Degradation of Atrazine, Propazine, and Simazine by *Rhodococcus* Strain B-30†

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Rhodococcus strain B-30 isolated from an agricultural soil degraded the herbicides atrazine, propazine, and simazine under aerobic conditions. Atrazine was degraded very rapidly. Almost 16 mg/L of atrazine was metabolized in 72 h, resulting in the formation of mono- and di-N-dealkylated products. Formation of 2-chloro-4,6-diamino-*s*-triazine was most likely caused by further degradation of deethylatrazine at the late stages of atrazine metabolism. The loss of atrazine from the incubation medium with *Rhodococcus* strain B-30 was fully accounted for by formation of metabolites. The bacterium degraded propazine to produce two major metabolites, 2-chloro-4-amino-6-(isopropylamino)-s-triazine and a new compound, 2-chloro-4-[(1-hydroxyprop-2-yl)amino]-6-(isopropylamino)-*s*-triazine, derived by the oxidative route. *Rhodococcus* strain B-30 metabolized stoichiometrically simazine to one of its mono-N-dealkylated products, namely 2-chloro-4-(ethylamino)-6-amino-*s*-triazine, which was not further degraded.

INTRODUCTION

Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine] is one of the most widely used herbicides in North America, mainly in corn-growing areas (Moxley, 1989). In recent years there has been considerable interest in atrazine biodegradation due to its potential as a groundwater pollutant (Goodrich et al., 1991) and for the development of technology for bioremediation of the herbicide-containing wastes (Cook, 1987a). Considerable information exists in the literature on the identification of processes that affect atrazine fate and transport in soils. For example, atrazine metabolism in soil has been shown to involve hydroxylation, N-dealkylation, and ring cleavage (Esser et al., 1975; Cook, 1987b; Erickson and Lee, 1989). N-Dealkylation of atrazine has been shown mainly due to fungi (Kaufman and Blake, 1970; Wolf and Martin, 1975). Giardina et al. (1979) isolated a *Nocardia* sp. from a soil enrichment culture that could N-dealkylate and deaminate atrazine using the herbicide as sole source of carbon and nitrogen. N-Dealkylation and deamination of atrazine occurred with this bacterium (Giardina et al., 1980). In other studies *Pseudomonas* species slowly N-dealkylated and dechlorinated atrazine (Behki and Khan, 1986).

Rhodococcus strains are ubiquitous in natural environments and are well-known for their diverse metabolic activities (Golovlev, 1980; Peczynska-Czoch and Mordarski, 1983; Finnerty, 1992). We recently reported that *Rhodococcus* soil isolates that degraded the herbicide EPTC (*S*-ethyl dipropylthiocarbamate) could also metabolize atrazine (Behki et al., 1993). The metabolism of atrazine by *Rhodococcus* TE1, like the degradation of EPTC (Tam et al., 1987), was associated with a relatively unstable plasmid (Behki et al., 1993) and involved only dealkylation. About 35% of the metabolized herbicide was not accounted for. *Rhodococcus* strain B-30 (Abou-Assaf, 1991) exhibited a stable EPTC-degrading phenotype and degraded EPTC by a mechanism different from that of *Rhodococcus* TE1 (*R*.TE1) (Dick et al., 1990; Behki and Khan, 1990). We investigated the metabolism of atrazine, simazine [2-chloro-4,6-bis(ethylamino)-*s*-triazine], and propazine [2-chloro-4,6-bis(isopropylamino)-*s*-triazine] by this bacterium and report on some of the additional catabolic functions of *R*.B-30, which unlike *R*.TE1, fully accounted for the metabolized atrazine.

MATERIALS AND METHODS

Bacteria. *Rhodococcus* strain B-30, kindly provided by Prof. W. A. Dick, is a soil isolate obtained by enrichment techniques from an EPTC-adapted soil at Ohio State University. It is an efficient EPTC degrader. The bacteria were routinely grown in a basal salts medium, BMN (Behki and Khan, 1986), supplemented with 1 g/L glycerol or glucose (BMNG) at 30 °C with shaking.

Chemicals. The three herbicides, atrazine, simazine, and propazine, were purchased from Chem Service (Westchester, PA) and were 96%+ pure. Analytical standards of *s*-triazine metabolites were a gift from Ciba Geigy (Greensboro, NC). Radiolabeled atrazine (U-ring-^{14}C, specific activity 4.5 mCimmol) was purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of the herbicides (5 mg/mL) were prepared in acetone or methanol.

Metabolic Studies. Aliquots of the *s*-triazine herbicides were taken from the stock solution into sterile Erlenmeyer flasks. The solvent was removed under a gentle stream of sterile air, and BMNG medium, to give a final concentration of 10–15 mg/L, was added to dissolve the herbicides. The medium was inoculated with B-30 cells grown overnight at 30 °C with shaking. For some experiments the bacteria were centrifuged and resuspended in BMNG before use. Cell-free controls containing the *s*-triazines were simultaneously included with all incubations. Aliquots were withdrawn from the incubation mixtures at various time intervals for measurement of cell growth, determined spectrophotometrically at 600 nm, and for analyses to determine the metabolic products. Atrazine metabolism under anaerobic conditions was tested with B-30 cells incubated with the herbicide under helium as described previously (Behki et al., 1993). The mass balance of atrazine metabolism was also determined by incubating [U-*ring*-^{14}C]atrazine (10 mg/L) in BMNG medium inoculated with B-30 cells for 84 h. The total ^{14}C in the incubation mixture was determined by liquid scintillation counting at 0 and 84 h. The radioactivity associated with the cell pellet was counted after the mixture was centrifuged at 12000*g* for 12 min. The mineralization of [^{14}C]atrazine was tested by acidification of the incubation medium to pH 2 to drive off any $^{14}CO_2$ and counting the radioactivity in the mixture. The cell-free radioactive incubation mixture after 84 h of incubation was chromatographed on an HPLC fitted with a radioactivity detector to quantitate atrazine and the metabolites.

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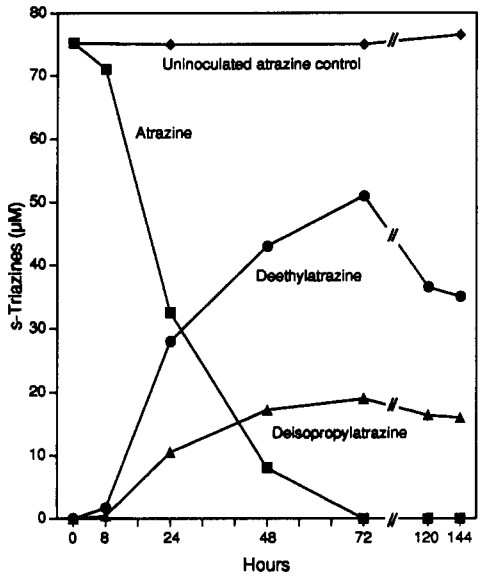

Figure 1. Degradation of atrazine by *R.*B-30 in BMNG medium. Initial OD (600 nm) of the incubation mixture was 0.14.

Analysis: High-Pressure Liquid Chromatography (HPLC). The high-pressure liquid chromatograph used was a Varian Model 5500 equipped with a variable-wavelength UV detector and a radioactivity monitor and data station (Berthold LB 504 and LB 512). The column was a Whatman Partisil 10 ODS-2 (25 cm long × 9.4 mm i.d.). Samples for analysis of atrazine, propazine, and simazine and their metabolites were obtained by centrifuging bacteria from the incubation mixture (12000*g*, 12 min) and analyzing aliquots of the supernatants by isocratic reversed HPLC using a methanol–water (70:30) solvent system (Behki et al., 1993).

Gas Chromatography (GC). Cell-free supernatants were extracted three times with 2 volumes of dichloromethane. The pooled fractions were dried over sodium sulfate, evaporated to just dryness, and dissolved in a small volume of methanol for analysis by GC. A portion of the extract was methylated with diazomethane. A Varian Model 3700 gas chromatograph equipped with a thermionic specific detector and a 15 m × 0.53 mm silica Megabore column coated with Carbowax (1.5 μm) was used. The column temperature was programmed from 170 to 220 °C at a rate of 2 °C/min. The injector and detector temperatures were 190 and 310 °C, respectively. The flow rate of the helium carrier gas was 20 mL/min. The identity of the compounds was confirmed by comparison of retention times (Rt) with those of authentic samples, by cochromatography, and finally by gas chromatography–mass spectrometry (GC–MS). Electron ionization (EI) and chemical ionization (CI) mass spectra were recorded on a Finnigan MAT 312 double-focusing reversed-geometry instrument coupled to an INCOS data system. Methane (research grade) was used as the "reactant" gas for the CI spectrum. For the EI mass spectrum the electron energy was set at 70 eV.

The amount of each compound in the sample was calculated on the basis of the peak areas obtained with standardized authentic samples analyzed under the same HPLC or GC conditions.

RESULTS AND DISCUSSION

Degradation of Atrazine by *Rhodococcus* Strain B-30. Atrazine was rapidly degraded by *R.*B-30 (Figure 1). Most of the herbicide was metabolized in less than 72 h, resulting in the formation of N-dealkylated metabolites, namely deethyl- and deisopropylatrazine. Atrazine and its two N-dealkylated metabolites accounted for over 90% of the initial amount of the herbicide added at zero time. A substantial decrease in the amount of deethylatrazine was observed in the incubation mixture after about 120 h. This was accompanied by the appearance of a new metabolite indicated by a peak at Rt 2.8 min on the HPLC and at Rt 24.9 min on the GC chromatograms. The material represented by this peak was further identified by GC and GC–MS as 2-chloro-4,6-diamino-*s*-triazine with a retention time (Rt 24.9 min) and mass spectrum identical to those of the authentic compound. This metabolite was apparently not degraded further by B-30 cells. The amounts of the metabolite at 120 and 144 h were found to be 9 and 11 μM. The formation of the three metabolites noted above was further confirmed by using [^{14}C]atrazine in the incubation mixture and analyzing the degradation products by HPLC using a radioactivity monitoring detector. These results differ from those obtained with *R.*TE1 (Behki et al., 1993) in that only 67% of the atrazine added at zero time could be accounted for during the incubation. Furthermore, there was no detectable decrease in the amount of deethylatrazine formed from atrazine for up to 1 week, and the formation of 2-chloro-4,6-diamino-*s*-triazine was not observed. The mass balances of atrazine and the metabolites at 0, 8, 24, 48, 72, 120, and 144 h were 100, 96, 96, 88, 93, 84, and 81% with *R.*B-30 compared with 100, 95, 95, 68, 65, 59, and 61% with *R.*TE1 (Behki et al., 1993). In addition, about 3.5–5.5% of the radioactivity was associated with the cell pellet as determined in experiments with [^{14}C]atrazine.

Table 1. Degradation of Atrazine by *Rhodococcus* Strains B-30 and TE1 at Various Stages of Cell Growth

Rhodococcus strain	OD (600 nm)		atrazine[a] degraded (%)	
	0 h	20 h	20 h	66 h
B-30	0.11	0.69	55.2	99.4
TE1	0.12	1.08	15.2	97.6
B-30	0.90	1.66[b]	97.1	ND[c]
TE1	0.85	1.48[b]	16.3	98.7
B-30	3.19	3.21	96.8	ND
TE1	3.05	2.97	74.1	98.9

[a] Initial atrazine concentration was 75 μM. [b] Optimal OD value in BMNG before the stationary phase. [c] ND, not determined.

Rhodococcus TE1 degrades atrazine primarily when the bacteria are in the stationary phase of growth (Behki et al., 1993). However, *R.*B-30 was found to degrade atrazine throughout the growth cycle (Table 1). This was demonstrated by centrifuging the overnight cultures of the *Rhodococcus* strains, resuspending the cells in BMNG containing 75 μM atrazine, and incubating for 66 h. The amount of atrazine remaining in the incubation mixture was determined at 20 and 66 h. The results showed that the amount of atrazine degraded by *R.*B-30 was mainly dependent on the number of bacteria in the incubation mixture rather than on the stage of cell growth.

Rhodococcus strains TE1 and B-30 showed some similar characteristics for atrazine degradation. The addition of EPTC (up to 100 μg/mL) to the incubation medium containing atrazine (15 μg/mL) did not affect either the rate or the extent of atrazine degradation or vice versa. Neither of the herbicides alone or in combination had any effect on the growth of the bacteria. Atrazine degradation was not due to the induction phenomenon since the extent of atrazine degradation was the same by bacteria grown in the presence or absence of atrazine in the medium. There was no atrazine degradation if the incubations were carried out under anaerobic conditions. No mineralization of U-^{14}C-ring-labeled atrazine was observed, indicating that the triazine ring remained intact. *Rhodococcus* B-30 also differed from *R.*TE1 in some other aspects. For example, the formation of 2-chloro-4,6-diamino-*s*-triazine from atrazine was not detected with *R.*TE1 but was evident with *R.*B-30. Furthermore, the metabolites formed from atrazine almost fully accounted for the loss of the herbicide

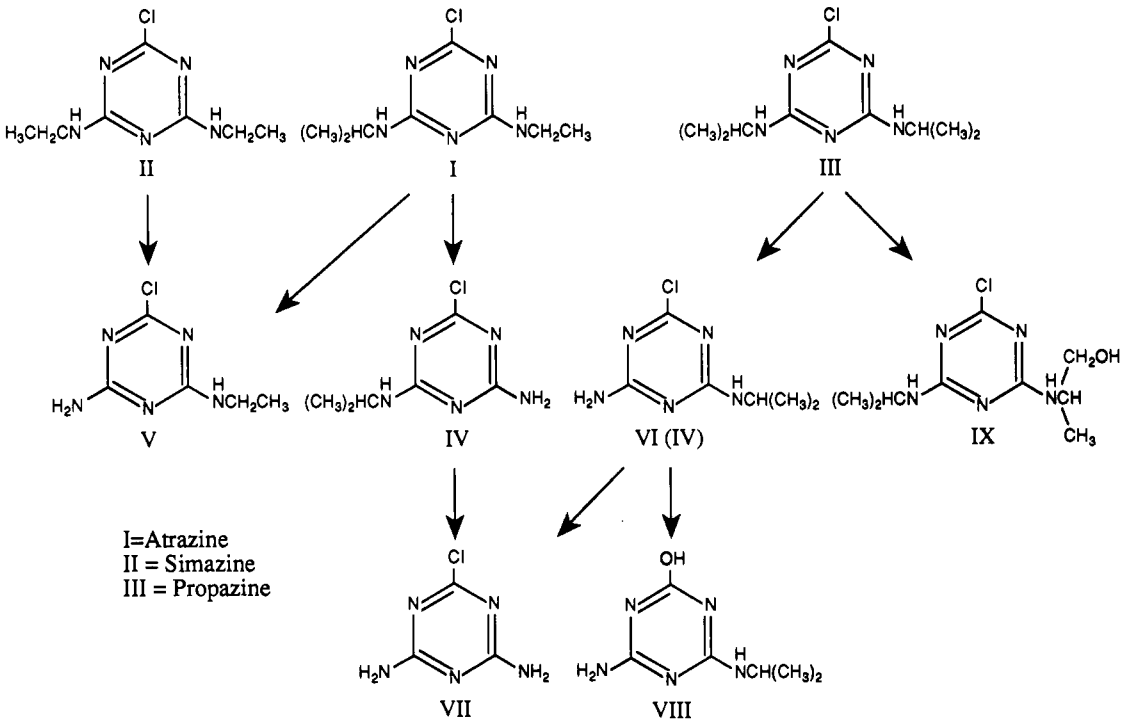

Figure 2. Schematic presentation of atrazine, propazine, and simazine degradation by *R*.B-30.

from the incubation medium, and the degradation was not primarily limited to the cells in the stationary phase.

Degradation of Simazine and Propazine. Simazine was degraded by *R*.B-30 at a slower rate in BMNG medium than atrazine. Under the experimental conditions used for atrazine metabolism, 2.9, 9.7, and 20.2 μM simazine of 70 μM added to the incubation medium at zero time was degraded in 24, 48, and 72 h, respectively. An almost stoichiometric amount of deisopropylatrazine was formed to account for the amount of simazine metabolized. The presence of 2-chloro-4,6-diamino-*s*-triazine was not detected after 6 days of incubation with simazine.

Propazine was degraded at a slower rate than atrazine or simazine by *R*.B-30 cells in BMNG medium. The amounts of propazine metabolized in 24, 48, 72, and 144 h were 1.1, 4.3, 12.4, and 23.2 μM when propazine (60 μM at zero time) degradation was determined under incubation conditions similar to those used for atrazine (Figure 1). Two major metabolites, monodealkylated propazine and compound **IX** (Figure 2), were formed from propazine. In addition, two minor metabolites were also recovered from the incubation mixture after 5–6 days of incubation. They were identified as 2-hydroxydeisopropylpropazine and 2-chloro-4,6-diamino-*s*-triazine by GC and GC–MS. There was no transformation of propazine in uninoculated control incubated under these conditions for 12 days.

The degradation pathways for atrazine, simazine, and propazine incubated with B-30 are shown in Figure 2. GC analyses of extracts of incubation mixtures showed peaks having retention times identical with those of authentic reference standards. Thus, GC analysis of the extract of the incubation mixture containing atrazine (**I**) with B-30 showed peaks at retention times (Rt) of 9.1, 15.0, 17.6, and 24.9 min. Furthermore, GC–MS of these peaks showed molecular ions at *m/e* 215, 187, 173, and 144, respectively, in addition to the chlorine isotopic peaks ($M^{\bullet+}$ + 2). The fragmentation spectra were analogous to those of the authentic compounds **I** (Rt 9.1 min), **IV** (Rt 15.0 min), **V** (Rt 17.6 min), and **VII** (Rt 24.9 min). Similarly, incubation of simazine (**II**) with B-30 resulted in the formation of **V** (Rt 17.6 min). Analysis of the extract from the incubation mixture containing propazine (**III**) with B-30 revealed the presence of **III** (Rt 7.3 min), **VI** or **IV** (Rt 15.0 min), and two other peaks at Rt 10.8 min and Rt 27.0 min. The compound at Rt 10.8 min showed a molecular ion at *m/e* 169 and was found to be a hydroxy derivative of **VI** or **IV** (Figure 2). The product represented by a peak at Rt 27.0 min was further analyzed in detail by CI and EI mass spectrometry. The CI mass spectra (Figure 3a) showed a base peak at *m/e* 246 ($M^{\bullet+}$ + 1) with chlorine isotopic peaks ($M^{\bullet+}$ + 2) at *m/e* 248. The CI mass spectra (methane) also showed peaks at *m/e* 274 ($M^{\bullet+}$ + 29) and 286 ($M^{\bullet+}$ + 41). The EI mass spectra (Figure 3b) did not show a distinctive $M^{\bullet+}$ ion, but it exhibited peaks at *m/e* 230 ($M^{\bullet+} - CH_3$, 4.2%) and 214 ($M^{\bullet+} - CH_2OH$, 100%) with a chlorine isotopic peak at *m/e* 216 ($M^{\bullet+}$ + 2). The mass of the compound ($M^{\bullet+}$) was found to be 245.1043, which is compatible with that calculated (245.10432) for $C_9H_{16}N_5ClO$. Thus, the compound represented by the peak at Rt 27.0 min was assigned the structure shown for compound **IX** (Figure 2).

The results of this study show that atrazine was very rapidly degraded by *R*.B-30 and the degradation was dependent upon cell density rather than the stage of cell growth. *Rhodococcus* TE1 degrades the herbicide primarily in stationary phase cells. Also, unlike the results reported previously with *R*.TE1 (Behki et al., 1993), the metabolites formed with B-30 culture fully accounted for the amount of atrazine degraded up to 72 h. Slightly lower values at later times may represent undetected metabolites or intermediates. The formation of 2-chloro-4,6-diamino-*s*-triazine was evident only after 4–5 days of incubation of atrazine with B-30 and coincided with the decrease in the amount of deethylatrazine (Figure 1). This compound was not formed with *R*.TE1. *Rhodococcus* B-30 did not degrade deisopropylatrazine even after incubation for 12 days. Deethylatrazine was degraded by B-30 extremely slowly and to a limited extent after a lag of about 5 days when 2-chloro-4,6-diamino-*s*-triazine formation was detected. *Rhodococcus* TE1 did not degrade either of the monoalkylated (or dealkylated) *s*-triazines. This indicates that the presence of both alkyl groups is important for

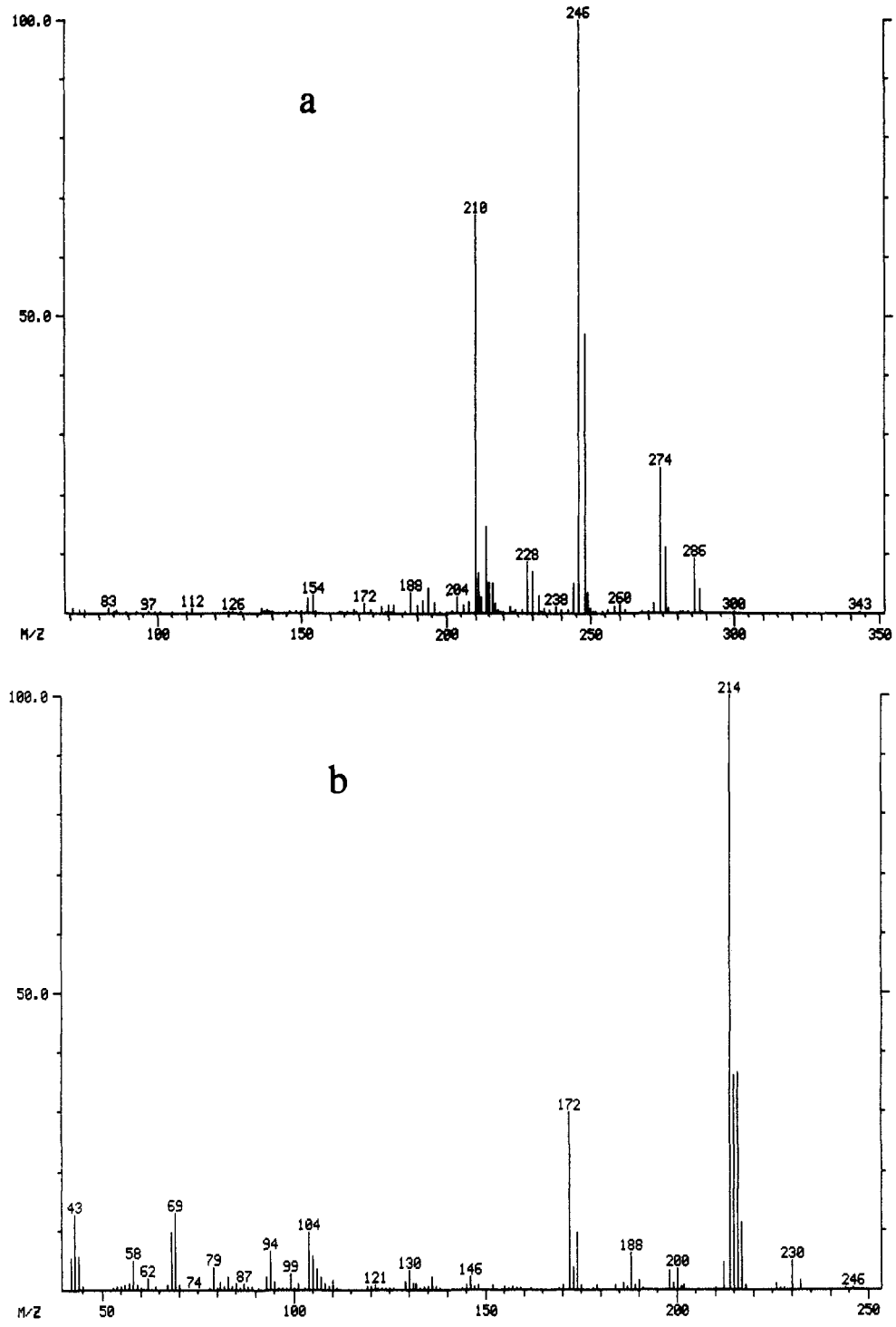

Figure 3. CI (a) and EI (b) mass spectra of compound **IX** (Figure 2).

N-dealkylation of either of the alkyl group. This was not applicable to hydroxyatrazine, which was not degraded or dealkylated at all by either of the two *Rhodococcus* strains. Thus, it appears that the presence of Cl at the 2-position is necessary for dealkylation of atrazine by the two bacterial strains.

Atrazine dealkylation is the dominant degradative pathway in soil (Erickson and Lee, 1989), and dealkylated atrazine derivatives were shown to be more readily degraded than the parent compound (Cook and Hutter, 1984). Cook and Hutter (1984) reported dechlorination and deamination of deethylsimazine by a *Rhodococcus* species. However, their *Rhodococcus* isolate did not degrade atrazine. The results reported here extend the range of diverse metabolic activities of various strains of this genus. These bacteria belonging to the actinomycetes group, abundant in soil, may play an important role in catalyzing atrazine degradation in situ.

Simazine degradation by *R*.B-30 similar to the results obtained with *R*.TE1 produced a stoichiometric amount of deisopropylatrazine which accumulated in the medium and was stable for up to 2 weeks. However, *R*.B-30 catalyzed propazine degradation quite differently from *R*.TE1. The latter converted propazine to its monodealkylated product only (Behki et al., 1993). With B-30, an additional metabolite (**IX** in Figure 2) was found to accumulate in the culture medium. Hydroxylation of the propyl group during EPTC degradation by this *Rhodococcus* strain has been reported (Dick et al., 1990). Small amounts of 2-chloro-4,6-diamino-*s*-triazine and hydroxy-

deethylatrazine were produced at the later stages of incubation. The formation of the former was also observed with atrazine (Figure 1). Atrazine and its metabolites were not used as sole nitrogen sources by *R*.B-30, indicating that the molecules are not deaminated.

None of the *s*-triazine herbicides used in the studies were metabolized by *R*.B-30 under anaerobic conditions, suggesting the involvement of an oxidative mechanism for dealkylation reactions similar to that observed for *R*.TE1. The enzymological studies of N-dealkylation of atrazine with the *Rhodococcus* strains have not as yet been successful.

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