequently transferred to media with various nitrogen source for 24 h pre-incubation. Such pre-incubated cultures were then shifted to experimental media consisting of respective nitrogen sources and infected as per requirement. Cultures supplemented with equivalent volumes of heat killed viral lysates (healthy cultures) were considered as controls. Viral yield in infected cultures were estimated after complete cell lysis.

Both nitrite and ammonia levels in extra cellular media were detected throughout the incubation period. For this, 5 ml aliquots of cultures (healthy and infected) were centrifuged and supernatant used for colorimetric detection of nitrite[25] and ammonia[26]. Chlorophyll content was estimated by following the method of[17]. Exogenous nitrogen did not interfere $NO_2$ and $NH_4^+$ detections.

### *Estimation of "Ammonia Oxidase" Enzyme*

The samples (healthy and infected cultures), at times indicated, were harvested by centrifugation (10,000 × g for 15 min), washed and suspended in 50 mM Tris/ HCl buffer (pH 7.5) containing $NaCl_2$ (5 mM). The in-situ and in-vivo enzyme activities were detected respectively in

permeabilized cell and cell-free preparations. For assaying cellular enzyme, toluene (2% v/v) was added to above cell suspension and vigorously shaken for 4 min in order to obtain permeabilized cells[3]. A portion of this preparation was added to a reaction mixture containing in a final volume of 1 ml: Tris/ HCl buffer pH 7.5, 100 µmol; $NH_4Cl$, 10 µmol; 220 µmol. Assays were carried out at 30 ± 2°C in open test tubes after adding $NH_4Cl$ (substrate) and $H_2O_2$ (Oxidant), for a period of 10 min. Appearance of nitrite (product) was colorimetrically estimated[25] against a blank (assay mixture without $NH_4Cl$) as a reference.

Cell-free extract (crude) of above mentioned cell suspension was prepared and used as a source of enzyme. Cells were sonicated for 10 min at 4°C at an interval of 2 min after every 2 min of sonication using a MSE-MK Ultrasonicator and subsequently centrifuged at 20,000 × g for 20 min at 4°C. A portion of the supernatant was added to the reaction mixture as stated above and enzyme activity detected.

Specific enzyme activity was represented as µmol $NO_2$ formed in one unit of time per mg protein of extract or per mg chlorophyll of permeabilized cells. Protein content of extract was measured using bovine serum albumin as standard[16].

Asparagine and glutamine were freshly prepared when required and sterilized by filtering through membrane filter (0.45 µm pore size, Sartorius), before being added to sterilized medium.

All chemicals were purchased at highest purity grade from M/S. Sigma Chemicals Co. (U.S.A.) and B.D.H. (U.K.).

## RESULTS AND DISCUSSION

The reproductive cycle of AS-1 in *Anacystis nidulans* completed within 18 h, N-1 in *Nostoc muscorum* and LPP-1 in *Phormidium uncinatum* and *Plectonema boryanum* established after 48 h of infection. Furthermore, the cyanophage growth pattern was almost identical in all cultures irrespective of the nitrogen source used (data not shown). The lysates obtained from above experiments were used for detecting the final burst size (virus yield).

Nitrite in the culture medium supported maximum virus yield whereas its replacement by ammonia, glutamine or asparagine slightly reduced the final burst size. Virus production in nitrogen starved *Plectonema boryanum*, *Phormidium uncinatum* and *Anacystis nidulans* cultures (incubated in $N_2$ medium) reduced significantly when compared with their combined nitrogen cultures. However, the burst size of N-1 in N-2 grown cultures which is apparently due to the $N_2$ fixing ability of the host, thereby supplying nitrogen for phage multiplication (Table -1).

**Table -1: Yield of cyanophage LPP-1 and N-1 in host cyanobacteria *Phormidium uncinatum, Plectonema boryanum*, *Anacystis nidulans* and *Nostoc muscorum* , grown/ incubated with different nitrogen sources**

| Cyanobacteria and Nitrogen source | Virus yield PFU/ Infected cell |
|---|---|
| *Phormidium uncinatum*/ LPP-1 | |
| Chu 10 ($N_2$ – medium) | 15 |
| Chu 10 + $NO_3$ medium | 265 |
| Chu 10 + $NH_4$ medium | 225 |
| Chu 10 + glutamine medium | 216 |
| Chu 10 + asparagine medium | 218 |
| *Plectonema boryanum*/ LPP-1 | |
| Chu 10 ($N_2$ – medium) | 26 |
| Chu 10 + $NO_3$ medium | 325 |
| Chu 10 + asparagine | 298 |
| *Anacystis nidulans*/ AS-1 | |
| Chu 10 ($N_2$ – medium) | 5 |
| Chu 10 + $NO_3$ medium | 91 |
| Chu 10 + asparagine | 85 |
| *Nostoc muscorum*/ N-1 | |
| Chu 10 ($N_2$ – medium) | 102 |
| Chu 10 + $NO_3$ medium | 126 |
| Chu 10 + asparagine | 114 |

Preincubation and experimental conditions are described in “Materials and Methods” section. The values presented are mean of three independent experiments.

PFU- Plaque Forming Units

**Table - 2: Liberation of Nitrite and ammonia by healthy and virus infected *Plectonema boryanum*, *Anacystis nidulans* and *Nostoc muscorum* cultures grown/ incubated with different nitrogen sources**

| Cyanobacteria and Nitrogen source | | Inorganic Nitrogen in the medium (μM) | |
|---|---|---|---|
| | | Ammonia | Nitrite |
| *Plectonema boryanum* | Healthy | nd | 4 |
| Chu 10 + asparagine | Infected (LPP-1) | 3600 | 250 |
| *Anacystis nidulans* | Healthy | 98 | 4.6 |
| Chu 10 + asparagine | Infected (AS-1) | 3500 | 335 |
| *Nostoc muscorum* | Healthy | nd | 4.7 |
| Chu 10 + asparagine | Infected (N-1) | 3400 | 216 |

Healthy and infected cultures were incubated for complete cyanophage multiplication keeping the initial culture density 1.5 ug chl $ml^{-1}$. Changes in nitrate and ammonia levels of cultures were measured after incubation.

nd- not detectable
Values given are average of three parallel readings.

Fig. -1 (A and B) presents the data on ammonia and nitrite liberation kinetics of healthy and infected *Phormidium uncinatum* sustained with asparagine as nitrogen source. The uninfected cyanobacterium released substantial amounts of ammonia and nitrite in the medium (Fig. -1A). In comparison, much higher levels of nitrite (3.4 fold) and ammonia (1.8 fold) were detected in LPP-1 infected cultures under similar conditions (Fig. -1B). Furthermore, the nitrite and ammonia liberation kinetics of healthy and infected cultures were nearly identical. To examine, whether the characteristics release of two inorganic nitrogen is governed by the nature of nitrogen nutrition, similar experiments were conducted with healthy and LPP-1 infected *Phormidium uncinatum* cultures incubated under nitrogen deficient condition ($N_2$ grown cultures) or grown with $NO^-_3$, $NH^+_4$ or glutamine nitrogen source. Glutamine grown healthy *P. uncinatum* cultures liberated 20μM and 237μM of ammonium and nitrite. Such cultures, when infected, generated appreciable levels of inorganic nitrogen (400μM of $NH^+_4$ and 990μM of $NO^-_2$). Ammonium grown healthy and infected cultures showed rather similar ammonium uptakes rate (30-40μmol $NH^+_4$ uptake $min^{-1}$ $mg^{-1}$ chlorophyll). However, under these conditions low but consistent level of nitrite was detected in surrounding medium (healthy 58 μM and infected 250 μM). Nitrite grown and nitrogen starved ($N_2$ cultures) *Phormidium uncinatum* cells (infected and normal) failed to liberate inorganic nitrogen.

Table -2 illustrates ammonia and nitrite liberation activities of normal and phage infected *Nostoc muscorum*, *Plectonema boryanum* and *Anacystis nidulans* cultures grown with asparagine nitrogen. Unlike *Phormidium uncinatum*, the three cyanobacteria showed comparatively poor to negligible amounts of extracellular ammonia and nitrite, however, infection appreciably enhanced the inorganic nitrogen levels. Under latter conditions especially ammonia contents were substantially increased 3.5 mM when compared with that of *Phormidium uncinatum*/ LPP-1 system (0.9 mM). Healthy and virus infected three cyanobacteria failed to liberate any of the inorganic nitrogen when grown/ incubated in $N_2$ or $NO^-_3$ medium (data not shown). In general, the infected cyanobacterial cells sustained with ammonia and amino acids (amines)

liberated considerably higher levels of inorganic nitrogen than the respective healthy cultures. In any situation, $NO^{-}_{3}$ was not detected in culture media (data not shown).

An attempt was further made to determine the cause of biological conversion of amino acids (amines) to inorganic nitrogen. Repression of $N_2$ fixation by *Nostoc muscorum* in presence of amino acids (amines) and ammonia[23,24]) and lack of $N_2$ fixing ability in rest of the three cyanobacteria led us to consider that both inorganic nitrogen are derived from exogenous organic nitrogen (asparagine and glutamine) rather than from atmospheric nitrogen. Hence, we decided to look for evidence of nitrification in healthy and infected cyanobacteria maintained with asparagine nitrogen. An assay procedure for the enzyme(s) involved in ammonia oxidation to nitrite (designated by a hypothetical name “ammonia oxidase”) was established. Substantial enzyme activity was detected both in permeabilized cells and cell free preparations (crude) with $H_2O_2$ (*i.e.* oxidized glutathione or potassium ferricyanide) or after omitting $NH_4Cl$ or $H_2O_2$ in the assay mixture also gave negative results. The physico-chemical parameters for optimum enzymatic activity described under Materials and Methods were standardized in advance. Among healthy cultures of all four cyanobacteria, the maximum enzymatic activity was experienced in *Phormidium uncinatum*. Permeabilized preparations of *Plectonema boryanum*, *Anacystis nidulans* and *Nostoc muscorum* had low but consistent levels of ammonia oxidase, however, activity was negligible in cell-free preparations. The activity of the cyanobacterial cultures substantially enhanced following virus infection (Table -3). The cellular and cell-free enzyme activities of uninfected *Phormidium uncinatum* were approximately 65% and 30% of infected cyanobacterium. In comparison, the activity levels in phage infected *Plectonema boryanum*; *Anacystis nidulans* and *Nostoc muscorum* cells were respectively 17, 7.9 and 10 folds higher than corresponding healthy cultures (Table -3). Similarly, alleviated enzyme levels were detected in cell-free preparations, obtained from virus infected three cyanobacterial hosts.

The study of cyanophage reproductive cycle in nitrogen sufficient or deficient cyanobacterial cells, shows a direct dependence of the process on availability of exogenous fixed nitrogen source. It is suggested that combined nitrogen in any assimilable form plays tremendous role in supporting phage protein synthesis. Our data on final burst size of virions in combined nitrogen supplemented cyanobacterial cells (Table -1) is in accord with the earlier reports[2,19]. However, a small yield of virions in nitrogen starved *Phormidium uncinatum*, *Plectonema boryanum* and *Anacystis nidulans* cells are attributed to the internal protein breakdown. A protease mediated cells proteins and phycocyanin degradation has been made evident in nitrogen starved *Anacystis nidulans* $R_2$[11]. $N_2$ grown *Nostoc muscorum* cultures apparently support the nitrogen demand of N-1 development through efficient diazotrophy.

**Table 3: Cellular and cell free activities of “ammonia oxidase” enzyme system(s) of healthy and infected cyanobacteria with asparagines nitrogen**

| Cyanobacteria | | Enzyme activity (n mol $NO_2$ – formed/ min) Cellular | Cell-free |
|---|---|---|---|
| *P. uncinatum* | Healthy | 30.6 | 16.2 |
| | Infected | 46.8 | 57.8 |
| *P. boryanum* | Healthy | 2.2 | nd |
| | Infected | 37.9 | 34.0 |
| *A. nidulans* | Healthy | 3.6 | nd |
| | Infected | 27.0 | 28.8 |
| *N. muscorum* | Healthy | 4.6 | nd |
| | Infected | 40.8 | 56.6 |

Inocula preparations were as in Table -1. Healthy and infected cultures with initial culture density 1.6 ug Chl/ ml were incubated for half the period needed for a complete cyanophage growth cycle, and enzyme activities were represented in terms of per mg chlorophyll and protein, respectively. An average of three independent readings were taken into consideration.

nd- not detectable

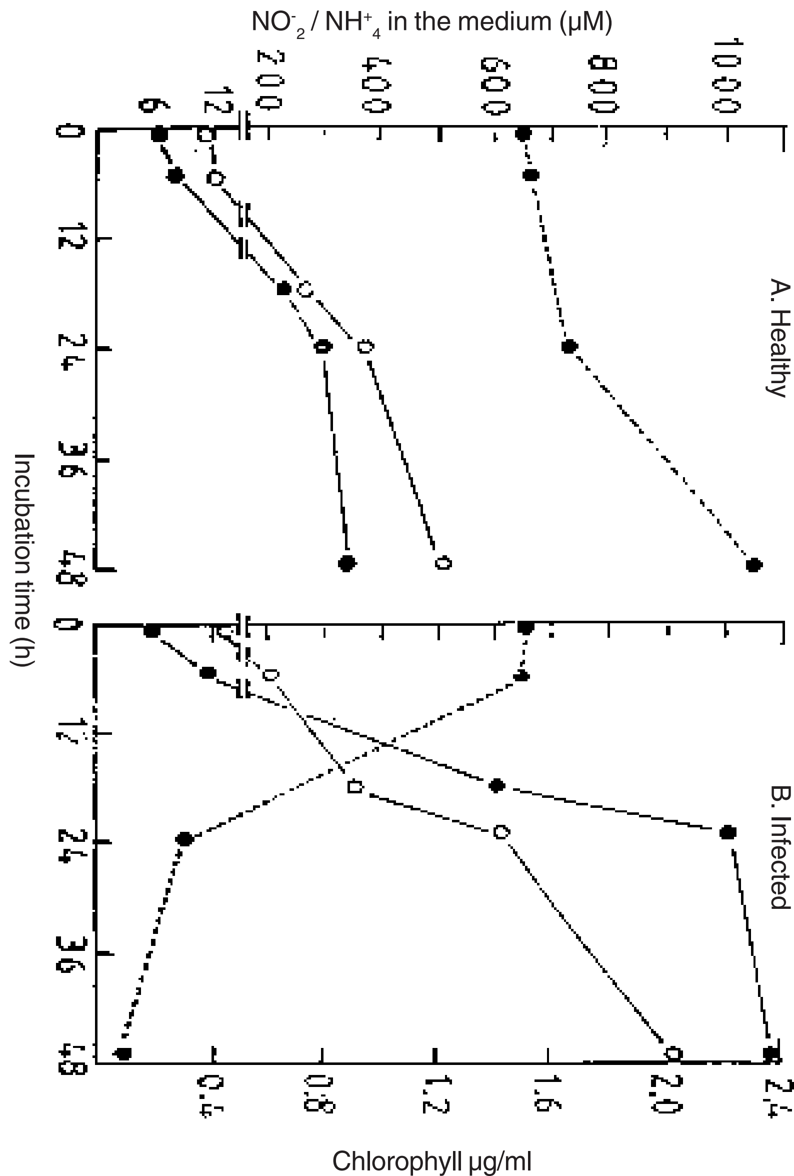


**Fig. -1**

The unusually high levels of nitrite and ammonia in glutamine and asparagines sustained *Phormidium uncinatum* cultures were rather unexpected. It is suggested that efficient oxidative deamination of exogenous amino acids (amines) seems to be the first step of oxidation, leading to ammonia release. The findings of[10] on the mechanism of ammonia release by *Anabaena cylindrica* through deamination of amino acids supports our views. A stimulatory role of phage multiplication on host deamination reaction is based on the observed alleviated $NH^+_4$ levels in the medium of infected cyanobacteria (Fig.-1). The possibility of ammonia liberation following lysis of infected cells cannot be ruled out, however, the significant fraction of this nitrogen appears to be released out of intact cells.

The second step of oxidation reaction, leading to the conversion of endogenously generated or externally supplied ammonia to nitrate is conventionally known as nitrification.

A novel enzyme system has been identified in cyanobacteria, showing the catalytic function of ammonia oxidation to nitrite (ammonia oxidase) (Table 3). Such oxidative deamination and ammonia oxidation are the processes, quite analogous to nitrification and related oxidation reaction[1, 13, 18] detected in various organisms including green algae[15, 27]. In fact, enzyme capable of heterotrophic nitrification has been isolated and catalytic activity analyzed in green algae[29]. To data, nitrate has been claimed to be the final oxidation product of nitrification. Therefore, it can be postulated that the cyanobacterial "nitrification" stops at the level of nitrite without its further oxidation to nitrite, may be due to the absence of nitrite oxidase enzyme system in cyanobacteria.

Oxidative deamination of amino acids (amines) to ammonia and its subsequent oxidation to nitrite, two characteristic features of cyanobacterial metabolism, appear to be lifted by cyanophage attack. Under artificial assay conditions, $H_2O_2$ serves as the best oxidant for "ammonia oxidase" enzymatic activity. Speculations can be made that NADP is the physiological oxidant for this enzyme. Nitrification reaction in crude extracts of green algae has been coupled with continuous reaction of NADP to $NADPH_2$ [29]. In such conditions, virus infection may enhance cellular $NADPH_2$ production of host cyanobacteria through lifting the process of "nitrification". Enhancement of glucose-6-phosphate dehydrogenase activity in *Anacystis nidulans* after cyanophage AS-1 infection has been proposed to be the sole cause for the alleviated production of $NADPH_2$ in order to support phage development[6]. $NADPH_2$ may either undergo reoxidation by highly active NADPH-oxidase system[6] or get consumed for intensive viral DNA synthesis[6, 22, 28].

In viral infected cyanobacterial cell, external amino acids (amines) and ammonia may, therefore, be metabolized mainly via two important pathways. Most of the nitrogen is assimilated for biosynthetic reaction to meet phage protein demand. A fraction of these nitrogen, however, diverts to "nitrification" like reactions for generating additional cellular reactants.

Sherman and Pauw[22] while characterizing the nature of new proteins of AS-1M infected *Synechococcus cedrorum* cells, clearly indicated the possibility of a number of enzymatic activities yet undetected. In this connection, it is noteworthy to identify set of enzyme(s) in cyanobacteria involved in "nitrification" like reactions. Further, the implication of such metabolic processes of host in viral multiplication would be a major line of future investigations.

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