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Siderophore Production in Two Species of Nostoc as Influenced by the Toxicity of Nitrophenolics

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The acquisition of iron by microorganisms requires the production of high-affinity chelating agents known as siderophores to solubilize iron in biologically available form (Lankford 1973; Neilands 1973). Many microbes produce siderphores as well as membrane-transport proteins that facilitate uptake of hydrophilic siderophore complexes across nonpolar cell membranes (Neilands 1973). Most of the siderophore transport systems require an input of cellular energy for iron in the cells (Davis and Byers 1971; Emery 1971; Lankford 1973). In cyanobacteria, siderophore-mediated iron uptake is a contributing factor in their ability to dominate eucaryotic algae (Murphy et al. 1976; Bailey and Taub 1980). A number of cyanobacteria have been found to produce hydroxamate type siderophores, but the only one which has been structurally identified in Anabaena is schizokinen (Murphy et al. 1976). Schizokinen is further known to facilitate iron uptake in *Anabaena* spp. strain 6411 and 7120 (Simpson and Neilands 1976; Goldman et al. 1983).

Nitrophenolics have been listed as priority pollutants by the U.S. Environmental Protection Agency (Keith and Telliard 1973; Spain et al. 1984) Ecologically beneficial cyanobacteria form a major component of soil microflora (Shields and Durell 1964), and hence are likely to interact with nitrophenolics. However, there are virtually no reports on the nontarget effects of these phenolics on siderophore production in species of cyanobacteria. The present study was, therefore, undertaken, undertaken to establish the nontarget effects of selected nitrophenols (*o*-nitrophenol, ONP; *m*-nitrophenol, MNP; *p*-nitrophenol, PNP; and 2,4-dinitrophenol, DNP) and *p*-nitrosophenol (PSNP) and paminophenol (PAP), the reduction metabolities of PNP towards two soil isolates of cyanobacteria, *Nostoc muscorum* and *N.linckia* employing siderophore production as the toxicity criterion, and also to determine whether glucose or succinate amendment at 0.5% and addition of 10µpM adenosine triphosphate (ATP) would annul the toxicity by the selected phenolics on siderophore accumulation.

MATERIALS AND METHODS

Axenic cultures of *N.muscorum* (Ag), B & F and *N.linckia* (Roth) B & F obtained from a semi-and agricultural soil, were maintained in liquid Allen's medium at 28 ± 4°C and a photon per flux density (PPFD) of 65 µE $m^{-2} s^{-1}$ provided by flourescent tubes (Megharaj et al. 1991). The test nitophenols, ONP, MNP, DNP and PNP, and PNSP and PAP, the reduction metabolites of PNP, were purchased from Sigma Chemical Company, St.Louis MO., USA. Aliquots (25ml) of Allen's medium lacking iron (Lammers and Loehr 1982) taken in 50 x 200 mm culture tubes, were sterilized by autoclaving and then inoculated with exponentially growing cultures of *N.muscorum* and *N.linckia* (Megharaj et al. 1992). The culture tubes were maintained at a PPFD of 65 µE and a temperature of 28 ± 4°C, and were shaken thrice a day for aeration. Triplicate samples were withdrawn after 5, 10, 15,

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20,22,25 and 27 days of incubation for siderophore assay (Clarke et al. 1987) Attempts have been made to characterize the siderophore produced by the two species of *Nostoc* following thin-layer chromatography and infrared spectrophotometry (Mullis et al. 1971).

In another experiment , the culture medium was supplemented with iron in the form of $FeCl_3$ (Lammers and Loehr 1982) at desired concentrations ranging from 0.5 to 5.0 µM. Aliquots of medium without iron served as controls. The medium in the culture tubes was inoculated with exponentially-growing cultures of *N.muscorum* and *N.linckia.* The cultures used as inocula were grown initially in iron-lacking medium. Fifteen days after inoculation, triplicates samples of each treatment were assayed for siderophore.

Portions (50ml) of Allen's medium lacking iron were treated with technical grade ONP, MNP, DNP or PNP, or the reduction metabolities of PNP, Viz., PNSP and PAP at levels of EC25 and EC50, determined by linear regression analysis as described earlier (Madhavi et al. 1995). Untreated medium without iron served as a control. After incubation for 15 days, triplicate samples of each treatment were withdrawn for assaying siderophore.

In a repeat experiment, iron-lacking culture medium with or without a phenol was supplemented with glucose (0.5%), succinate (0.5%), or ATP (10µM) to determine whether these sources could reverse the phenol toxicity towards siderophore production. Aliquots of iron lacking medium without the addition of a phenol and with no source of carbon and or energy served as controls. Exponentially-growing cultures were used as inocula. The extent of siderophore production was determined in triplicates of each treatment after 25 days of incubation.

The data were subjected to analysis of variance, and the means were compared with the use of Duncan's new multiple range test at the 5% level (Megharaj et al.1991).

RESULTS AND DISCUSSION

The Siderophore production in *N.muscorum* and *N.linckia* was monitored in iron-limited conditions almost until the stationary phase of their growth cycle. The accumulation of siderophore was maximal during the exponential phase , and gradually decreased with

Table 1. Culture yield and siderophore production in *Nostoc* spp.

Incubation,	*N. muscorum*		*N. linckia*	
Days	OD 650 nm	Siderophore	OD 650 nm	Siderophore
0	0.019^{a}	n.d.	0.014^{a}	n.d.
5	0.084^{b}	26^{a}	0.096^{b}	69^{a}
10	0.185^{e}	67^{b}	0.122^{c}	72^{b}
15	0.352^{h}	206^{g}	0.365^{h}	218^{g}
20	0.261^{g}	187^{f}	0.273^{g}	194^{f}
22	0.220^{f}	143^{e}	0.240^{f}	162^{e}
25	0.163^{d}	98^{d}	0.145^{e}	87^{d}
27	0.128^{c}	75^{c}	0.130^{d}	77^{c}

Siderophore concentration, in µM.
n.d., not determined.
Mean values (n = 3) in each column followed by the same letter are not significantly different (p>0.05) according to Duncan new multiple range (DMR) test

Table 2. Influence of iron amendment to cluture medium on siderophore production

Iron Concentration	*N. muscorum*			*N. linckia*		
	Day 10	Day 15	Day 22	Day 10	Day 15	Day 22
0	68.83[d]	204.20[d]	74.26[d]	78.50[d]	216.50[d]	167.90[d]
0.5 μM	75.46[e]	209.40[e]	94.86[e]	84.40[e]	223.00[e]	184.30[e]
1.0 μM	59.16[c]	192.50[c]	53.70[c]	54.50[c]	163.30[c]	75.80[c]
3.0 μM	55.20[b]	165.72[b]	50.50[b]	49.32[b]	145.20[b]	58.20[b]
5.0 μM	52.50[a]	156.60[a]	48.23[a]	46.70[a]	121.75[a]	37.26[a]

Refer toTable 1 for footnote.

further incubation of the culture (Table 1). Initiation in synthesis of siderophore by the selected cultures in iron-limited cyanophycean medium indicated that the siderophore system develops primarily as a response to iron starvation in cyanobacteria (Clarke et al.1987). The extent of siderophore accumulation in the growth medium was directly proportional to the culture yield throughout the growth phase. Such a secretion pattern was shown to be typical for hydroxamate siderophores in cyanobacteria and fungi (Neilands 1983; Clarke et al. 1987). The present data also showed that *N. linckia* produces more siderophore than *N. muscorum.* The observed differences in the siderophore production appears to have arisen due to species and strain diversity in cyanobacteria which might contribute to the siderophore accumulation and transport systems as suggested for bacteria (Neilands 1966) and fungi (Emery 1978).

Table 3. Toxicity of nitrophenolics towards siderophore production in *Nostoc* spp. grown in absence or presence of iron

Nitrophenolic/EC	No Iron		0.5 μM iron	
	N. muscorum	*N. linckia*	*N. muscorum*	*N. linckia*
Untreated	205.5[m]	218.0[m]	209.5[l]	223.6[l]
ONP 25	129.7[i]	145.5[i]	173.5[e]	201.5[i]
50	76.5[d]	93.3[e]	124.6[a]	180.7[e]
MNP 25	121.1[h]	88.5[d]	193.2[h]	195.3[g]
50	72.7[c]	35.8[b]	137.5[b]	125.6[a]
DNP 25	138.7[j]	158.2[j]	183.4[f]	199.0[h]
50	85.3[e]	95.7[f]	140.6[d]	150.3[b]
PNP 25	119.2[g]	85.5[c]	185.4[g]	199.7[h]
50	58.8a	31.7[a]	138.5[c]	173.8[d]
PNSP25	142.5[k]	205.9[k]	200.0[j]	209.3[j]
50	68.8b	138.5[g]	185.4[g]	185.4[f]
PAP 25	150.6[l]	208.8[l]	202.4[k]	212.5[k]
50	88.7[f]	139.1[h]	195.2[i]	160.0[c]

Refer to Table 1 for footnote.

Table 4. Effect of glucose, succinate, and adenosine triphosphate (ATP) amendment on nitrophenol toxicity towards siderophore production in *Nostoc* spp.

Nitrophenolic EC	Glucose		Succinate		ATP	
	N.muscorum	*N. linckia*	*N.muscorum*	*N.linckia*	*N.muscorum*	*N.linckia*
Control	225.7^{l}	244.2^{l}	218.3^{l}	221.2^{m}	218.9^{f}	225.3^{g}
ONP 25	150.3^{d}	222.5^{h}	185.3^{g}	201.5^{j}	n.d.	n.d
50	107.2^{a}	189.5^{e}	105.7^{e}	138.6^{e}	212.0^{d}	205.0^{e}
MNP 25	208.6^{j}	225.6^{l}	192.3^{h}	180.2^{g}	n.d.	n.d.
50	126.0^{b}	193.4^{f}	126.1^{d}	125.2^{b}	215.3^{e}	210.5^{d}
DNP 25	220.5^{k}	230.2^{k}	200.7^{k}	189.7^{i}	n.d.	n.d.
50	130.3^{c}	185.6^{d}	168.8^{f}	149.2^{f}	196.9^{a}	185.4^{a}
PNP 25	205.9^{h}	217.0^{g}	195.5^{i}	212.5^{k}	n.d.	n.d.
50	166.2^{e}	138.7^{a}	154.5^{d}	126.6^{c}	199.4^{b}	201.6^{b}
PNSP 25	208.3^{i}	228.9^{j}	194.3^{i}	201.6^{j}	n.d.	n.d.
50	178.3^{f}	143.4^{b}	151.3^{c}	128.8^{d}	212.0^{d}	220.4^{f}
PAP 25	207.4^{j}	226.2^{i}	198.7^{j}	187.7^{h}	n.d.	n.d.
50	183.9^{g}	148.0^{c}	158.4^{e}	114.9^{a}	210.6^{c}	205.8^{c}

nd., not determined.
Refer to Table 1 for other footnote

The siderophore extracted from the culture medium gave an Rf value of 0.60 in butanol-water-acetic acid. Also, the infrared spectrum obtained from air-dried samples of ether extract (Clarke et al. 1987) exhibited major peaks at 1604, 1412, 1034 cm^{-1}. In view of the close agreement of the above values with those reported (Mullis et al. 1971), the siderophore produced by the selected cultures of cyanobacteria has been tentatively identified as schizokinen.

The data on siderophore production in the species of *Nostoc* as a response to the iron status in the growth medium are presented in Table 2. The cultures, grown initially in a medium devoid of iron, when transferred to growth medium supplemented with 0.5 M iron, produced significantly large quantities of siderophore. However, increasing concentrations of iron in the culture medium greatly reduced the amount of siderophore accumulated in cultures. Thus, the highest concentrations of 5µM iron significantly inhibited the synthesis of siderophore in both cultures. The present observation of a drop in siderophore production beyond 0.5µM iron may reflect the satisfaction of cellular iron requirement in the test cultures at 0.5µM iron added to the medium, and consequent repression of iron uptake systems. Clarke et al. (1987) reported a similar production of schizokinen by Anabaena spp. grown with varying concentrations of iron. Our result suggests that the culture does not build-up significant iron reserves but takes up iron from the environment when required by the organism (Clarke et al. 1987).

To determine the nontarget effect of selected nitrophenols (ONP, MNP, DNP, and PNP) and the reduction metabolites of PNP (PNSP and PAP) towards the species of *Nostoc* siderophore produced was assayed on day 15 of culture growth. In iron limited growth conditions, the selected nitrophenols, at both EC25 and EC50 levels, significantly inhibited the synthesis of siderophore in test cultures (Table 3). Higher concentrations of EC50 in all cases exerted greater toxicity towards siderophore production. A comparison of the toxicities of four

nitrophenols clearly indicated that PNP was more toxic, and that the toxicity followed the order. PNP MNP ONP DNP. The present observation of differential toxicity among the selected nitrophenolics could be attributed largely to the position of nitrogroup on the ring moiety. However, siderophore production was less affected by PNSP and PAP than it was by their parent compound, PNP. Similarly, soil isolates of *Nostoc* spp. were found to be more sensitive to PNP than to PAP (Megharaj et al. 1991). Likewise, our results conform to the generalization that parent compounds are more toxic than their products of metabolism to the nontarget cyanobacteria (Venkateswarlu 1993).

Interestingly, the nitrophenol toxicity towards siderophore production was significantly alleviated, when the test cultures were grown in presence of 0.5µM iron along with the specified concentrations of the toxicants. Thus, the percent inhibition of 71.4 observed with PNP at EC50 towards siderophore production in *N.muscorum* turned out to be 33.9 when the culture medium was supplemented with iron. The corresponding percent inhibition values in *case* of *N.linckia* grown in presence of EC50 PNP and 0.5µM iron were 85.5 and 22.3, respectively. It is therefore quite obvious that *N.linckia* could resist nitrophenol toxicity in presence of iron more efficiently than *N.muscorum*

A comparison of data presented in Tables 3 and 4 indicate that supplementing the culture medium with carbon and/or energy sources significantly enhances siderophore production in test cultures. Furthermore, the toxicity exerted by the selected phenolics at EC25 was greatly reversed under the impact of glucose, succinate and ATP supplemeted to the medium (Table 4) Even the pronounced toxicity observed with EC50 values of the test chemicals was also alleviated significantly. Of the three amendments employed in the present study, the energy-rich source ATP significantly annulled the nitrophenol toxicity towards the test criterion of siderophore production. Such a response (reversal of nitrophenolics in presence of ATP) could be expected in view of the fact that most siderophore transport systems require the input of cellular energy for iron uptake into cells (Negrin et al. 1978; Pungsley et al. 1977). On the other hand, the alleviation of nitrophenol toxicity was significantly greater in presence of glucose than succinate. This differential response associated with the two carbon sources used could be due to their possible implication in different pathways of energetics. The exogenous glucose is shown to be metabolized by glycolytic and/or pathway, contributing up to 46% of the total cell material, while the remaining 54% is being utilized to withstand the toxicant's stress in cyanobacteria (Wildon and Rees 1965). The utilization of succinate by the test species of *Nostoc* is seemingly less effective due to its probable use in sluggish production of succinyl-CoA during the incomplete TCA cycle being operative among cyanobacteria (Rippka et al. 1971). The preferential utilization of glucose over acetate by *N.linckia* subjected to carbofuran toxicity was reported recently (Megharaj et al. 1993). The inhibitory effect of propanil on photoautotrophic growth of *Nostoc calcicola* was reversed with the addition of glucose (Pandey 1985). Likewise, amendments of organic carbon sources were shown to provide additional energies necessary to supress the inhibitory action of heavy metals towards metabolic activities of phytoplantkton (Wu and Lorenzen 1984)

The present study clearly indicates that the siderophore production in cyanobacteria could be used as one of the indices while establishing the toxicity pattern of environmental pollutants such as nitrophenols. This is particularly true in view of the fact that the siderophore production is greatly beneficial to ecologically important organisms like *Nostoc* spp., and that it may drastically be affected in presence of the toxicants. Also, our results suggest that the amendment of organic carbon such as glucose or succinate, or energy-rich sources like ATP to the culture medium would annul the toxicity of nitrophenolics towards siderophore production.

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