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Metronidazole as a specific inhibitor of nitrogenase activity in the heterocystous cyanobacterium *Nostoc* ANTH, an isolate from *Anthoceros punctatus*

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The effect of metronidazole on growth and nitrogen metabolism of the N_2-fixing heterocystous cyanobacterium *Nostoc* ANTH and its paraquat-resistant (PQ-R), chlorate-resistant (Clo-R) and Het$^-$Nif$^-$ mutants was studied. Metronidazole inhibited the diazotrophic growth of *Nostoc* ANTH and its PQ-R and Clo-R mutants when grown in the absence of combined nitrogen. The drug inhibited nitrogenase activity completely within 3h of treatment but did not affect the activities of nitrate reductase and glutamine synthetase, and ammonium transport. All the strains grew well in presence of metronidazole when the growth medium was supplemented with sources of combined nitrogen. The Het$^-$Nif$^-$ strain, which lacked heterocysts and was unable to fix N_2 remained unaffected by metronidazole in medium supplemented with combined nitrogen. The inhibition of diazotrophic growth by metronidazole was ascribed to the impairment of nitrogenase activity.

Key words: glutamine synthetase, metronidazole, nitrate reductase, nitrogenase, *Nostoc*.

Metronidazole (2-methyl-5-nitroimidazole-1-ethanol) inhibits microbial growth. The breakdown products of reduced metronidazole are suggested to be responsible for this antimicrobial activity[1,2,3]. The drug specifically blocks ferredoxin- and flavodoxin-linked reactions, including nitrogen fixation in phototrophs by serving as an electron acceptor from reduced ferredoxin/flavodoxin[4,5,6].

Reports of metronidazole toxicity to N_2-fixing organisms are few and sometimes contradictory. The inhibitory effect of metronidazole on growth and N_2 fixation by bacteria growing on molecular nitrogen is reversed in presence of combined sources of nitrogen[5,7]. However, such studies are inconclusive in the case of diazotrophic cyanobacteria. While some have reported growth inhibition of N_2 fixation and diazotrophic growth in *Anabaena* spp.[8,9], there is one report indicating growth inhibition in *Nostoc muscorum* even in ammonium-containing medium[10]. Furthermore, high concentrations of metronidazole used (0.6 to 2 mM) in these studies can not preclude the adverse effect on cellular metabolism[8,9,10]. Thus, effects of metronidazole on diazotrophy in cyanobacteria merit further study.

The present study on *Nostoc* ANTH suggests that at a concentration of 0.2 mM, metronidazole or a product of its break-down specifically inhibits nitrogenase activity and that the drug has no effect on the activities of nitrate reductase, glutamine synthetase or ammonium transport.

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Materials and Methods

Nostoc ANTH (isolated from *Nostoc-Anthoceros* symbiosis) and its paraquat-resistant (PQ-R), and chlorate-resistant (Clo-R) mutant strains were grown in batch cultures in BG-11_0 (N-medium) and Het$^-$Nif$^-$ mutant strains in BG-11_0 medium supplemented with 5 mM KNO_3, at 25^{0}C and a photon fluence rate of 50 µmol.m^{-2}.s^{-1} as described in Rippka *et al.*[11]. Where ever needed BG 11_0-medium was supplemented with a source of combined nitrogen (in the form of 2 mM NH_4Cl, 5 mM KNO_2, 5 mM KNO_3, 1 mM glutamine, 1 mM asparagine, 1 mM arginine and 1 mM urea and buffered with equimolar concentration of HEPES (pH 7.5). The Chlorate-resistant (Clo-R) and Het$^-$Nif$^-$ mutant strains of *Nostoc* ANTH were those isolated earlier[12,13]. Spontaneously occurring paraquat-resistant (PQ-R) mutants of *Nostoc* ANTH were obtained by plating approximately 4x10^8 colony forming units (CFU) on solid N-medium containing 0.4 µM paraquat (lethal concentration, 0.06 µM). After three weeks of incubation, the few surviving colonies were picked up and re-streaked on plates containing 0.4 µM paraquat. The PQ-R mutants arose at a frequency of 1.9x10^{-8}. One such mutant Clone was then selected and used in the present study.

Phycocyanin and allophycocyanin content was measured by using the method of Bennett and Bogorad[14] Chlorophyll *a* content was measured as described by Mackinney[15] and protein content as described by Lowry *et al*[16]. Heterocyst frequency was determined by counting number of heterocysts per 100 vegetative cells under a light microscope.

Nitrogenase activity was determined as before[17]. Glutamine synthetase (transferase) and nitrate reductase activities were estimated in alkyltrimethyl-ammoniumbromide (CTAB) permeabilised cells as described by Sampio *et al*[18] and Manzano *et al*[19], respectively.

Ammonium transport activity was measured using the ammonium analogue [^{14}C] methylammonium as described earlier[20]. Exponentially growing *Nostoc* ANTH cells were harvested by centrifugation, washed twice in 10 mM HEPES-NaOH buffer (pH 7.0) and resuspended in the same buffer to a chlorophyll concentration of 5 µg mL^{-1}. They were equilibrated for 30 min under culture growth condition. Methylammonium uptake activity experiments were carried out by adding [^{14}C] methylammonium (Sp. Activity 172 $KBq.\mu mol^{-1}$) to a final concentration of 50 µM. 400 µL samples were withdrawn at specific time intervals and cells were separated from their bathing medium by centrifugation through silicon oil DC 550/dinonylphthalate (40/60, v/v) into perchloric acid/water (15/85, v/v)[21]. The [^{14}C] quantity in perchloric acid fraction was determined by using a liquid scintillation counter (Model 1801, Beckman Instruments). Non-specific binding of radioactivity was determined by measuring its incorporation in toluene treated cells[20]. These values were always subtracted from the values obtained for toluene-untreated samples. Nitrogen content of *Nostoc* ANTH cells was determined using an automated CHNS Analyser (Elementar/vario EL-III).

Results

When transferred to N-medium, *Nostoc* ANTH grew at the expense of N_2 as the sole nitrogen source. This was evident from the increase in chl *a*, phycocyanin, allophycocyanin and protein content over the three days experimental period. Addition of metronidazole (0.2 mM) caused cessation of growth. There was no increase in chl *a* content while phycocyanin, allophycocyanin and protein content declined. The sharpest decline was noted in phycocyanin followed by allophycocyanin and protein content (Table 1). These results suggested that cells incubated in presence of metronidazole faced nitrogen limitation because they could not use N_2 as nitrogen source and that internal reserves of phycocyanin, allophycocyanin and proteins in cells were mobilized as nitrogen source.

Nostoc ANTH is known to utilize a variety of organic and inorganic compounds of nitrogen, in addition to molecular nitrogen[13,22]. While metronidazole inhibited the growth of *Nostoc* ANTH in N-medium, it failed to do so in presence of combined nitrogen such as nitrate, ammonium, urea, arginine, asparagine and glutamine (Table 2). This showed that metronidazole toxicity was nitrogen source dependent. These observations further suggested that metronidazole inhibited the cyanobacterial diazotrophic growth by inhibiting N_2-fixation.

The effect of metronidazole on the growth rate of different mutant strains isolated in our laboratory was also examined. As in the case of *Nostoc* ANTH parent strain, the PQ-R mutant was also susceptible to metronidazole toxicity only under diazotrophic growth condition and not in the presence of other utilizable inorganic or organic nitrogen sources. The fact that PQ-R mutant strain was as susceptible to growth inhibitory action of metronidazole as the wild type strain, suggests that the mode of action of metronidazole

Table. 1. Effect of metronidazole on the growth of cyanobacterium *Nostoc* ANTH.

Time (h)	N-medium				N-medium + metronidazole			
	Chl *a*	PC	APC	Protein	Chl *a*	PC	APC	Protein
0	1.00±0.05	8.22±0.5	4.57± 0.3	112±4.0	1.00±0.04	8.22±0.6	4.57±0.4	112±4.0
24	1.34± 0.05	9.86±0.7	5.81± 0.35	120±5.0	1.08±0.04	9.1±0.8	5.62±0.5	74±3.0
48	1.98±0.06	21.6±0.9	12.6± 0.6	160±7.0	1.10±0.05	2.3±0.04	2.6±0.08	69±2.8
72	2.9±0.18	18.64±0.8	19.4± 0.8	231±8.0	1.04±0.03	0.98±0.02	1.55±0.02	62±2.7

Nostoc ANTH was inoculated into $BG\text{-}11_0$ with and without 0.2 mM metronidazole. Changes in chlorophyll *a* (Chl *a*), phycocyanin (PC), allophycocyanin (APC) and protein were recorded at regular intervals. All values are given as $\mu g.mL^{-1}$. Values presented are means ± SE from two independent experiments, each with two replicates

Table 2. Growth of *Nostoc* ANTH and its paraquat-resistant (PQ-R), chlorate-resistant (Clo-R) and Het$^-$Nif$^-$ mutants in different nitrogen media in presence or absence of 0.2 mM metronidazole (Met).

Medium	*Nostoc* ANTH	PQ-R	Clo-R	Het$^-$Nif$^-$
N_2 (BG-11$_0$)	2.54±0.1	2.3±0.1	2.41±0.2	0.1±0.0
+Met	0.30±0.0	0.25±0.0	0.28±0.0	0.1±0.0
+5 mM NO_3^- with or without Met	3.30±0.2	3.01±0.2	0.17±0.0	3.27±0.2
+5 mM NO_2^- with or without Met	3.20±0.2	2.9±0.2	2.98±0.2	2.97±0.1
+2 mM NH_4^+ with or without Met	3.10±0.15	3.0±0.1	3.15±0.1	3.2±0.1
+1 mM Glutamine with or without Met	4.10±0.2	3.6±0.2	3.66±0.2	3.85±0.2
+1 mM Asparagine with or without Met	3.50±0.2	3.2±0.2	3.1±0.2	3.0±0.1
+1 mM Arginine with or without Met	2.80±0.1	2.55±0.1	2.6±0.1	2.5±0.1
+1 mM Urea with or without Met	2.90±0.2	2.88±0.1	2.77±0.2	2.92±0.1

Nostoc ANTH and its mutant strains were inoculated at an initial chlorophyll concentration of 0.5 µg chl *a*.mL^{-1}. Increase in chlorophyll content (µg chl *a*.mL^{-1}) was determined after 4d of growth in different nitrogen media. Values presented are means ± SE from two independent experiments, each with two replicates.

and paraquat are different. Furthermore, unlike metronidazole toxicity, paraquat toxicity is not dependent on nitrogen source.

The Clo-R mutant defective in nitrate reductase activity and nitrate uptake activity[12] and therefore unable to use nitrate as nitrogen source, was also used for checking specificity of metronidazole toxicity. It was susceptible to metronidazole both in N- and nitrate-media, since this strain depends on N_2 as nitrogen source and is unable to utilize nitrate from the medium[12]. However, it grew well in the presence of nitrite, ammonium, asparagine, glutamine, urea and arginine with or without metronidazole by utilizing them as nitrogen sources (Table 2).

Metronidazole had no effect on the growth of Het$^-$Nif$^-$ mutant since this strain does not form heterocysts and lacks N_2-fixation. It grows only in presence of a source of combined nitrogen. The above studies indicate that growth inhibition by metronidazole under diazotrophic growth conditions is the result of the inhibition of N_2-fixation leading to nitrogen limitation in the cell. Indeed lower nitrogen content was observed in *Nostoc* cells susceptible to metronidazole (Table 3). Addition of combined nitrogen into the growth medium reversed the metronidazole toxicity. As shown in Fig. 1, diazotrophically growing parent *Nostoc* ANTH culture treated with 0.2 mM metronidazole for 72h showed full recovery of the growth after addition of 5 mM nitrate. Similar results were obtained with other nitrogen sources also (data not shown).

Since metronidazole inhibited diazotrophic growth by inhibiting utilization of N_2 we proceeded to examine its effect on the activity of nitrogenase and other nitrogen

Table 3. Effect of metronidazole on the nitrogen content of *Nostoc* ANTH.

Medium	Nitrogen Content (%N)
N_2 (BG-11$_0$)	6.10± 0.3
+Met	4.60± 0.2
+5 mM NO_3^- with or without Met	7.05± 0.3
+5 mM NO_2 with or without Met	6.76± 0.35
+2 mM NH_4^+ with or without Met	5.96± 0.32
+1 mM Glutamine with or without Met	9.08± 0.42
+1 mM Asparagine with or without Met	8.76± 0.4
+1 mM Arginine with or without Met	8.37± 0.42
+1 mM Urea with or without Met	7.50± 0.35

Nostoc ANTH was inoculated in fresh medium at an initial concentration of 2 µg chl *a*.mL^{-1}. Nitrogen content was analyzed in cells harvested after 4 days of growth in different nitrogen media with or without metronidazole (0.2 mM). Values are means ± SE from two independent experiments, each with two replicates.

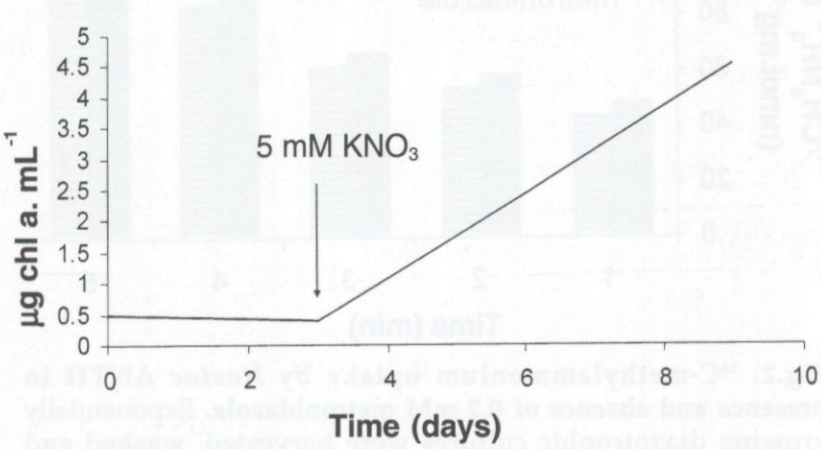

Fig.1. Nitrogen source supported recovery of *Nostoc* ANTH growth against metronidazole toxicity. Exponentially growing *Nostoc* ANTH was transferred to fresh N_2-medium (BG-11$_0$) containing metronidazole (0.2mM) to a concentration of 0.5 µg chl *a*.mL^{-1}. After 72 h, 5 mM nitrate was added to the growth medium. Increase in chlorophyll *a* concentration was recorded before and after the addition of nitrate to culture (3rd day and 9th day, respectively).

metabolizing enzymes in parent *Nostoc* ANTH and its Clo-R mutant. As shown in Table 4, nitrogenase activity in both the strains was inhibited within three hours of treatment with 0.2 mM metronidazole. However, there was no inhibition of nitrate reductase and glutamine synthetase activities. Nitrogenase activity of Clo-R mutant (derepressed for nitrogenase activity in nitrate medium) was inhibited in N-medium as well as in nitrate medium in the presence of metronidazole (Table 4). As expected, level of nitrate reductase activity in Clo-R strain was insignificant which could not be induced by nitrate in the medium due to the inability of the mutant strain to transport nitrate into the cell. Effect of metronidazole on ammonium transport activity of wild type strain was also analyzed (Fig 2). The activity in cells treated with metronidazole for 6 h was similar to that of metronidazole untreated cells. This suggested that ammonium transport activity was also insensitive to the inhibitory action of metronidazole.

Discussion

Our observation on metronidazole inhibition of diazotrophic growth of *Nostoc* ANTH is consistent with the earlier reports[4,7]. In addition, we have shown here that the action of metronidazole on diazotrophic growth was due to the inhibition of nitrogenase and that it did not affect the activity of nitrate reductase, glutamine synthetase, ammonium transport and utilization of such combined nitrogen sources as nitrate, nitrite, ammonium, urea, glutamine, asparagine or arginine.

Table 4. Effect of metronidazole on heterocyst frequency and activities of nitrogenase (N_2 ase), nitrate reductase (NR) and glutamine synthetase (GS) of the parent *Nostoc* ANTH and its Clo-R mutant.

Growth medium	Wild type				Clo-R strain			
	N_2ase	HF (%)	NR	GS	N_2ase	HF (%)	NR	GS
N-medium	9±0.6	5-6	1.7±0.1	701±15	9.5±0.7	5-6	1.3±0.1	692±13
N-medium+Met	0.82±0.0	0.0	1.6±0.1	690±18	0.54±0.0	0.0	1.2±0.1	699±20
NO_3^--medium	0.9±0.0	0.0	4.7±0.6	695±21	10.8±0.5	5-6	1.5±0.1	685±12
NO_3^--medium + Met	0.6±0.0	0.0	4.6±0.5	690±15	0.6±0.0	0.0	1.6±0.1	686±20

Activities of N_2 ase (nmole C_2H_4 formed.μg^{-1} chl *a*.h^{-1}) and NR and GS (nmol product formed.min^{-1}.mg^{-1} protein) were measured in N_2- and NO_3-grown cultures. Metronidazole (0.2mM) was added 3h prior to measurement of N_2 ase and 6h prior to measurement of NR and GS. Values presented are means ± SE from two independent experiments, each with two replicates.

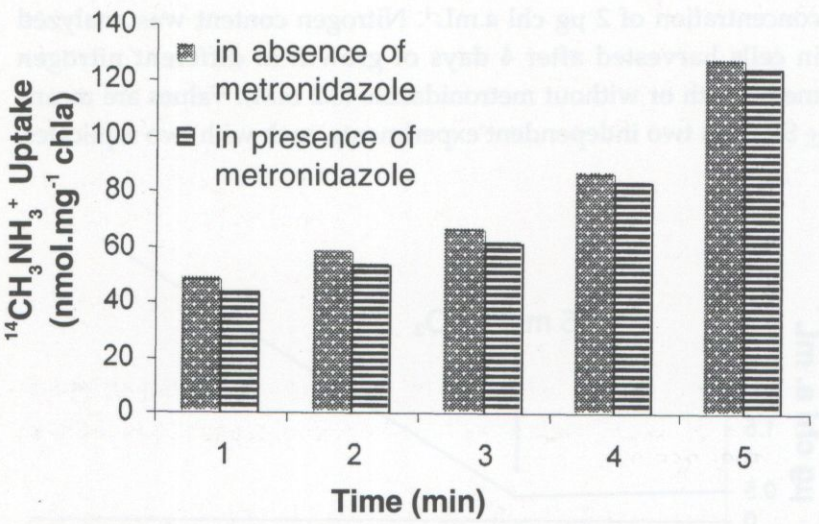

Fig.2. ^{14}C-methylammonium uptake by *Nostoc* ANTH in presence and absence of 0.2 mM metronidazole. Exponentially growing diazotrophic cultures were harvested, washed and transferred to fresh N_2-media with or without 0.2 mM metronidazole. Such cultures were incubated for 6 h under culture growth conditions. Thereafter cells were washed and transferred to HEPES buffer (pH 7.0) without metronidazole. After an equilibration period of 30 min, uptake experiment was started by adding ^{14}C-methylammonium at a final concentration of 50 nmol.ml^{-1}. The values presented are means of two independent experiments, each with two replicates.

Metronidazole is a nitrophenolic drug that enters the cell through passive diffusion. In the cell, the drug is reduced, producing cytotoxic nitro radicals, by reduced ferredoxin or flavodoxin under low redox conditions[6]. Under anaerobic intracellular conditions the cytotoxic nitro radicals remain in reduced form and exhibit their toxic effect. In aerobic environment the cytotoxic nitro radicals are oxidized by oxygen leading to a futile cycle[6]. The superoxide radical anions formed during this process may still be toxic for the anaerobic organisms but organisms such as cyanobacteria can detoxify superoxide radicals using superoxide dismutase and hydrogen peroxidase[23]. In *Nostoc* ANTH, nitrogenase is located only in heterocysts[24,25]. Under the microaerobic conditions that prevail in heterocysts [26,27], reduced nitro radicals produced from metronidazole are not detoxified by oxidation and they inhibit N_2-fixation activity. Reduced ferredoxin is the reductant for nitrogenase as well as metronidazole in the heterocysts and this may lead to competition for reductant between nitrogenase and metronidazole resulting in inhibition of nitrogenase activity.

This explains why diazotrophic growth is inhibited by metronidazole. The lack of inhibitory effect on nitrate reductase may be explained by the fact that nitrate reductase is absent in heterocyst and located only in vegetative cells [28,29] that are aerobic. The glutamine synthetase and ammonium transport activity are present in all cells and even if these are affected in heterocysts the overall impact is negligible because heterocysts are only 5-10% of the total cell population.

In presence of combined sources of nitrogen, heterocyst formation and nitrogenase activity are repressed. The filaments consist of vegetative cells only. Unlike heterocysts, intracellular environment in vegetative cells is aerobic because of photolysis of H_2O by PS II in these cells. This allows oxidation and detoxification of the drug. The above results show that metronidazole exerts its toxic effect selectively to diazotrophically growing heterocystous cyanobacteria by generating nitrogen limiting condition due to inhibition of nitrogenase and probably other enzymes under the microaerobic environment prevailing in heterocysts. To the best of our knowledge there is only one report of metronidazole toxicity to *Nostoc muscorum* growing in the presence of ammonium nitrogen[10]. The reason for this toxicity could be the inability of *N. muscorum* vegetative cells to detoxify the relatively high concentration of reactive nitro radicals generated by reduction of the drug used at relatively higher concentration (0.6 mM)[10].

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