Chemotaxis and Biodegradation of 3-Methyl-4-Nitrophenol by Ralstonia sp. SJ 98

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Received June 20, 2000

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. SJ 98 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. SJ 98 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. SJ 98; 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 organophosphorus 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. SJ 98 (9). In the present study the involvement of this Ralstonia sp. SJ 98 in chemotaxis and biodegradation of 3-methyl-4-nitrophenol has been elucidated.

MATERIALS AND METHODS

Microorganism and culture conditions. A Ralstonia sp. SJ 98 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-
**RESULTS AND DISCUSSION**

A NACs degrading, gram negative, motile bacterium *Ralstonia* sp. SJ 98 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. SJ 98 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 Rf 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 $\text{M}^+ - \text{CO}$ and $\text{M}^+ - \text{CHO}$ 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 Rf 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. SJ 98.

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Number of cells in the capillary</th>
<th>Chemotaxis index (C.I.) ± S.D.</th>
</tr>
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<tbody>
<tr>
<td>3-Methyl-4-nitrophenol</td>
<td>8300</td>
<td>9.0 ± 0.8</td>
</tr>
<tr>
<td>Aspartic acid^a</td>
<td>55000</td>
<td>60 ± 5.8</td>
</tr>
<tr>
<td>Negative control</td>
<td>920</td>
<td>1 ± 0.2</td>
</tr>
</tbody>
</table>

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

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. SJ 98 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. SJ 98 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.
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. Sj 98 is chemotactic towards several NACs (9), the chemotactic behavior of Ralstonia sp. Sj 98 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. Sj 98 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. Sj 98 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. Sj 98 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. Sj 98 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. Sj 98.

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. Sj 98 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. Sj 98 in efficient degradation of 3-methyl-4-nitrophenol.

ACKNOWLEDGMENTS

We are thankful to Mr. Dhan Prakash and Mr. R. Sureshkumar for technical help. We are also grateful to Mr. Vikas Grower and Mrs. Kamaljeet Kaur, NIPER, Mohali for help in recording the HPLC and GC-MS spectra. This work was supported by CSIR and DBT, India. S.K.S. and A.C. were supported by Senior Research Fellowships awarded by CSIR, Government of India. This is IMTECH communication number 014/2000.

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