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Chemotaxis of a *Ralstonia* sp. SJ98 toward Different Nitroaromatic Compounds and Their Degradation

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A *Ralstonia* sp. SJ98, isolated by a chemotactic enrichment technique, was capable of utilizing different nitroaromatic compounds (NACs). It utilized *p*-nitrophenol, 4-nitrocatechol, *o*-nitrobenzoic acid, and *p*-nitrobenzoic acid as the sole source of carbon and energy. It was observed that *Ralstonia* sp. SJ98 was chemotactic to the above-mentioned NACs as tested by the drop assay, swarm plate assay, and capillary assay. However, it failed to show chemotactic behavior toward those compounds which were not degraded by the microorganism. This is the first report which shows the chemotaxis of a microorganism toward different NACs and their subsequent degradation. Some of the intermediates of the NACs' degradative pathways have been identified using TLC, GC, and GC-MS studies. The results presented here indicate a correlation between chemotaxis and biodegradation of NACs.

The microbial degradation of environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and nitroaromatic compounds (NACs) has been studied extensively (1–4). The pathways involved in the degradation of these compounds have been investigated and in many cases genes encoding for these pathways have been cloned and sequenced. However, an important aspect related to biodegradation which have received little attention is chemotaxis which is the movement of microorganisms toward chemical attractants and away from chemical repellents (5, 6). Chemotaxis could be very helpful in enhancing the ability of motile microorganisms to locate and subsequently degrade the toxic and recalcitrant compounds present in a contaminated environment.

NACs are widely distributed in the environment because of their extensive use in the manufacturing of pharmaceuticals, pesticides, plasticizers, azo-dyes and explosives (3, 4). Once released into the environment, NACs undergo complex physical, chemical and biological changes. These NACs and their incomplete degradative products have a high toxicity and some of them are potential carcinogens (4, 7). Nitrophenolic compounds can also accumulate in the soil as a result of hydrolysis of several organophosphorous insecticides such as parathion, methyl parathion and nitrophenolic herbicides (4, 8, 9) and may enter the ground water resources where they cause deleterious effects. Although there are several reports on the biodegradation of different NACs in the literature (3, 4, 10), relatively less work has been carried out on the identification of metabolites and the degradative pathways involved. Several microorganisms such as *Escherichia coli*, *Rhizobium* sp., *Azospirillum* sp., and *Pseudomonas* sp. have been reported to show chemotaxis toward many kinds of sugars, amino acids and aromatic compounds (6, 11, 12). It has been shown that the naphthalene-degrading *Pseudomonas putida* strains are chemotactic toward naphthalene and salicylate and this phenomenon was found to be plasmid-encoded (13, 14).

In the present study, a NACs-degrading, gram-negative and motile bacterium *Ralstonia* sp. SJ98 has been isolated and characterized from soil contaminated with pesticides. The microorganism is capable of utilizing several NACs as sole source of carbon and energy. Furthermore, for the first time, we have demonstrated that the microorganism exhibited chemotaxis toward those NACs which were degraded by it.

MATERIALS AND METHODS

Isolation and culture conditions of strain SJ98. Strain SJ98 was isolated from a pesticide contaminated soil sample from Assam agricultural fields, India by using an enrichment technique developed in our laboratory and termed as 'chemotactic enrichment technique'. In this technique, first the selective microbial communities were enriched for *p*-nitrophenol degradation by using standard enrichment method in which a soil sample (1 g) was suspended into 100 ml minimal medium (MM, 15) supplemented with *p*-nitrophenol at a final concentration of 0.5 mM and incubated at 30°C under shaking conditions (150 rpm). The fading of yellow color to colorless indicated

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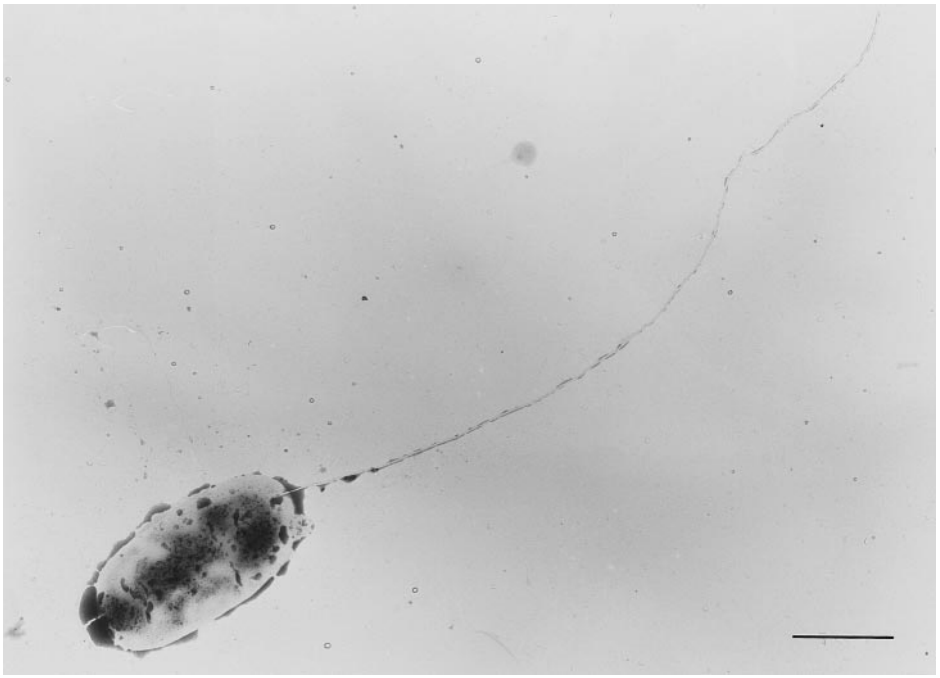

FIG. 1. Transmission electron microscopy of *Ralstonia* sp. SJ98 showing a long flagellum at 8000× magnification. Bar indicates 1 micron.

the degradation of *p*-nitrophenol. Thereafter, the enriched but mixed population of *p*-nitrophenol-degrading microorganisms was subjected to swarm plate assay of chemotaxis (see later). In this assay the microorganisms accumulated at the migrating turbid rings were taken out with the help of a micropipet, grown further and again subjected to the same swarm plate assay. After repeating three such exercises, the samples were plated onto MM agar containing *p*-nitrophenol and thus a pure culture of a microorganism was obtained which was motile, chemotactic and was capable of utilizing *p*-nitrophenol as the sole source of carbon and energy.

Identification of strain SJ98. The identification of strain SJ98 was carried out by its morphological, physiological and biochemical features using the Bergey's Manual of Systematic Bacteriology (16). The transmission electron microscopy (TEM) was performed to visualize the flagellar structures using standard negative staining procedure of TEM. In this method the cells were grown in nutrient broth (NB) till mid-log phase and subsequently washed three times with potassium phosphate buffer (pH 7.2) and finally suspended in distilled water. One drop of this suspension was mixed with a drop of 2% phosphotungstic acid (pH 7.0). After 2–3 min of incubation at room temperature, a drop of sample was transferred on to a formvar (polyvinyl formaldehyde)-coated grid (mesh size 200), air-dried, and finally observed under transmission electron microscope.

Growth conditions and extraction of intermediates of biodegradative pathways. The composition of the MM used in the present study was same as described earlier (15). The NACs were added as the filter sterilized solution into the MM at a final concentration of 0.5 mM. The media were inoculated with overnight grown seed cultures and incubated at 30°C under shaking conditions (200 rpm). Growth on different NACs in liquid culture was monitored by measuring absorbance (O.D.) at 600 nm. The depletion of *p*-nitrophenol (17), 4-nitrocatechol (18) and *o*-nitrobenzoic acid (19) was determined as described previously. For *p*-nitrobenzoic acid, the absorbance at 272 nm (absorbance maxima at pH 7.0) was monitored and the residual *p*-nitrobenzoic acid was calculated from a standard curve prepared using authentic *p*-nitrobenzoic acid. Nitrite release assay was performed as described earlier (20, 21); nitrite concentrations were determined by comparison of values with those of a standard calibration curve prepared using sodium nitrite. Ammonia was detected by measuring the oxidation of NADH in the presence of 2-oxoglutarate and L-glutamate dehydrogenase using ammonia detection kit purchased from Sigma Chemicals Co. (St. Louis, MO).

In order to elucidate the degradative pathways the extractions of intermediates were carried out. Following growth of the microorganism as described above, the cell-free supernatants were extracted with equal volume of ethyl acetate (neutral extraction) and then the pH of the aqueous phase was adjusted to 2.0 with HCl and again extracted with ethyl acetate (acidic extraction) (22). The neutral and acidic extracts were mixed and evaporated to dryness using a rotary evaporator and dissolved in 200 μl of methanol for its further analyses to identify the possible intermediates in the degradative pathways of NACs.

Analytical methods. Thin layer chromatography (TLC) and gas chromatography (GC) were carried out to study the degradation and identify the possible intermediates of the degradative pathways of NACs as described previously (23). Gas chromatography-mass spectrometry (GC-MS) analysis was performed using a Shimadzu QP5000 GC-MS equipped with quadruple mass filter and DB-1 (100% dimethylpolysilaxane) capillary column (30 m × 0.25 mm), ionization of 70 eV, scan interval 1.5 sec and mass range 40 to 500. The oven temperature was 120°C for 5 min with 10°C increase per minute to a final temperature of 200°C for 20 min and the injector temperature was kept at 200°C. The parameters for the detection of *o*-nitrobenzoic acid were the same as reported earlier (19). Authentic NACs (10 mg/ml final concentration) were used for comparative studies.

Chemotaxis assays. The chemotaxis behavior of the strain SJ98 was tested by three well-established methods, i.e., drop assay (13, 24), swarm plate assay (25, 26) and capillary assay (26, 27). All these methods are well established in our laboratory (14).

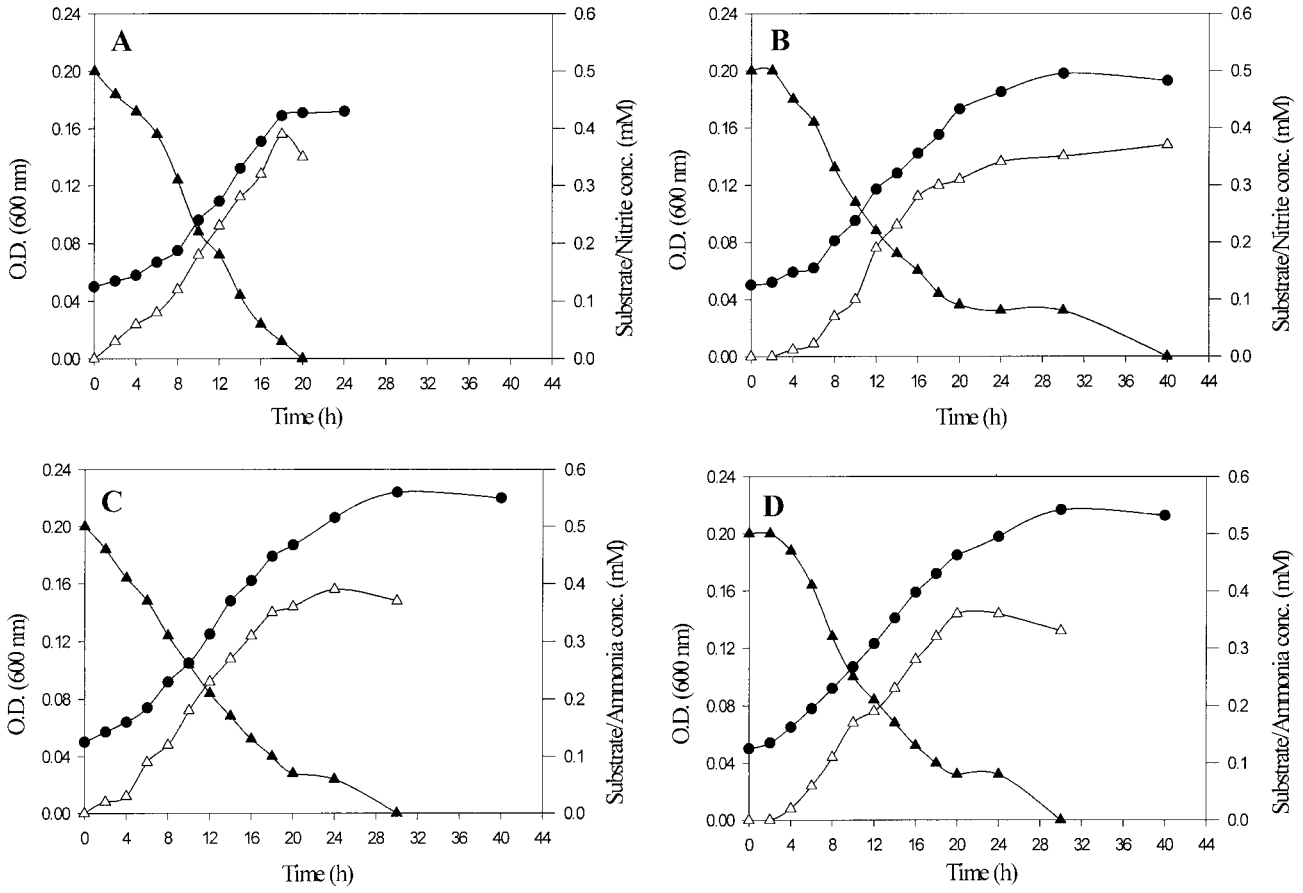

FIG. 2. Degradation of NACs by *Ralstonia* sp. SJ98 grown on *p*-nitrophenol (A), 4-nitrocatechol (B), *o*-nitrobenzoic acid (C), and *p*-nitrobenzoic acid (D) as sole source of carbon and energy. The inoculum used here was taken from cells grown on respective NACs. ●, $O.D._{600}$ of the culture; ▲, substrate depletion; △, nitrite/ammonia release.

Drop assay. The medium used for drop assay consisted of MM containing 0.30% bacto-agar and 1 mM glucose as carbon source. The cells were grown in NB and induced at their early log-phase with 0.2 mM *p*-nitrophenol and grown further for 3–4 h. Thereafter, the cells were pelleted, washed with MM and suspended in the drop assay medium, and then poured into petri plates. The chemotactic response was determined by placing the crystals of NAC at the center of the plate. Aspartic acid was used as positive control. The turbid rings were observed around the center within 3 h of incubation at room temperature (25°C).

Swarm plate assay. In this assay, an appropriate NAC (final concentration 0.2 mM) was added to MM medium containing 0.16% bacto agar and plates were poured. About 75 μl of washed cell suspension (OD_{600} ~2.0) in MM, earlier induced with 0.2 mM *p*-nitrophenol (as mentioned above) was gently poured at the center of the plate and incubated at room temperature (25°C). Aspartic acid (0.2 mM) was used as positive control. Here, 1 mM glucose was added to the cell suspension for providing energy to the cells. The formation of rings was observed after 12–16 h of incubation.

Capillary assay. Capillary tubes (Drummond Scientific, U.S.A.) of 1 μl capacity were used for this assay. The chemotaxis buffer consisted of 100 mM potassium phosphate buffer (pH 7.2) and 20 μM EDTA. The appropriate NAC was added to the buffer to give the final concentrations of 10, 20 and 200 μM. Aspartic acid (200 μM) was used as positive control. These solutions were filled into the capillaries which were then inserted into the cell suspension (10^6–10^7 cells/ml of chemotaxis buffer) on a glass slide. After a 30 min incubation at room temperature (25°C), the cells in the capillaries were plated out onto the nutrient agar plates following serial dilutions. The plates were incubated at 30°C for 24–48 h and, thereafter, the total number of colonies were determined. The capillary tube containing buffer alone was used as control. The chemotaxis index (C.I.) (11) was determined as the ratio of the number of bacterial cells accumulated in the test capillaries containing NAC to that of control.

Chemicals. All the NACs used were purchased either from Sigma or Aldrich, U.S.A. Analytical and spectroscopic grade ethyl acetate and methanol were purchased from E. Merck, Germany. All other chemicals were of highest purity grade commercially available.

RESULTS

Isolation and Identification of Strain SJ98

Strain SJ98 was isolated in our laboratory by employing a technique termed as "chemotactic enrichment technique" (see Materials and Methods) using *p*-nitrophenol as the sole source of carbon and energy as well as a chemoattractant. The morphological, physiological and biochemical features of strain SJ98 showed that it was a motile, gram negative, aerobic, short rods, oxidase and catalase positive. It grew on MacConkey agar as a lactose fermenting microorgan-

TABLE 1

Biodegradation of NACs by *Ralstonia* sp. SJ98

NACs used	TLC analysis: Intermediate identified (R_f value)	GC analysis: Intermediate identified (R_t value in min)	GC-MS analysis: Intermediates identified; Retention time (min); Mass spectral properties: {M/Z (% relative intensity [molecular ion]}
p-Nitrophenol	1,2,4-Benzenetriol (0.39)	1,2,4-Benzenetriol (3.78)	1,2,4-Benzenetriol; 9.475; {126 (72[M^+]), 97 (14), 80 (37), 55 (22), 52* (100)}
4-Nitrocatechol	1,2,4-Benzenetriol (0.39)	1,2,4-Benzenetriol (3.76)	1,2,4-Benzenetriol; 9.475; {126 (72[M^+]), 97 (14), 80 (37), 55 (22), 52* (100)}
o-Nitrobenzoic acid	Anthranilic acid (0.46)	Anthranilic acid (2.30)	Anthranilic acid; 4.292; {137 (60 [$M]^+$), 119* (100), 92 (77), 81 (4), 65 (52), 52 (40)}
p-Nitrobenzoic acid	Protocatechuic acid (0.20)	Protocatechuic acid (3.23)	Protocatechuic acid; 14.300; {154 (68[M^+]), 137* (100), 109 (41), 81 (35), 63 (52), 53 (76)}

Note. Intermediates were identified by comparing with authentic compounds.

ism and utilized citrate as the carbon source. Casein, starch and urea were not hydrolyzed, indole was not produced and gelatin was not liquefied. It showed the absence of arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase. The microorganism did not produce acid from sugars such as glucose, lactose, sucrose, xylose, arabinose, maltose, dulcitol, raffinose, salicin, adonitol, trehalose, rhamnose and inulin. On the basis of these results strain SJ98 was tentatively identified as *Ralstonia* sp. The transmission electron microscopic studies revealed the presence of a long flagellum (Fig. 1).

Biodegradation of NACs by Ralstonia sp. SJ98

It was observed that *Ralstonia* sp. SJ98 was capable of utilizing *p*-nitrophenol, 4-nitrocatechol, *o*-nitrobenzoic acid and *p*-nitrobenzoic acid as the sole source of carbon and energy with the total degradation of these compounds (Fig. 2). Since it is known in the literature that a NAC may be degraded either by an oxidative or a reductive route (3, 4, 10), attempts were made to determine the same in the present study. The results indicated that *p*-nitrophenol and 4-nitrocatechol were degraded oxidatively as determined by the release of nitrite molecules in the medium, whereas *o*-nitrobenzoic acid and *p*-nitrobenzoic acid were degraded reductively as determined by the release of ammonia in the medium (Fig. 2). Other tested compounds such as *o*-nitrophenol, *p*-nitroaniline, 2,3-dinitrotoluene, naphthalane, phenanthrene and salicylate were not degraded by the microorganism.

In order to identify the intermediates of the degradative pathways of NACs, TLC, and GC studies were performed on the extracted samples and these results were confirmed by GC-MS studies (Table 1). Taken together it was shown that 1,2,4-benzenetriol is an intermediate in the biodegradation pathway of *p*-nitrophenol and 4-nitrocatechol, anthranilic acid is an intermediate in the degradation pathway of *o*-nitrobenzoic acid and protocatechuic acid is an intermediate in the degradation pathway of *p*-nitrobenzoic acid (Fig. 3). The detailed analyses of the degradative pathways involved were not carried out for the reason that the primary objective of the present study was to correlate the phenomenon of chemotaxis with the microbial degradation of NACs.

Chemotaxis of Ralstonia sp. SJ98 toward NACs

The chemotaxis behavior of *Ralstonia* sp. SJ98 toward different NACs was tested by using three different methods. The qualitative methods included drop assay and swarm plate assay, and the quantitative

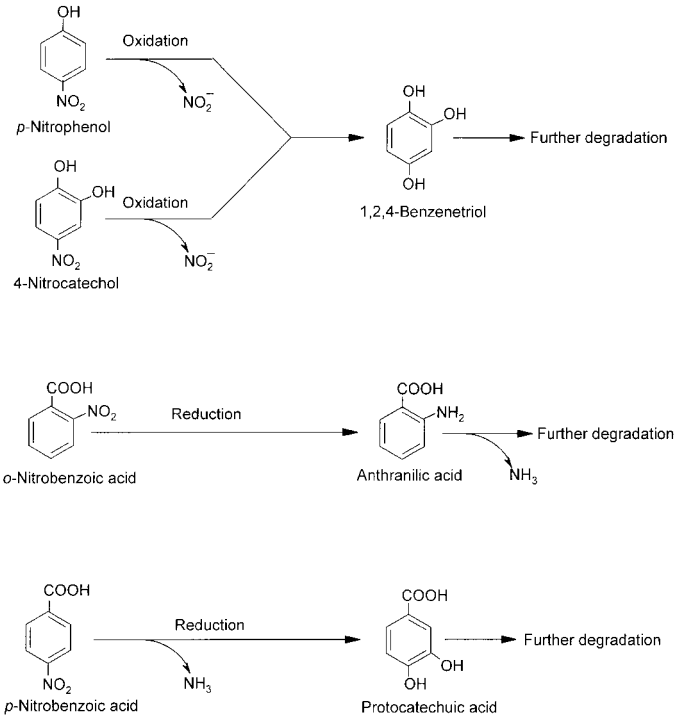

FIG. 3. The intermediates formed via oxidative and reductive routes during the biodegradation of NACs by *Ralstonia* sp. SJ98.

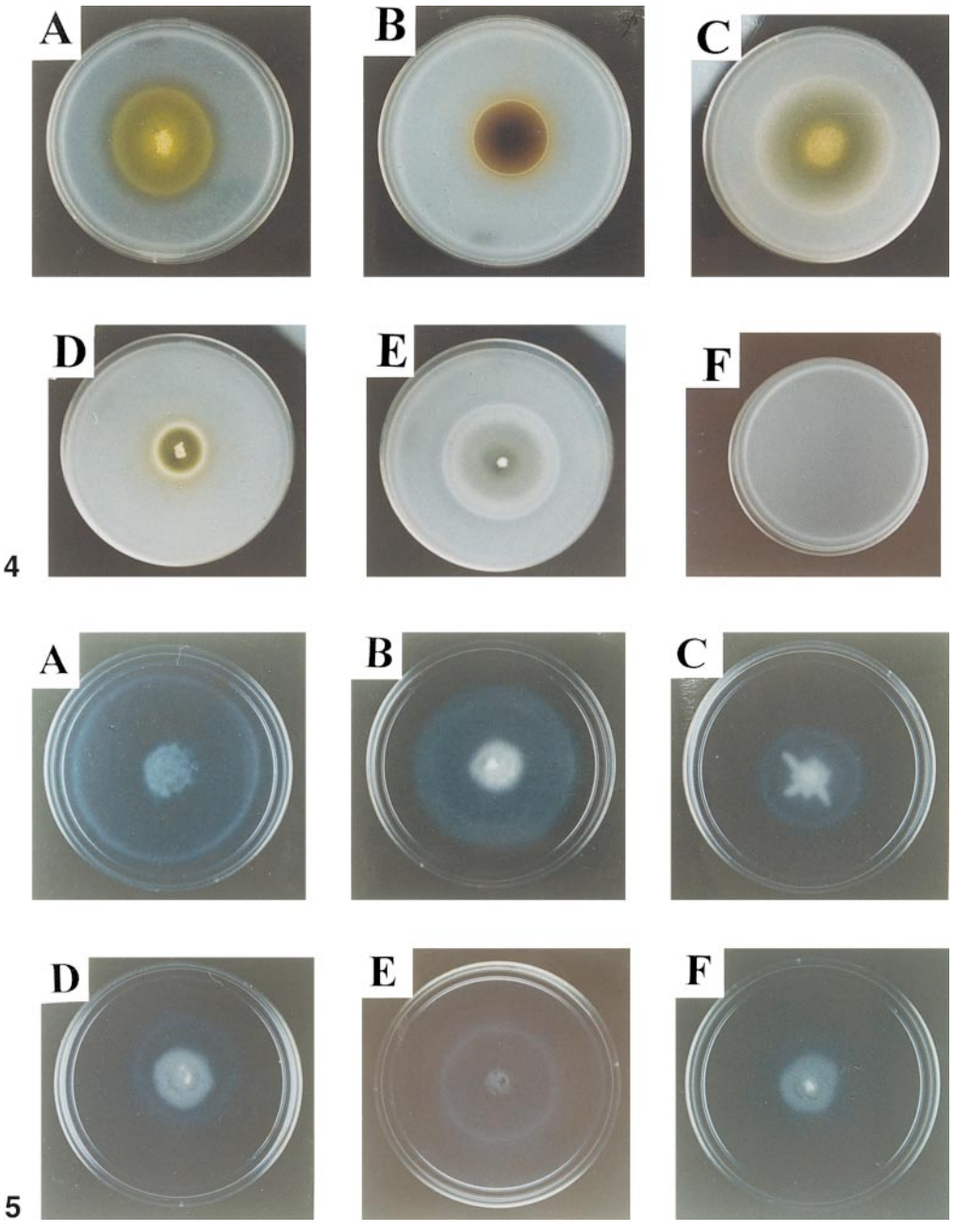

FIG. 4. Chemotaxis response of *Ralstonia* sp. SJ98 in drop assay toward different NACs: A, *p*-nitrophenol; B, 4-nitrocatechol; C, *o*-nitrobenzoic acid; D, *p*-nitrobenzoic acid; E, aspartic acid as positive control; F, negative control without any compound. Photographs were taken within 3 h of incubation.

FIG. 5. Chemotaxis response of *Ralstonia* sp. SJ98 in swarm plate assay toward different NACs: A, *p*-nitrophenol; B, 4-nitrocatechol; C, *o*-nitrobenzoic acid; D, *p*-nitrobenzoic acid; E, aspartic acid as positive control; F, negative control without any compound. Photographs were taken between 12 to 16 h of incubation. The outside rings of the cells were very sharp; however, they were disturbed during the shifting for photography.

method was performed by capillary assay. *Ralstonia* sp. SJ98 exhibited chemotaxis toward only those NACs which were utilized by the microorganism. The results of drop and swarm plate assays of chemotaxis in the form of migrating rings of the microorganism are shown in Figs. 4 and 5, respectively. The chemotaxis behavior of *Ralstonia* sp. SJ98 was quantitated by the capillary assay. It was observed that *Ralstonia* sp. SJ98 was chemotactic toward different NACs with a C.I. between 3- to 16-fold at their optimum concentration of 200 μM (Table 2). When aspartic acid was used as positive control, it showed very strong chemotaxis with a C.I. of 60. However, *Ralstonia* sp. SJ98 neither utilized *o*-nitrophenol, *p*-nitroaniline, 2,3-dini-

TABLE 2

Capillary Assay of Chemotaxis of *Ralstonia* sp. SJ98 toward Different NACs

NACs used	Number of cells in the capillary	Chemotaxis index (C.I.) ± S.D.
p-Nitrophenol	2700	3 ± 0.4
4-Nitrocatechol	14700	16 ± 2.5
o-Nitrobenzoic acid	6400	7 ± 1.4
p-Nitrobenzoic acid	3600	4 ± 0.3
Aspartic acid	55000	60 ± 5.8
Negative control	920	1 ± 0.2

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

trotoluene, naphthalene, phenanthrene and salicylic acid and nor it showed the chemotaxis toward these compounds which indicated correlation between biodegradation and chemotaxis phenomena. This is the first report showing the chemotaxis behavior of a microorganism toward NACs.

DISCUSSION

Ralstonia sp. SJ98, a chemotactic and NACs-degrading microorganism, was isolated from a pesticide contaminated soil by "chemotactic enrichment technique." This technique could be very useful in screening a selective chemotactic microorganism from a mixed microbial community. It was shown that *p*-nitrophenol, 4-nitrocatechol, *o*-nitrobenzoic acid and *p*-nitrobenzoic acid were completely degraded by *Ralstonia* sp. SJ98. Although we have identified 1,2,4-benzenetriol as an intermediate in the degradative pathway of *p*-nitrophenol and 4-nitrocatechol, further work is currently underway to determine the metabolic pathways for their degradation. 1,2,4-benzenetriol has earlier been shown to be an intermediate in the degradation of these compounds (4, 28) (Fig. 3). Anthranilic acid and protocatechuic acid were identified as intermediates in the degradative pathways of *o*-nitrobenzoic acid and *p*-nitrobenzoic acid, respectively. Earlier reports have also suggested the involvement of these compounds as intermediates (19, 29, 30).

Microorganisms have the ability to use different strategies to navigate to various ecological niches where environmental factors are favorable for their growth and survival. The present study has clearly revealed that in case of *Ralstonia* sp. SJ98, the chemotaxis phenomenon is metabolism-dependent since the microorganism exhibited chemotaxis toward those NACs which are completely utilized as sole source of carbon and energy. On the other hand, this microorganism neither showed the chemotaxis toward *o*-nitrophenol, naphthalene and phenanthrene, nor did it utilize these compounds. Therefore, there could be two possibilities for this physiological response. First, the chemotactic behavior is metabolism-dependent which means that the degradation of a compound results in a change in the cellular energy level which in turn gives rise to a signal to the flagellar motor responsible for the chemotaxis behavior. This metabolism dependent behavior is a wide spread phenomenon in microorganisms such as *E. coli*, *Salmonella typhimurium*, and *Rhodobacter sphaeroides* (31–33). The other possibility is the recognition of NACs or their degradative intermediates by the chemoreceptor(s) which ultimately transfers the signal to the flagellar motor. Work is currently underway in this direction to investigate the mechanism of 'biodegradation induced chemotaxis' phenomenon in our laboratory. However, on the basis of present study it can be concluded that biodegradation of NACs is essential for the chemotaxis of *Ralstonia* sp. SJ98 toward these compounds. Since the *Ralstonia* sp. SJ98 completely degraded the above said four NACs and also showed chemotaxis toward these compounds, hence it could prove to be useful in eliminating these NACs directly from the polluted environments.

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