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Toxicity of N-Containing Heterocyclic Aromatic Compounds and Their Utilization for Growth by a Few Purple Non-Sulfur Bacteria

N. Rajasekhar, Ch. Sasikala, Ch. V. Ramana

Environmental Microbial Biotechnology Laboratory, Center for Environment, IPGS and R, J. N. T. University, Mahaveer Marg, Hyderabad 500 028, India

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Frank and Gaffron (1941) first discovered the existence of aromatic carbon metabolism in purple non-sulfur bacteria. Since then, a number of aromatic compounds are shown to be assimilated by purple non-sulfur bacteria (Sasikala and Ramana 1998). While photometabolism of homocyclic aromatic compounds by purple non-sulfur bacteria is widely studied and largely focussed on the studies of naturally occurring aromatics, in recent years interest has increased on the biodegradation of xenobiotic aromatic compounds also (Blasco and Castillo 1992) because of their environmental impact (Razo-Flores et al. 1997). Heterocyclic aromatic compounds represent two-thirds of known organic compounds chemically (xenobiotic) synthesized. They are extensively used as industrial solvents, dyes, explosives, pharmaceuticals and pesticides (Kaiser et al. 1996). Accumulation of these compounds is a major threat to microorganisms, since many of these compounds are antimicrobial (Vance et al. 1986) and the problem is more severe under subsurface environments where anaerobic conditions prevail. Since purple non-sulfur bacteria play an important role in anaerobic environments, it is of interest to investigate the probable utilization of some of the heterocyclic aromatic compounds for growth by a few purple non-sulfur bacteria. The earlier studies were restricted only to the photobiodegradation of a few naturally occurring heterocyclic aromatic compounds like purines (Busse et al. 1984), pyrimidines (Kaspari 1979) by *Rhodobacter capsulatus*, indole by *Rb. sphaeroides* (Rajasekhar et al. 1999) and pyridines/pyrazines by *Rhodopseudomonas palustris* (Sasikala et al. 1994). In the present communication we report the utilization of other heterocyclic aromatic compounds (quinolines, imidazole, captan and carbendizam) also by a few purple non-sulfur bacteria along with toxicity studies which helped in understanding the structural basis of toxicity of these compounds on purple non-sulfur bacteria.

MATERIALS AND METHODS

Rhodobacter sphaeroides OU5 (ATCC 49885; DSM 7066), *Rhodopseudomonas palustris* OU 11 (ATCC - 51186; DSM - 7375) and *Rhodopseudomonas* species JA1 (a new isolate from Dairy effluent) were grown photoheterotrophically (anaerobic/light (2,400 lux) in fully filled reagent bottles (500 mL) on a modified

Correspondence to: Ch. Sasikala

Biebl and Pfennig's (1981) medium containing (g/L): KH_2PO_4 – 0.5; MgSO4. $7H_2O$ – 0.2; NaCl – 0.4; $CaCl_2$. $2H_2O$ - 0.05; Yeast extract – 0.6; Ferric citrate (0.1 % w/v) – 5 mL; Trace elements – 1 mL (The composition of the trace element solution SL7 is as follows (mg/L) : HCl (25 % v/v) - 1 mL; $ZnCl_2$ – 70; $MnCl_2.4H_2O$ – 100; H_3BO_3 – 60; $CoCl_2.6H_2O$ – 200; $CuCl_2.H_2O$ – 20; $NiCl_2.6H_2O$ – 20; $NaMoO_4.2H_2O$ – 40). Either malate (22 mM)/ succinate (25 mM) or pyruvate (27 mM) were used as sole carbon source for *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris* and *Rhodopseudomonas* species JA1, respectively. Ammonium chloride (7mM) served as nitrogen source in the above medium and the cultures were incubated at 30 + 2 °C, under light (2,400 lux) anaerobic conditions.

Logarithmically growing cultures of purple non-sulfur bacteria were inoculated (5% v/v) into an assay medium containing the above basal salts with heterocyclic compound (1 mM) (nitrogen source) [heterocyclic compounds (purity w/v or v/v): pyridine (98%), nicotinic acid (98%), 4-(Dimethyl amino) pyridine (99%), 2,4,6-trimethyl pyridine (99%), pyrazines (99%), quinolines (98%), imidazole (99%), indole (98%) adenine (98%), guanine (98%), uracil (98%), captan (50%) and carbendizam (50%)] and malate or pyruvate or succinate (as the case may be) as sole carbon source in fully filled screw cap test tubes (15 X 125 mm), incubated phototrophically (anaerobic light 2,400 lux). Growth was measured turbidometrically at 660 nm in Systronics colorimeter every day until two consistent optical density values were observed. Fifty percent growth inhibitory concentration (IC_{50}) and Minimum Inhibitory Concentration (MIC) of N-containing heterocyclic aromatic compounds on the purple non-sulfur bacteria were studied in a medium containing above mineral salts with malate (22 mM) and ammonium chloride (7 mM) as carbon and nitrogen sources, respectively, in fully filled screw cap test tubes (12 × 100 mm). Disappearance of heterocyclic aromatic compound was studied in the culture supernatant after centrifuging (10,000 rpm for 15 min) the stationary phase (15 days) culture of *Rhodopseudomonas* sp JA1. The initial and the final absorption spectra of the compounds after required dilutions were analyzed using a ECIL spectrophotometer (model GS5703 AT) and the difference in the optical density values at the absorption maxima of each compound was considered for calculating the disappearance of the compound.

RESULTS AND DISCUSSION

Rps. palustris, which is known to degrade a variety of homocyclic aromatic compounds (Harwood and Gibson 1988) and *Rb. sphaeroides* and a newly isolated *Rhodopseudomonas* sp JA1 represented the test organisms in the present study on toxicity and utilization of N-containing heterocyclic aromatic compounds. A number of heterocyclic aromatic compounds were selected for the present study based on the variation in the number and position of 'N' atom in the heterocyclic ring. Thus, pyridine, pyrazine, quinoline, imidazole, indole, purine (guanine), pyrimidine (uracil), benzimidazole (Carbendazim) and isoindole

(Captan) were used as representatives of N-containing heterocyclic aromatic compounds. Their effect on purple non-sulfur bacteria (PNSB) is shown in Table 1. A threshold of 10 mM was kept as an upper limit in assessing the toxicity of N-substituted heterocyclic aromatic compounds on purple non-sulfur bacteria, because toxicity on microorganisms by N-substitutions of aromatic compounds fall within this range (Razo-Flores et al. 1997). The organisms were not inhibited by pyridine, pyrazine, imidazole, uracil (except *Rps. palustris*) and guanine (except *Rps. palustris*) suggesting these compounds are not toxic to PNSB at low concentrations. The highest toxicity was observed with indole, which is known for its antimicrobial activity against gram negative bacteria (Kubo 1993).

Toxicity of N-containing heterocyclic aromatic compounds was observed only with those compounds having a homocyclic ring attached to the heterocyclic ring (Fig. 1). Toxicity among these compounds increased in the following order: benzimidazole<quinoline<isoindole<indole for *Rb. sphaeroides* (Fig. 1) and near

Table 1. IC_{50} and MIC values of various N-containing heterocyclic aromatic compounds on purple non-sulfur bacteria.

Compounds	*Rb. sphaeroides* OU5		*Rps. palustris* OU11		*Rhodopseudomonas* sp JA1	
	IC_{50} (mM)	MIC (mM)	IC_{50} (mM)	MIC (mM)	IC_{50} (mM)	MIC (mM)
Pyridine	>10	>10	>10	>10	>10	>10
3-pyridine carboxylic acid (Nicotinic acid)	>10	>10	9.5	>10	7.3	>10
4-(Dimethyl Amino) pyridine	6	>10	2.6	3	>10	>10
Pyrazine	>10	>10	>10	>10	>10	>10
2-Amino pyrazine	>10	>10	>10	>10	>10	>10
Pyrazine-2-Carboxylic acid	>10	>10	>10	>10	>10	>10
Quinoline	1.5	5	0.6	2	1.2	7
8, Hydroxy quinoline	0.5	4	0.5	1	0.6	2
Imidazole	>10	>10	5.9	>10	>10	>10
Indole	0.6	4	0.5	1	0.2	2
Guanine	>10	>10	5.3	>10	7.3	>10
Uracil	>10	>10	4.2	>10	>10	>10
Carbendizam	5	7	3.2	7	5.3	9
Captan	1	1	0.4	<1	0.5	2

Results are average values of an experiment done in duplicates expressed after 48 h of light [2,400 lux] anaerobic incubation at 30±2°C. IC_{50} = 50% growth inhibitory concentration (mM). MIC = Minimum Inhibitory Concentration (mM) of growth.

similar pattern was also observed with the other two species also (Fig. 1), suggesting common toxicity pattern among PNSB. Thus, pyridine and imidazole were not toxic, while benzimidazole and quinoline were found to be toxic at low concentrations. The reason for such toxicity with respect to the compounds with homocyclic ring may be due to several reasons. While the major reason appears to be the hydrophobicity of the homocyclic ring (Razo-Flores et al. 1997) and its effect on the bacterial membranes (Sikkema et al. 1994; Razo-Flores et al. 1997), the other could be their effect on the photosynthetic pigments in this group of microorganisms (Rajasekhar et al. 1999; Wright and Madigan 1991).

All N-containing heterocyclic aromatic compounds were used as sole 'N' source in the presence of an organic carbon compounds (malate/succinate/pyruvate). Growth was not observed on most of the compounds by *Rb. sphaeroides* and *Rps. palustris*. Growth was observed on 4-dimethyl amino pyridine, pyrazine, 2-amino pyrazine and captan by *Rb. sphaeroides* (Table 2). Growth by *Rps. palustris* was

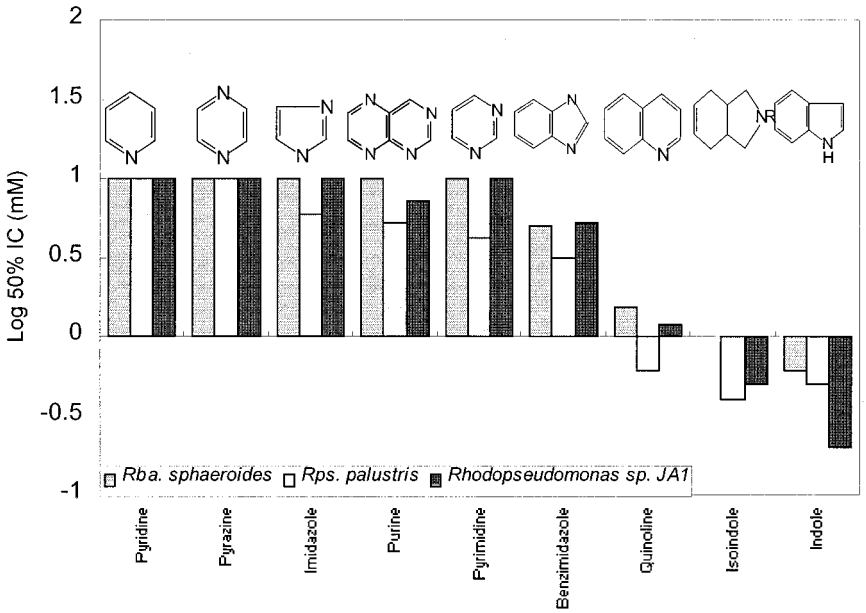

Figure 1. Comparison of toxicity of various N-containing heterocyclic aromatic compounds on purple non-sulfur bacteria.

observed with pyridine, nicotinic acid, 4-dimethyl amino pyridine, imidazole and captan (Table 2). *Rhodopseudomonas* sp JA1was able to grow on pyridine, nicotinic acid, 4-dimethyl amino pyridine, pyrazine, 2-amino pyrazine, quinoline, 8-hydroxy quinoline, guanine, uracil and carbendazim (Table 2).

Since *Rhodopseudomonas* sp JA1 showed a wide utilization of N-containing heterocyclic aromatic compounds, disappearance of these compounds was studied

only with respect to this species and data presented in Table 2. Almost 100% disappearance of the compounds (Figs. 2-3) was observed from the culture supernatant with all the compounds tested except pyrazine-2-carboxylic acid and indole. The disappearance of the compounds can be correlated with increase in the biomass of *Rhodopseudomonas* sp JA1, suggesting the biodegradation and its incorporation as cell mass. Thus, increased cell mass compared to other species also may be due to the degradation of the compound. However, though disappearance of the compound was observed (Figs. 2-3) with reference to indole, imidazole, captan, pyrazine-2-carboxylic acid, 2,4,6-trimethyl pyridine, their

Table 2. Biomass yields of various purple non-sulfur bacteria on various N-containing heterocyclic aromatic compounds and their photobiodegradation by *Rhodopseudomonas* sp JA1.

Compound (1 mM)	Mol. wt.	†Biomass yield (mg dry wt. ml^{-1})			♠Photo-bio-degrada-tion (mM)
		Rb. sphaeroides OU5	*Rps. palustris* OU 11	*Rhodo*pseudomonas sp JA1	
Pyridine	79	0.15 (0)	0.14 (0.05)	2.66(0.7)	0.9
3-pyridine carboxylic acid (Nicotinic acid)	123	0.21(0)	0.17(0.08)	2.26(0.3)	0.9
4-(Dimethyl Amino) pyridine	122	0.36(0.09)	0.05(0)	2.46(0.5)	1
2,4,6-Trimethyl pyridine	121	0.06(0)	0.11(0.02)	1.96(0)	0.85
Pyrazine	80	0.34(0.07)	0.23(0.14)	2.06(0.2)	0.9
2-Amino pyrazine	86	0.43(0.16)	0.22(0.13)	2.56(0.6)	1
Pyrazine-2-Carboxylic acid	124	0.19(0)	0.04(0)	1.86(0)	0.25
Quinoline	129	0.07(0)	0.01(0)	2.66(0.7)	0.7
8, Hydroxy quinoline	147	0(0)	0(0)	2.06(0.1)	0.8
Imidazole	68	0.22(0)	0.17(0.08)	1.76(0)	0.9
Indole	117	0.07(0)	0.01(0)	0.16(0)	0.36
Guanine	151	0.15(0)	0.05(0)	2.76(0.8)	NT
Uracil	112	0.10(0)	0.07(0)	2.66(0.7)	NT
Carbendizam	191	0.09(0)	0.07(0)	2.06(0.1)	0.98
Captan	600	0.36(0.09)	0.15(0.06)	0.66(0)	0.94
Control (without Compound)	NA	0.27	0.09	1.96	NA

NT = Not tested; NA = Not applicable. Results are average values of an experiment done in duplicates expressed after 15 days of light [2,400 lux] anaerobic incubation at 30±2°C. †Data expressed in parentheses are the values of biomass gain over the control (without organic compound). ♠Photobiodegradation of N-containing heterocyclic aromatic compounds by *Rhodopseudomonas* sp JA1 after 15 days of assay.

incorporation into the cell mass must not have occurred, since there was no increase in biomass when compared to that of control. Furthermore, there was no noticeable change in initial and final absorption spectra (Figs. 2-3), clearly indicating that there was no photobiotransformation. Under such conditions, the disappearance of the compound may presumably be explained as due to the passive absorption by the organism. When transferred to regular growth medium,

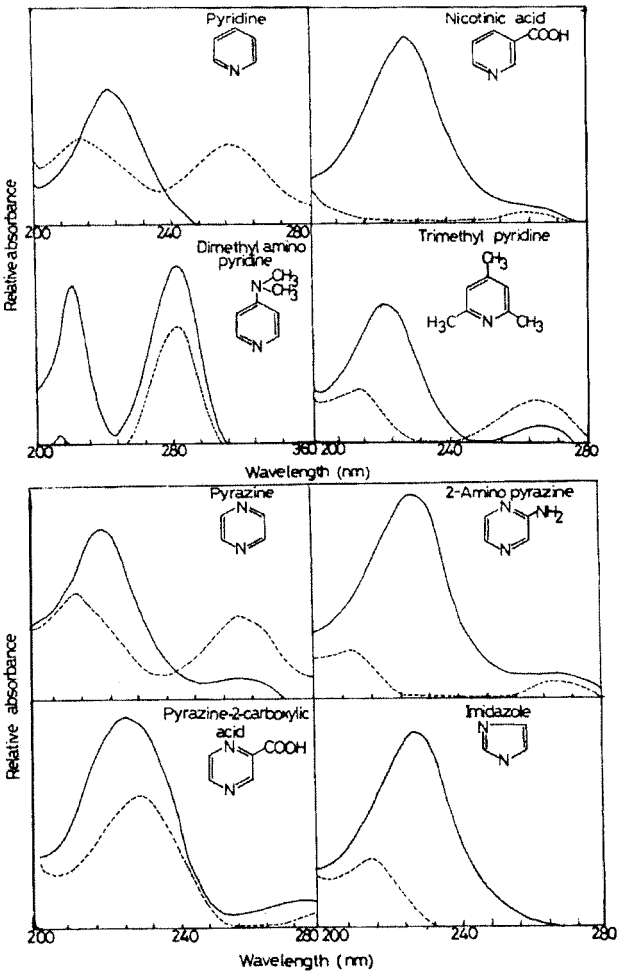

Figure. 2. Ultra violet absorption spectra of the heterocyclic compounds. Supernatants after the assay were analyzed for absorption spectra (----) and compared with initial (—).

the organism grew equally with that of control (without heterocyclic compound) (data not shown), indicating the effect of these compounds as reversible and not lethal.

This study indicates wide spread utilization of N-containing heterocyclic aromatic compounds for growth by PNSB, indicating their role under natural anoxic phototrophic conditions as de-pollutants of heterocyclic rings. On the other hand, the study opens a scope in evaluating the various biochemical pathways in this group of microorganisms, which was scantily studied.

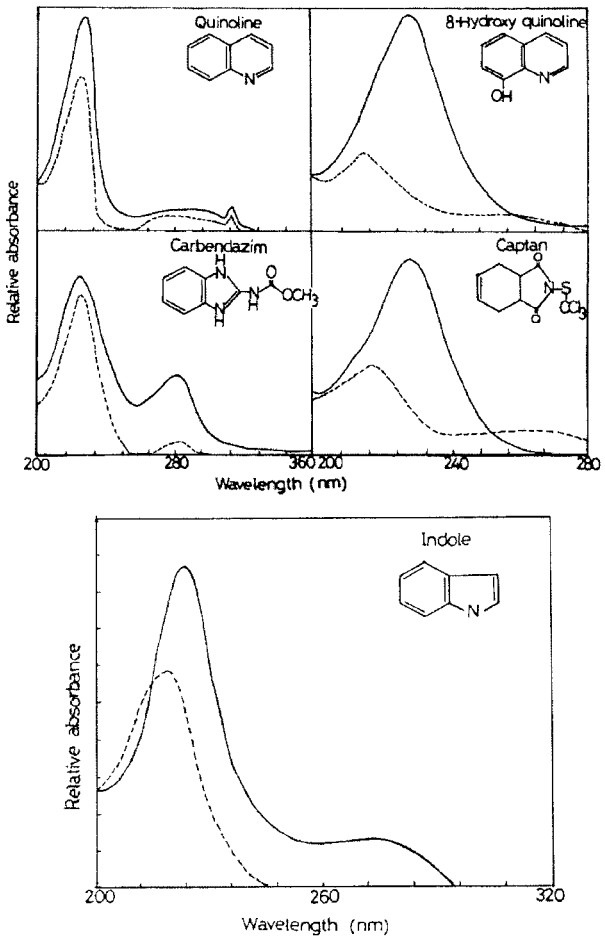

Figure 3. Ultra violet absorption spectra of the heterocyclic compounds. Supernatants after the assay were analyzed for absorption spectra (----) and compared with initial (—).

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