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Chemotaxis and Biodegradation of 3-Methyl-4-Nitrophenol by *Ralstonia* sp. SJ98

Bharat Bhushan,*[,1] Sudip K. Samanta,*[,2] Ashvini Chauhan,*
Asit K. Chakraborti,† and Rakesh K. Jain*[,3]

**Institute of Microbial Technology, Sector-39A, Chandigarh 160036, India; and †Department of Medicinal Chemistry, National Institute of Pharmaceutical Education and Research, Sector-67, S.A.S. Nagar 160062, India*

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3-Methyl-4-nitrophenol is one of the major breakdown products of fenitrothion [*O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl) thiophosphate], a recalcitrant organophosphate insecticide used in agriculture. Being the non-polar methylated aromatic compound, 3-methyl-4-nitrophenol is highly toxic and, therefore, a complete degradation of this compound is important for environmental decontamination/bioremediation purposes. A gram negative, motile *Ralstonia* sp. SJ98 was isolated by selective screening from a soil sample contaminated with pesticides. The microorganism was capable of utilizing 3-methyl-4-nitrophenol as the sole source of carbon and energy. Thin layer chromatography (TLC), gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), and high performance liquid chromatography (HPLC) were performed to determine the possible intermediates in the degradative pathway of this compound. Taken together, catechol was found to be one of the major intermediate of the pathway. Furthermore, the chemotactic behavior of *Ralstonia* sp. SJ98 towards 3-methyl-4-nitrophenol was tested using three different methods i.e., drop assay, swarm plate assay and capillary assay, which were found to be positive towards this compound. This is the first report clearly indicating the involvement of a microorganism in the chemotaxis and biodegradation of methyl-4-nitrophenol and formation of catechol as an intermediate in the degradative pathway.

***Key Words:* *Ralstonia* sp. SJ98; 3-methyl-4-nitrophenol degradation; catechol formation; chemotaxis.**

Nitroaromatic compounds (NACs) are widely spread in the environment because of their extensive use in the manufacturing of pharmaceuticals, pesticides, plasticizers, azo dyes, and explosives (1–3). The NACs and their incomplete degradative products have a high level of toxicity and some of them are potential carcinogens (1, 2, 4). Once released into the environment, NACs undergo complex physical, chemical, and biological changes. Nitrophenolic compounds can also accumulate in the soil as a result of hydrolysis of several organophophorus insecticides such as parathion, methyl parathion, and fenitrothion (3, 5–7), and may enter the ground water resources where they cause adverse effects to the biological systems.

Although, there are several reports on biodegradation of different NACs (1–4), little is known on the biodegradation of 3-methyl-4-nitrophenol which is one of the major breakdown products of fenitrothion, a recalcitrant organophosphate insecticide, and is highly toxic. Only recently, the involvement of a plasmid in the degradation of fenitrothion has been reported in which a *Burkholderia* sp. strain NF100 was shown to first hydrolyze the organophosphate bond of fenitrothion forming 3-methyl-4-nitrophenol which was further converted to methylhydroquinone, the substrate for oxygenase-catalyzed ring fission (8). We have recently reported the degradation and chemotactic activity towards four NACs viz- *p*-nitrophenol, 4-nitrocatechol, *o*-nitrobenzoate and *p*-nitrobenzoate by a *Ralstonia* sp. SJ98 (9). In the present study the involvement of this *Ralstonia* sp. SJ98 in chemotaxis and biodegradation of 3-methyl-4-nitrophenol has been elucidated.

MATERIALS AND METHODS

Microorganism and culture conditions. A *Ralstonia* sp. SJ98 was isolated in our laboratory by "chemotactic enrichment technique" from pesticide contaminated soil sample (9). The composition of the minimal medium (MM) used in the present study was same as described earlier (10). 3-Methyl-4-nitrophenol was added as the filter sterilized solution into MM at a final concentration of 0.5 mM. The medium was inocu-

[1] Current address: Department of Biochemistry and Molecular Biology, Wayne State University, School of Medicine, 540, East Canfield, Detroit, MI 48201.
[2] Current address: Department of Microbiology, University of Iowa, 3-401 Bowen Science Building, Iowa City, IA 52242.
[3] To whom correspondence should be addressed. Fax: +91-172-690585/690632. E-mail: rkj@imtech.ernet.in.

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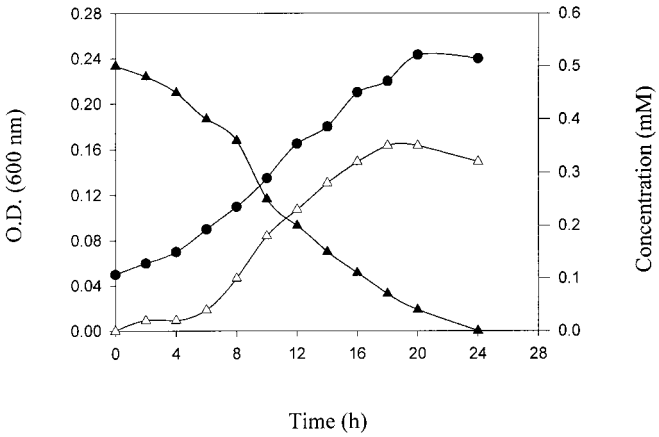

FIG. 1. Degradation of 3-methyl-4-nitrophenol by *Ralstonia* sp. SJ98. 3-methyl-4-nitrophenol is metabolized with concomitant release of nitrite. The inoculum used was obtained from washed cells of *Ralstonia* sp. SJ98 grown overnight on 3-methyl-4-nitrophenol. ●, The optical density (OD) of culture; ▲, 3-methyl-4-nitrophenol concentration; △, nitrite concentration.

lated with overnight grown seed culture and incubated at 30°C under shaking conditions (200 rpm). Growth was monitored by measuring absorbance (OD) at 600 nm. The depletion of 3-methyl-4-nitrophenol was monitored by measuring absorbance at 320 nm which is the absorbance maximum of 3-methyl-4-nitrophenol at pH 7.0, and the residual compound was calculated from a standard curve prepared using authentic 3-methyl-4-nitrophenol as shown earlier in case of *o*-nitrobenzoate (11). Nitrite concentrations in the samples were determined by comparison of values with those of a standard calibration curve prepared using sodium nitrite as described earlier (12).

Extraction of intermediates, analytical methods, and chemotaxis assays. The extraction of intermediates of 3-methyl-4-nitrophenol degradative pathway, analytical methods (TLC, GC, and GC-MS) and the chemotaxis methods used in the present study were exactly same as used in our previous studies (9, 11, 13).

In order to test whether the energy source of flagellar motors in *Ralstonia* sp. SJ98 was Na^+ or H^+ driven motive force, amiloride, a specific inhibitor of Na^+ driven flagellar motors (14, 15), was mixed in the drop assay and swarm plate assay media of chemotaxis. The stock solution of amiloride was prepared in dimethyl sulfoxide (DMSO) and then added to chemotaxis medium at a final concentration in the range of 1 to 5 mM. In motility restoration experiment, sodium chloride was supplemented in the chemotaxis medium at different levels between 50 to 400 mM and the threshold inhibitory concentration (2 mM) of amiloride was kept constant in the medium.

High performance liquid chromatography (HPLC) analysis. Quantitative analysis of intermediates in 3-methyl-4-nitrophenol degradation was performed by HPLC as shown earlier (13, 16). The culture was harvested during late log-phase following growth of *Ralstonia* sp. SJ98 on MM supplemented with 3-methyl-4-nitrophenol (0.5 mM) and succinate (10 mM), and the washed concentrated cell suspension ($OD_{600} \sim 1.5$) was incubated with 3-methyl-4-nitrophenol (0.5 mM). 2,2-Dipyridyl was also added in order to detect the accumulating intermediates from 3-methyl-4-nitrophenol (13). Acetonitrile-water containing 13.5 mM trifluoroacetic acid (20:80) was the mobile phase at a flow rate of 1 ml/min. Compounds were identified and quantified by comparison of HPLC retention times and UV-visible spectra to those of standards.

Chemicals. 3-Methyl-4-nitrophenol was purchased from Aldrich Chemical Co. whereas catechol, amiloride, and 2,2′-dipyridyl were purchased from Sigma Chemical Co. (USA). All other chemicals used were of highest purity available.

RESULTS AND DISCUSSION

A NACs degrading, gram negative, motile bacterium *Ralstonia* sp. SJ98 was isolated in our laboratory (9). Its degradation capacity and chemotactic ability were tested for a methylated nitroaromatic compound, 3-methyl-4-nitrophenol, which was utilized as the sole source of carbon and energy. The complete degradation of this compound occurred via oxidative route with corresponding release of nitrite molecules (Fig. 1). In order to identify the intermediates of the degradative pathway of 3-methyl-4-nitrophenol, TLC, GC, and GC-MS studies were performed on the extracted samples following growth of *Ralstonia* sp. SJ98 on 3-methyl-4-nitrophenol. These studies showed the presence of two major compounds in the degradative pathway along with 3-methyl-4-nitrophenol. Compound I with an R_f value of 0.46 in TLC studies and retention time of 2.23 min in GC studies was apparent in this study which corresponded well with the authentic catechol indicating that this may be an intermediate in the degradation pathway. The GC-MS studies also revealed the presence of catechol with a retention time of 4.22 min and molecular ion at *m*/*z* 110 corresponding to the molecular mass of catechol and fragmentation ion at *m*/*z* 82 and 81 corresponding to the losses of $M^+ - CO$ and $M^+ - CHO$ were identical to that produced by authentic catechol. These results therefore clearly showed the presence of catechol in the degradative pathway of 3-methyl-4-nitrophenol. Compound II having an R_f value of 0.43 in TLC studies and retention time of 2.25 min in GC studies was also evident. However, attempts to identify this compound were unsuccessful as it could not be correlated to any of the likely intermediates before the formation of catechol in the biodegradation of 3-methyl-4-nitrophenol as checked by TLC, GC, and GC-MS studies (data not shown). A recent report by Hayatsu *et al.* (8) has shown the degradation of 3-methyl-4-nitrophenol via the formation of methylhydroquinone. However, we were unable to detect this compound as an intermediate in the degradative pathway indicating that methylhydroquinone is not involved in the degradation of 3-methyl-4-nitrophenol by *Ralstonia* sp. SJ98.

TABLE 1

Capillary Assay for Chemotaxis of *Ralstonia* sp. SJ98 towards 3-Methyl-4-nitrophenol

Compound	Number of cells in the capillary	Chemotaxis index (C.I.) ± S.D.
3-Methyl-4-nitrophenol	8300	9.0 ± 0.8
Aspartic acid[a]	55000	60 ± 5.8
Negative control	920	1 ± 0.2

Note. S.D., Standard deviation.
[a] Aspartic acid was used as positive control.

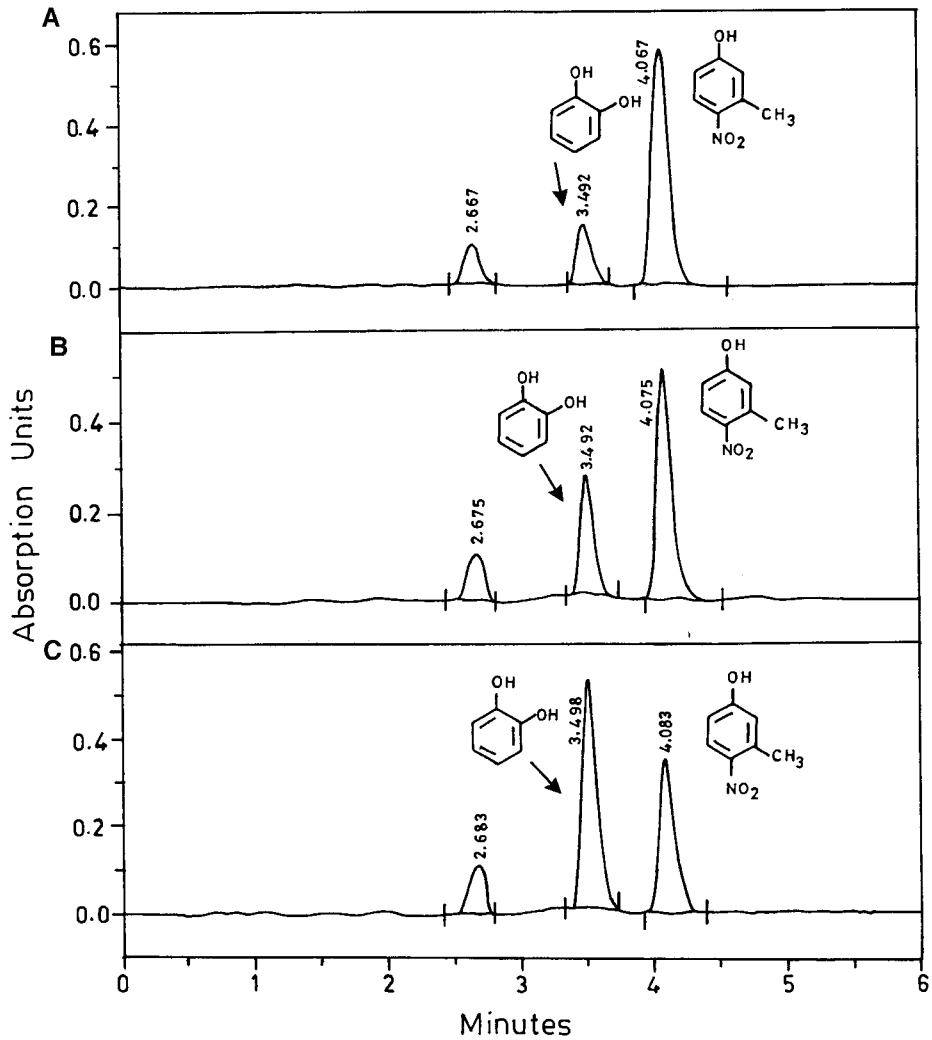

FIG. 2. HPLC chromatograms of the conversion of 3-methyl-4-nitrophenol by *Ralstonia* sp. SJ98. Concentrated cell suspensions grown on 3-methyl-4-nitrophenol and succinate were incubated with 3-methyl-4-nitrophenol and intermediates were detected when the ring cleavage was blocked using 2,2′-dipyridyl; (A) sample analyzed at 4 h growth interval; (B) sample analyzed at 6 h growth interval; and (C) sample analyzed at 10 h growth interval. The intermediate at retention time of 2.66 min is an unidentified metabolite.

Attempts were then made to determine the stoichiometry and rate of conversion of 3-methyl-4-nitrophenol into catechol by HPLC studies. The concentrated cell suspension of *Ralstonia* sp. SJ98 was incubated with 3-methyl-4-nitrophenol (0.5 mM) in the presence and absence of 2,2′-dipyridyl, a ring cleavage inhibitor (13, 16). HPLC studies revealed the formation of catechol from 3-methyl-4-nitrophenol during its degradation. In presence of 2,2′-dipyridyl, catechol (retention time of 3.49 min; Fig. 2A) started appearing after

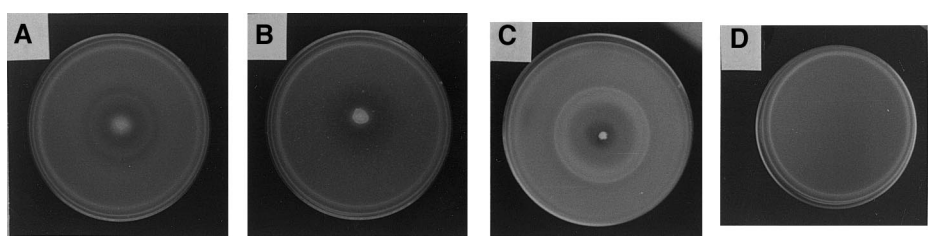

FIG. 3. Chemotactic response of *Ralstonia* sp. SJ98 in drop assay towards: (A) 3-methyl-4-nitrophenol; (B) 3-methyl-4-nitrophenol along with amiloride at a concentration of 2 mM in the medium; (C) aspartic acid used as positive control; (D) negative control.

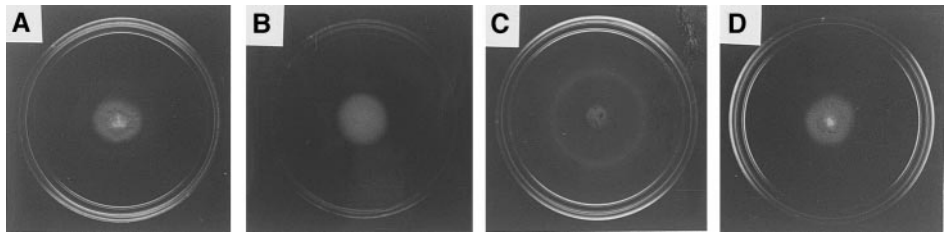

FIG. 4. Chemotactic response of *Ralstonia* sp. SJ98 in swarm plate assay towards: (A) 3-methyl-4-nitrophenol; (B) 3-methyl-4-nitrophenol along with amiloride at a concentration of 2 mM in the medium; (C) aspartic acid used as positive control; (D) negative control.

4 h of incubation (0.05 mM); after 6 h of incubation 0.10 mM of catechol was detected (Fig. 2B) and after 10 h its concentration in the medium was 0.20 mM (Fig. 2C). Catechol increased up to a concentration of 0.28 mM after 14 h with corresponding depletion of 3-methyl-4-nitrophenol (0.32 mM; retention time of 4.07 min). However, in absence of 2,2′-dipyridyl, there was a complete degradation of 3-methyl-4-nitrophenol within 10 h and no intermediates were detected; the maximum catechol released was 0.40 mM after 8 h of incubation which corresponded to the depletion of 3-methyl-4-nitrophenol (0.42 mM). Unidentified compound II as indicated above could not be identified and future investigations are necessary to identify the same. On the basis of studies carried out by TLC, GC, GC-MS, and HPLC, it could be established that catechol is one of the intermediates in the degradative pathway of 3-methyl-4-nitrophenol.

Since our group recently reported that *Ralstonia* sp. SJ98 is chemotactic towards several NACs (9), the chemotactic behavior of *Ralstonia* sp. SJ98 towards 3-methyl-4-nitrophenol was also tested by three different methods, i.e., drop assay, swarm plate assay, and capillary assay. All these methods demonstrated the chemotaxis of *Ralstonia* sp. SJ98 towards 3-methyl-4-nitrophenol. The results of drop and swarm plate assay in the form of migrating rings of the microorganism have been shown in Figs. 3 and 4, respectively. In capillary assay, it was observed that *Ralstonia* sp. SJ98 was chemotactic towards 3-methyl-4-nitrophenol at an optimum concentration of 200 μM with a chemotaxis index (C.I.) of 9.0 (Table 1).

In order to test whether chemotactic activity in *Ralstonia* sp. SJ98 is driven by Na^+ motive force, experiments were performed with amiloride which is a well known selective inhibitor of Na^+ driven flagellar motor (14, 15). When amiloride was mixed in the chemotaxis medium at a threshold inhibitory concentration of 2 mM, it inhibited the chemotactic activity of the microorganism towards 3-methyl-4-nitrophenol which indicated that motility in *Ralstonia* sp. SJ98 is driven by Na^+ motive force (Figs. 3 and 4). Although restoration of motility of microorganisms by increasing the concentration of Na^+ ions in the medium has been reported earlier in some cases (14, 15), in the present study, the restoration of motility and chemotaxis could not be achieved even up to a concentration of 400 mM of sodium chloride indicating that the motility inhibition phenomenon may be irreversible in *Ralstonia* sp. SJ98.

This is the first report in which 3-methyl-4-nitrophenol is shown to be degraded via the formation of catechol (Fig. 5). This indicates that *Ralstonia* sp. SJ98 converts the non-polar methylated nitroaromatic compound into highly polar catechol which is then degraded further by oxygenase(s) enzyme. Furthermore, chemotaxis of any microorganism towards 3-methyl-4-nitrophenol, an immediate byproduct of fenitrothion, has also been shown for the first time suggesting the role of *Ralstonia* sp. SJ98 in efficient degradation of 3-methyl-4-nitrophenol.

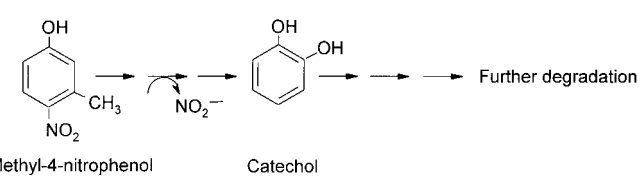

FIG. 5. Proposed initial pathway for the degradation of 3-methyl-4-nitrophenol by *Ralstonia* sp. SJ98.

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