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Metabolism of the Herbicide Bromoxynil by *Klebsiella pneumoniae* subsp. *ozaenae*

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Enrichment of soil samples for organisms able to utilize the herbicide bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) as a nitrogen source yielded bacterial isolates capable of rapidly metabolizing this compound. One isolate, identified as *Klebsiella pneumoniae* subsp. *ozaenae*, could completely convert 0.05% bromoxynil to 3,5-dibromo-4-hydroxybenzoic acid and use the liberated ammonia as a sole nitrogen source. Assays of cell extracts of this organism for the ability to produce ammonia from bromoxynil revealed the presence of a nitrilase (EC 3.5.51) activity. The enzyme could not utilize 3,5-dibromo-4-hydroxybenzamide as a substrate, and no 3,5-dibromo-4-hydroxybenzamide could be detected as a product of bromoxynil transformation. Comparison of related aromatic nitriles as substrates demonstrated that the *Klebsiella* enzyme is highly specific for bromoxynil.

Owing to the steady use of synthetically produced compounds over the last 20 years, enormous quantities of halogenated aromatic compounds have been produced, and a variety of these xenobiotic substances have been released into the environment by pesticide and herbicide application. Because of the recalcitrance of many of these compounds to degradation, their persistence in the environment can be lengthy. Recently, much attention has focused on the microbial degradation of the chlorophenoxyacetate herbicides 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid (1, 8). We focused our interest on the biodegradative process for the halogenated aromatic nitrile bromoxynil (3,5,-dibromo-4-hydroxybenzonitrile), a widely used herbicide that is selective against broad-leaved weeds in a number of tolerant plants. Results of previous work on the possible effects of this phytotoxic agent on natural microbial populations has shown this herbicide to be bactericidal and fungistatic (21).

Both microbial populations and tolerant plants can transform the cyano group of nitrilic herbicides. Soil perfusion studies have shown that the cyano group on both bromoxynil (7, 19) and 2,6-dichlorobenzonitrile (15, 17, 23) can be hydrolyzed to the corresponding amide and carboxylic acid derivatives. Bromoxynil degradation by a *Flexibacterium* sp. (20) revealed the presence of the amide and acid products of the herbicide, and a corresponding study in wheat with ring-labeled bromoxynil demonstrated that the cyano group is hydrolyzed to the corresponding amide and carboxylic acid products (4, 5). These observations suggest that the cyano moiety of the molecule is important for its toxic properties to microorganisms and plants and that removal of this constituent would essentially detoxify the compound.

The hydrolysis reactions described above, as depicted for bromoxynil in Fig. 1, are catalyzed by a class of enzymes termed nitrilases. Nitrilase has been partially purified from barley and shown to convert a variety of aromatic nitriles to their corresponding acid derivatives in the absence of any detectable amide intermediate (14, 22). In addition, nitrilases have been purified from two species of *Nocardia* sp. (*rhodochrous* group) (9, 11), *Fusarium solani* (10), and *Arthrobacter* sp. (2). These enzymes exhibit a broad substrate specificity for many aromatic nitriles, converting them to their corresponding acids. Despite the fact that the *F. solani* and one of the *Nocardia* sp. were isolated from bromoxynil-treated soil, bromoxynil proved to be a very poor substrate for the nitrilase enzymes synthesized by these organisms.

In this report we demonstrate that a natural soil isolate, identified as *Klebsiella pneumoniae* subsp. *ozaenae*, is able to completely transform 0.05% bromoxynil to the corresponding acid and to utilize the liberated ammonia as its sole nitrogen source. Transformation of bromoxynil by this organism was attributed to a nitrilase activity highly specific for bromoxynil.

MATERIALS AND METHODS

Chemicals. 3,5-Dibromo-4-hydroxybenzonitrile, 3-bromo 4-hydroxybenzonitrile, 3,5-dibromo-4-hydroxybenzamide, and 3,5-dibromo-4-hydroxybenzoic acid were kindly supplied by May and Baker Ltd., Essex, England. Benzonitrile and 4-hydroxybenzonitrile were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Agar, tryptone, yeast extract (YE), and the gram stain were from Difco Laboratories, Detroit, Mich. All indicator dyes were from Sigma Chemical Co., St. Louis, Mo. The acetonitrile and water used for high-performance liquid chromatography (HPLC) were purchased from J. T. Baker Chemical Co., Phillipsburg, Pa., and were of HPLC grade. All other chemicals were of reagent grade.

Media, cultures, and growth conditions. Enrichment media (nitrogen-deficient [N^-] YE–multiple carbon sources [multi C]–bromoxynil contained the following per liter: 3.5 g of K_2HPO_4, 1.5 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 50 mg of yeast extract, 1.0 g of glycerol, 1.0 g of sodium citrate, 1.0 g of succinic acid, 0.5 g of 3,5-dibromo-4-hydroxybenzonitrile (sodium salt). N^--bromoxynil medium was the same except that the YE was omitted and the multi C sources were replaced by glucose at 2 g/liter. For growth of *Nocardia* sp. strain (*rhodochrous* group) NCIB 11215, the basal salts mixture was the same as N^--YE-multi C-bromoxynil, except that the multi C sources and bromoxynil were replaced with 2 g of mannitol and 0.5 g of 4-hydroxybenzonitrile per liter, respectively. The rich medium utilized was L-broth, which was modified by leaving out NaCl. Cultures were grown in a

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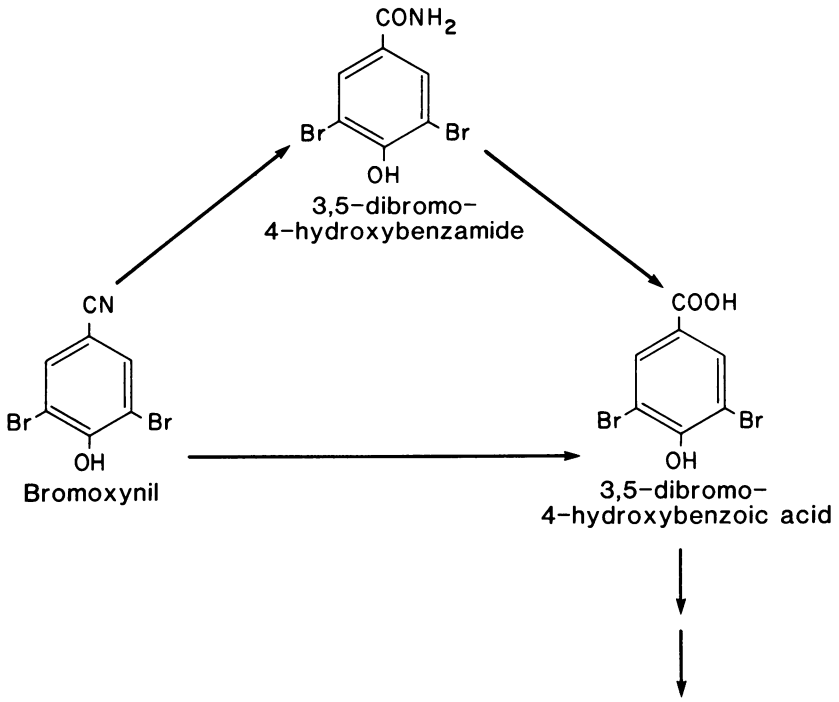

FIG. 1. Initial reactions in the metabolism of the herbicide bromoxynil by plants, microorganisms, or both.

Gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 30°C and 150 rpm. Agar-based media contained 15 g of Noble agar per liter.

K. pneumoniae subsp. *ozaenae* was obtained through the enrichment procedure described below. *Nocardia* sp. strain NCIB 11215 was generously provided by D. B. Harper of the Department of Agricultural and Food Chemistry, Queen's University, Belfast, Ireland.

Enrichment, isolation, and screening for bromoxynil degraders. The enrichment was initiated by the addition of 2 g of soil, obtained from an area contaminated with high bromoxynil levels, to 40 ml of N^- YE-multi C-bromoxynil medium in a 250-ml Erlenmeyer flask. Four serial transfers, each with a 1% inoculum, to 40-ml fractions of the same medium were conducted during a 19-day period. Cultures were fully grown at the time of each transfer.

After the final enrichment culture had grown to full density, a fraction of the suspension was streaked onto N^- YE-multi C-bromoxynil agar for the isolation of single colonies. Following incubation at 30°C 100 colonies were randomly picked from the population and restreaked twice onto modified LB plates to ensure purity. Each of the purified isolates was screened for the ability to transform bromoxynil in 1.5 ml of N^- YE-multi C-bromoxynil cultures. This was accomplished by inoculating each 1.5-ml fraction of medium with a single colony and shaking them for 24 h at 30°C, after which time the culture filtrates were analyzed by HPLC for the disappearance of bromoxynil against a noninoculated control sample (see below).

HPLC. Bacterial isolates were incubated at 30°C in either N^- YE-multi C-bromoxynil or N^--bromoxynil broth. After incubation culture filtrates were separated from the cells by centrifugation at 500 × *g* for 30 min. Culture filtrates were further clarified by passage through a 0.2-μgm-pore-size nylon-66 membrane. HPLC was performed on a system consisting of two pumps (100A; Beckman Instruments, Inc., Fullerton, Calif.), a 2.8-ml mixing chamber (Beckman), a 421 controller (421; Beckman), and a variable wavelength scanning spectrophotometer (1040A; Hewlett-Packard Co., Palo Alto, Calif.) controlled by a computer (85B; Hewlett-Packard). A C_{18} reverse-phase column (4.5 by 5.5 mm; Beckman) was used. Analysis of the filtrates proceeded under isocratic conditions. The aqueous solvent consisted of 60 mM triethylammonium bromide passed through a reverse-phase column (1 by 24 cm; RP-8; Lichroprep, Beckman) to remove trace contaminants and then filtered through a 0.2-μm-pore-size nylon-66 membrane and thoroughly degassed. The organic solvent was methanol. Solvents were mixed in a 2:3 ratio to result in an elution solvent that contained 60 mM triethylammonium bromide in 40% methanol. Flow rate was 1 ml/min. Five-microliter samples were analyzed for A_{286}.

Preparation of crude extracts and nitrilase assays. Overnight cultures (500 ml) were harvested by centrifugation at 8,000 × *g* for 15 min at 20°C. Cells were washed once in buffer containing 0.1 M potassium phosphate (pH 7.5), 5 mM dithiothreitol, and 1 mM EDTA and then suspended in 20 ml of the same buffer. The cell suspension was passed through a French pressure cell, and cell debris was removed by centrifugation at 60,000 × *g* and 2°C for 45 min. The resulting crude extract was dialyzed extensively against 0.1 M potassium phosphate (pH 7.5), 5 mM dithiothreitol, and 1 mM EDTA. Protein was assayed by the method of Bradford (3).

Nitrilase activity was assayed by the method of Harper (9). The reactions were initiated by the addition of 0.1 ml of crude extract samples to 0.9 ml containing 3 mM of each substrate in 0.1 M potassium phosphate (pH 7.5). After incubation for 1 h at 30°C the reactions were terminated by the addition of 1 ml of 0.33 M sodium phenoxide followed by the addition of 1 ml each of 0.01% sodium nitroferricyanide and 20 mM sodium hypochlorite. Each sample was then mixed thoroughly and placed in a boiling water bath for 3

min to allow complete color development. After dilution of each sample with 6 ml of water the A_{640} was measured. Ammonium chloride concentrations ranging from 0 to 1.5 μmol per assay tube were used to construct a standard curve. All samples were assayed in duplicate.

Identification of bacterial isolates. The preliminary classification of organisms capable of transforming bromoxynil is described below. The specific classification of the one organism chosen for further study was done by the method described in the 9th edition of *Bergey's Manual of Systematic Bacteriology* (13). All biochemical tests used have been described by Skerman (18).

RESULTS

Isolation and characterization of organisms able to metabolize bromoxynil. One hundred randomly chosen bacterial isolates were purified from enrichment cultures and screened individually for the ability to metabolize bromoxynil. The results revealed that 19 bacterial isolates are capable of metabolizing bromoxynil to unidentified metabolites shown by the appearance of new peaks on HPLC chromatographs (data not shown). Preliminary classification of these organisms by colony morphology and pigment production, cellular morphology, motility, Gram stain, and plasmid profiles revealed eight separate gram-negative isolates. One isolate exhibited facultative growth in the presence of oxygen. This organism, identified as *K. pneumoniae* subsp. *ozaenae* in accordance with the morphological and biochemical characters described in Table 1, was chosen for further study. To investigate the extent to which *K. pneumoniae* subsp. *ozaenae* was capable of utilizing bromoxynil as a sole nitrogen source, cells of this strain were incubated in N^- broth with and without 0.05% bromoxynil. The cell density of both cultures was monitored and correlated to the amount of bromoxynil remaining in the culture filtrate (Fig. 2). Growth of *K. pneumoniae* subsp. *ozaenae* in N^-–0.05% bromoxynil corresponded, for the most part, to the loss of bromoxynil from the medium, particularly during the 7 to 14 h of incubation when the culture was between the early-log and the early-stationary phases. When the cells reached the late-stationary phase (between 14.5 to 16.5 h of incubation), the rate of bromoxynil metabolism decreased. On further incubation (16.5 to 24 h) bromoxynil was virtually undetectable in the growth medium (data not shown).

TABLE 1. Characteristics of the bromoxynil-degrading organism *K. pneumoniae* subsp. *ozaenae*

Characteristic	Test result
Gram stain	−
Straight rods	+
Motility	−
Oxygen requirement	−[a]
Oxidase, Kovacs	−
Methyl red	+
Voges-Proskauer	−
Citrate, Simmons	+
Hydrogen sulfide on TSI Agar[b]	−
Urease, Christensen	−
Lysine decarboxylase	−
Ornithine decarboxylase	−
KCN, growth in	+
D-Glucose, acid production	+
D-Glucose, gas production	+

[a] Facultative growth
[b] BBL, Microbiology Systems, Cockeysville, Md.

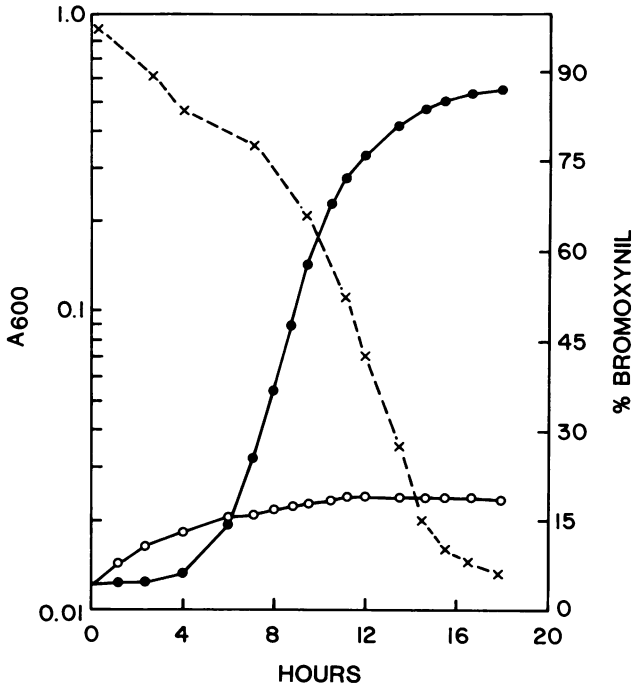

FIG. 2. Utilization of bromoxynil as a sole nitrogen source by *K. pneumoniae* subsp. *ozaenae*. An overnight culture of *K. pneumoniae* subsp. *ozaenae* was grown in the basal salts (N^-) medium described in the text, except that ammonium chloride was the nitrogen source. Fractions of the overnight culture were diluted to 10^7 cells per ml in two 50-ml cultures. These flasks contained N^- medium; one without bromoxynil and the other supplemented with 0.05% bromoxynil as a sole nitrogen source. The cultures were incubated at 30°C with shaking at 150 rpm, and the growth was monitored. Fractions (0.5 ml) were withdrawn at the indicated times and monitored for the disappearance of bromoxynil in the culture filtrate by HPLC, as described in the text. The area of the HPLC peaks were calculated and compared with a standard (0.5% bromoxynil in N^- medium). Symbols: ●, *K. pneumoniae* subsp. *ozaenae* supplemented with 0.5% bromoxynil as sole nitrogen source; ○, *K. pneumoniae* subsp. *ozaenae* without any nitrogen supplement; X, disappearance of bromoxynil in the culture filtrate.

By contrast, the culture without bromoxynil showed no signs of growth beyond the initial increase in culture density due to carry-over of exogenous ammonia from the fresh overnight inoculum. This phenomenon was not seen when the organism was incubated in the presence of bromoxynil because the bromoxynil was inhibitory in the initial growth phase. Thus, *K. pneumoniae* subsp. *ozaenae* is capable of utilizing bromoxynil as its sole source of nitrogen, presumably by liberating ammonia from the cyano group of the molecule. This organism, however, cannot utilize bromoxynil as a sole source of carbon.

Identification of the bromoxynil metabolite produced by *K. pneumoniae* subsp. *ozaenae*. In Fig. 3A are illustrated HPLC profiles of 3,5-dibromo-4-hydroxybenzoic acid, 3,5-dibromo-4-hydroxybenzamide, 4-hydroxybenzonitrile, and bromoxynil. Chromatography conditions enabled base-line separation of 4-hydroxybenzonitrile and bromoxynil and, therefore, their unambiguous identification. 3,5-Dibromo-4-hydroxybenzoic acid and 3,5-dibromo-4-hydroxybenzamide exhibited similar retention times and appeared as a single peak because of the amounts loaded. Retention time in this case could not be used as the sole identification criterion.

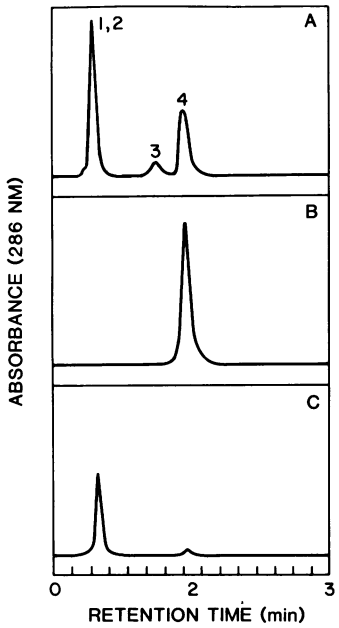

FIG. 3. HPLC analysis of the *K. pneumoniae* subsp. *ozaenae* culture filtrate containing bromoxynil. Media, growth of *K. ozaenae*, sample preparation, and HPLC conditions are described in the text. (A) Separation of standard compounds. Peak 1,2 contains 3,5-dibromo-4-hydroxybenzoic acid and 3,5-dibromo-4-hydroxybenzamide, while peaks 3 and 4 are the compounds 4-hydroxybenzonitrile and bromoxynil, respectively. The concentration of each standard component was 0.02%. (B) Analysis of a culture filtrate containing 0.05% bromoxynil in N^--YE medium after incubation for 16 h in the absence of *K. pneumoniae* subsp. *ozaenae*. (C) Analysis of a culture filtrate containing 0.05% bromoxynil in N^--YE medium after incubation for 16 h in the presence of *K. pneumoniae* subsp. *ozaenae*.

Spectral analysis along the peak followed by comparison of the spectra with purified standards enabled positioning of 3,5-dibromo-4-hydroxybenzoic acid and 3,5-dibromo-4-hydroxybenzamide within the single peak.

After 24 h of incubation of bromoxynil-supplemented medium in the absence of the organism, no degradation of the herbicide was evidenced (Fig. 3B). This same medium incubated in the presence of the *K. pneumoniae* subsp. *ozaenae* resulted in near complete conversion of bromoxynil to a new metabolic peak with a retention time similar to those of 3,5-dibromo-4-hydroxybenzoic acid and 3,5-dibromo-4-hydroxybenzamide (Fig. 3C).

Spectral analysis of this metabolite peak is presented in Fig. 4. In Fig. 4A are illustrated the spectra of the standard compounds 3,5-dibromo-4-hydroxybenzoic acid and 3,5-dibromo-4-hydroxybenzamide, while the spectral profile of the metabolite peak (Fig. 4B) matches the spectral profile of 3,5-dibromo-4-hydroxybenzoic acid. Both have peak maximums at 283 nm. It did not match with 3,5-dibromo-4-hydroxybenzamide, which has a maximum at 303 nm. We conclude that *K. pneumoniae* subsp. *ozaenae* uses bromoxynil as a sole nitrogen source by converting this compound directly to 3,5-dibromo-4-hydroxybenzoic acid, as no 3,5-dibromo-4-hydroxybenzamide could be detected in the metabolite peak.

Involvement of bromoxynil-specific nitrilase. To demonstrate that *K. pneumoniae* subsp. *ozaenae* possesses a nitrilase activity specific for bromoxynil, cell extracts from cells grown in N^--bromoxynil medium were prepared and assayed, with bromoxynil, 3-bromo-4-hydroxybenzonitrile, 4-hydroxybenzonitrile, benzonitrile, and 3,5-dibromo-4-hydroxybenzamide used as substrates (Table 2). For comparison purposes we also assayed cell extracts prepared from cells of *Nocardia* sp. strain (*rhodochrous* group) NCIB 11215 grown in N^- YE–0.2% mannitol–0.05% 4-hydroxybenzonitrile. This strain of *Nocardia* possesses a nitrilase activity which has been shown to have a substrate specificity of benzonitrile > 4-hydroxybenzonitrile >> bromoxynil (11).

The nitrilase activity of *K. pneumoniae* subsp. *ozaenae* produced 6- and 75-fold less ammonia from the substrates 3-bromo-4-hydroxybenzonitrile and 4-hydroxybenzonitrile, respectively, compared with that from a substrate of bromoxynil. Neither 3,5-dibromo-4-hydroxybenzamide nor benzonitrile acted as efficient substrates for this enzyme. The *Nocardia* nitrilase (11) exhibited a substrate specificity trend in the other direction. This enzyme formed 9-fold less ammonia with 4-hydroxybenzonitrile and 32-fold less ammonia with 3-bromo-4-hydroxybenzonitrile compared with benzonitrile. Both bromoxynil and 3,5-dibromo-4-hydroxybenzamide were not effective substrates for the *Nocardia* enzyme. These data demonstrate that the *Klebsiella* enzyme is highly specific for bromoxynil, and the order of specificity is bromoxynil > 3-bromo-4-hydroxybenzonitrile > 4-hydroxybenzonitrile ≫ 3,5-dibromo-4-hydroxybenzamide or benzonitrile.

DISCUSSION

Soil samples contaminated with high levels of bromoxynil were subjected to enrichment for organisms capable of utilizing the herbicide as a sole nitrogen source. Eight distinct bacterial isolates which could metabolize bromoxynil were isolated from the enrichment procedure. One of these organisms, an enteric bacterium, was classified as *K. pneumoniae* subsp. *ozaenae*. *K. pneumoniae* subsp. *ozaenae* was not only capable of rapidly metabolizing bromoxynil but, in addition, was found to utilize the herbicide effectively as a sole source of nitrogen. This was contrary to the results of a previous report (12) in which it was concluded that bromoxynil degradation by microor-

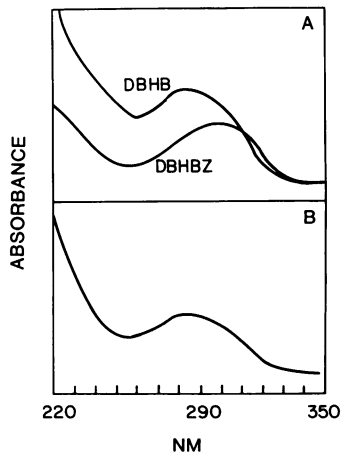

FIG. 4. Identification of the bromoxynil metabolite produced in culture filtrates by *K. pneumoniae* subsp. *ozaenae*. (A) Spectral analysis of the standard compounds 3,5-dibromo-4-hydroxybenzoic acid (DBHB) and 3,5-dibromo-4-hydroxybenzamide (DBHBZ). (B) Spectral analysis of the metabolite peak appearing in the *K. pneumoniae* subsp. *ozaenae* culture filtrate described in the legend to Fig. 3C.

TABLE 2. Substrate specificity of nitrilase activities

Substrate	μmol of NH_3 produced by[a]:	
	K. pneumoniae subsp. *ozaenae*	*Nocardia* sp.
Bromoxynil	1.490	<0.005
3-Bromo-4-hydroxybenzonitrile	0.250	0.045
4-Hydroxybenzonitrile	0.021	0.166
Benzonitrile	<0.005	1.460
3,5-Dibromo-4-hydroxybenzamide	<0.005	<0.005

[a] Assays were performed as described in the text. Approximately 400 μg of protein was added to each assay.

ganisms (including *K. pneumoniae* subsp. *ozaenae*) in pure culture requires the addition of exogenous nutrients. However, cultures of *K. pneumoniae* subsp. *ozaenae* routinely grew to maximum cell density in N^-–0.05% bromoxynil broth within a 24-h incubation period with complete conversion of the herbicide. Analysis of supernatant samples from bromoxynil-containing cultures of *K. pneumoniae* subsp. *ozaenae* by HPLC revealed complete turnover of the herbicide into a single metabolite (identified as 3,5-dibromo-4-hydroxybenzoic acid) present in the culture filtrate.

The metabolism of bromoxynil to 3,5-dibromo-4-hydroxybenzoic acid by *K. pneumoniae* subsp. *ozaenae* suggests the involvement of a nitrilase enzyme. This was confirmed by assaying cell extracts of this organism for the ability to release ammonia from bromoxynil. Comparison of this activity to that from *Nocardia* sp. strain (*rhodochrous* group) NCIB 11215 (11) with various related aromatic nitrile-containing substrates indicates that the enzyme activity from *K. pneumoniae* subsp. *ozaenae* exhibits high specificity for bromoxynil as substrate and that the *Nocardia* enzyme is highly specific for benzonitrile, as reported by Harper (11). Furthermore, the data demonstrate that the *Klebsiella* enzyme cannot utilize benzonitrile, the *Nocardia* enzyme cannot utilize bromoxynil, and that neither enzyme can utilize 3,5-dibromo-4-hydroxybenzamide at the substrate concentrations tested. Interestingly, the absence of the bromine substituents from the herbicide dramatically reduced the effectiveness of the *Klebsiella* enzyme and greatly enhanced the activity of the *Nocardia* enzyme. It is likely that the *meta*-positioned bromine atoms are required for recognition by the active site of the *Klebsiella* nitrilase yet impose steric hindrance on the active site of the *Nocardia* nitrilase. This observation is supported by the fact that 3-bromo-4-hydroxybenzonitrile acts as an intermediate substrate for both enzymes. It will be interesting to determine whether these two divergent nitrilases exhibit any cross-homology at the protein level.

Many organisms capable of transforming nitrile groups to their corresponding carboxylic acids do so in a two-step process involving an amide intermediate (6, 16). However, crude extracts of *K. pneumoniae* subsp. *ozaenae* could not catalyze the release of ammonia from 3,5-dibromo-4-hydroxybenzamide, and no 3,5-dibromo-4-hydroxybenzamide could be detected in bromoxynil-containing cultures from this organism. These results suggest that *K. pneumoniae* subsp. *ozaenae* transforms bromoxynil to 3,5-dibromo-4-hydroxybenzoic acid by a single nitrilase-catalyzed reaction.

Cometabolism has been implicated in the rapid degradation of nitrilic herbicides in the soil (12). It is possible that *K. pneumoniae* subsp. *ozaenae* plays an important role in this process, as has been suggested previously (12). This is supported by the observation that *K. pneumoniae* subsp. *ozaenae* can only convert bromoxynil to 3,5-dibromo-4-hydroxybenzoic acid, that this compound cannot be metabolized further by this organism, and that the metabolite is transported outside the cell. Preliminary experiments were performed with primary soil inoculations in a basal salts medium containing bromoxynil as the sole carbon and nitrogen source. After 3 days of incubation, approximately 90% of the bromoxynil was utilized and no metabolite peaks could be detected on the HPLC chromatogram. These data suggest that the herbicide is undergoing complete mineralization. It will be interesting to see if any single organism is capable of such a catabolic process or if the process is due solely to cometabolism. We are currently investigating this question by testing other bacterial isolates for their ability to use bromoxynil as a sole source of both carbon and nitrogen.

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