Cloning and Expression in *Escherichia coli* of a *Klebsiella ozaenae* Plasmid-Borne Gene Encoding a Nitrilase Specific for the Herbicide Bromoxynil

DAVID M. STALKER* AND KEVIN E. McBRIDE

Calgene, Inc., Davis, California 95616

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An enzyme (nitrilase) that converts the herbicide bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) to its metabolite 3,5-dibromo-4-hydroxybenzoic acid was shown to be plasmid encoded in the natural soil isolate *Klebsiella ozaenae*. The bromoxynil-specific nitrilase was expressed in *Escherichia coli* by direct transfer and stable maintenance in *E. coli* of a naturally occurring 82-kilobase *K. ozaenae* plasmid. Irreversible loss of the ability to metabolize bromoxynil both in *E. coli* and *K. ozaenae* was associated with the conversion of the 82-kilobase plasmid to a 68-kilobase species. In *E. coli* this conversion was the result of a host *recA*⁺-dependent recombinational event. A gene, designated *bxn*, encoding the bromoxynil-specific nitrilase was constitutively expressed in *K. ozaenae* and *E. coli* and subcloned on a 2.6-kilobase *Pst*I DNA segment. The polarity and the location of the gene were determined by assaying hybrid constructs of the bromoxynil-specific nitrilase gene fused with the heterologous *lac* promoter.

The ability of many naturally occurring soil microorganisms to metabolize a wide variety of organic compounds has been reviewed extensively (13, 16). Results of recent studies have shown that the genetic basis for the ability of soil bacteria to degrade many organic compounds resides on naturally occurring plasmid elements. The following systems are representative of plasmid-mediated degradation of specific organic compounds by microbes: 4-chlorobiphenyl by *Alcaligenes* and *Acinetobacter* spp. (27), nicotine by *Arthrobacter oxidans* (7), isopropylbenzene by *Pseudomonas putida* (11), and heterocyclic organic compounds such as dibenzothiophene by *Pseudomonas* sp. (25). Plasmid-encoded degradative systems have been described for the herbicide 2,4-dichlorophenoxyacetic acid (2, 10, 12) and related compounds such as 2,4,5-trichlorophenoxyacetic acid and 3-chlorobenzoate (14, 15, 20). TOL plasmid-mediated degradation of toluene, xylene, and related compounds by *P. putida* has also been investigated extensively (13, 21, 28, 30, 31).

We have been interested in nitrile-containing aromatic compounds represented by the herbicides bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), ioxynil (3,5-diiodo-4-hydroxybenzonitrile), and dichlobenil (2,6-dichlorobenzonitrile). Although bromoxynil and ioxynil are rapidly metabolized in soil, little is known about the microbial mechanism for such breakdown other than the fact that the cyano moiety of these compounds is subject to initial hydrolysis. A number of soil microbes that are capable of utilizing benzonitrile or 4-hydroxybenzonitrile as a sole source of carbon, nitrogen, or both have been identified (4, 17, 18). These organisms contain an enzyme, termed nitrilase, that converts benzonitrile and related compounds to their corresponding benzoic acid derivatives, releasing ammonia as a by-product (4, 17, 18). These nitrilases, however, cannot catalyze the conversion of bromoxynil. Recently, a soil bacterium, *Klebsiella ozaenae*, was isolated that utilizes bromoxynil as a sole source of nitrogen and that encodes a

nitrilase that rapidly converts bromoxynil to its metabolite 3,5-dibromo-4-hydroxybenzoic acid (23). The enzyme exhibits high specificity for bromoxynil as a substrate. In this report evidence is presented that the gene (*bxn*) encoding a bromoxynil-specific nitrilase is plasmid encoded in *K. ozaenae* and is constitutively expressed in *Escherichia coli* by direct transfer of a large, 82-kilobase (kb), naturally occurring *K. ozaenae* plasmid. Instability of the trait in *E. coli* and *K. ozaenae* is associated with a 14-kb excision event whereby the 82-kb *K. ozaenae* plasmid is converted to a plasmid of 68 kb. We also demonstrate subcloning and location of the *bxn* gene on a 2.6-kb *Pst*I fragment and transcription polarity by expressing the *bxn* gene under control of the *E. coli lac* promoter.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. *E. coli* strains utilized were MM294 (*thi endI gyrA96 hsdR17 supE44*), DH1 (*thi endI gyrA96 hsdR17 recA1 supE44*), 71-18 (*lac-pro thi Su2 F' lac ΔM15 lacI^q*). Characterization of the natural soil isolate *K. ozaenae* has been described previously (23).

Plasmids pACYC184, pACYC177, and pUC18 have been described previously (8, 29). *E. coli* strains harboring recombinant plasmids were grown in L-broth or on L-agar plates with appropriate antibiotic selection (tetracycline, 10 μg/ml; chloramphenicol, 20 μg/ml; penicillin, 350 μg/ml). Bromoxynil sodium (3,5-dibromo-4-hydroxybenzonitrile), a gift from Rhône-Poulenc Agrochimie, Lyon, France, was utilized as a selective agent at 0.05% in liquid and solid media. Selective medium (M9) utilizing 0.05% bromoxynil as a sole nitrogen source contained the following per liter: dibasic potassium phosphate, 3 g; monobasic sodium phosphate, 6 g; sodium chloride, 0.5 g; magnesium chloride, 0.1 g; calcium chloride, 0.01 g; glucose, 0.1%; thiamine, 0.005 g. For solid medium agarose was added at 0.8%.

DNA manipulations. Covalently closed circular DNA forms were purified from *E. coli* and *K. ozaenae* strains by sodium dodecyl sulfate-alkaline lysis and two centrifugations

* Corresponding author.

in cesium chloride-ethidium bromide gradients (9). Rapid DNA preparations (clone analysis DNAs) were prepared from 10-ml L-broth cultures as described previously (19). Plasmid DNAs were precipitated with ethanol and dissolved in TE buffer (0.02 M Tris [pH 8.0], 0.1 mM EDTA) prior to restriction analysis. Restriction enzymes were obtained from commercial sources and were used according to the specifications of the manufacturers. Undigested plasmids and plasmid bands were visualized on 0.4 and 0.7% agarose gels, respectively.

Ligation reactions were performed in a volume of 0.02 ml containing 0.02 M Tris hydrochloride (pH 8.0), 0.03 M sodium chloride, 0.01 M magnesium chloride, 0.01 M dithiothreitol, 0.5 mM ATP, and 0.15 U of T_4 ligase. *E. coli* strains were transformed with plasmid DNA as described previously (22).

Preparation of extracts and enzyme assays. Crude extracts were prepared from 200 ml of late log-phase cultures (5×10^8 cells per ml) by centrifugation at $8,000 \times g$ for 15 min at 4°C . Cells were washed in buffer containing 0.1 M potassium phosphate (pH 7.5), 5 mM dithiothreitol, and 1 mM EDTA and suspended in 10 ml of the same buffer. The cell suspension was passed through a French pressure cell, and debris was removed by centrifugation at $60,000 \times g$ at 2°C for 40 min. The resulting clear supernatant 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 described by Bradford (6).

Alternatively, whole bacterial cells could be analyzed for the presence of enzyme activity. Routinely, 1-ml culture samples were transferred to Eppendorf tubes, pelleted at $10,000 \times g$ for 2 min, washed with 1 ml of 0.1 M potassium phosphate (pH 7.5), pelleted, and suspended in 0.05 ml of the above buffer. Fractions were assayed for enzyme activity, with a minus substrate control included for each sample. Protein was determined as the optical density at 600 nm of $1.4 \text{ equal to } 10^9$ cells per ml equal to 150 μg of total protein.

Nitrilase activity was measured by determining the release of ammonia from the nitrile moiety of bromoxynil by the method described by Harper (17). Ammonium chloride concentrations ranging from zero to 1.5 μmol per assay served to construct a standard curve. All samples were assayed in duplicate, and specific activity was measured as micromoles of NH_3 released per minute per milligram of protein. Substrate concentrations in all assays was 3 mM bromoxynil.

RESULTS

The bromoxynil-specific nitrilase is plasmid encoded. The natural soil isolate *K. ozaenae* has been shown to convert the herbicide bromoxynil to its primary metabolite 3,5-dibromo-4-hydroxybenzoic acid by a bromoxynil-specific enzyme-catalyzed reaction (23). Plasmid DNA analysis of *K. ozaenae* cultures grown on 0.05% bromoxynil as sole nitrogen source indicated the presence of a single large plasmid element. To determine whether this large plasmid was involved in expression of the bromoxynil-specific nitrilase, plasmid DNA was prepared from *K. ozaenae* cultures and transformed in *E. coli* MM294 (*thi*). Selection for transformants on defined media supplemented with thiamine was applied by using bromoxynil as a sole source of nitrogen. Several colonies grew after 5 days of incubation at 30°C and were tested for retention of the *thi* auxotrophic marker. All colonies required thiamine for growth, indicating that they were authentic *E. coli* MM294. The *E. coli* transformants

FIG. 1. The bromoxynil-specific nitrilase is encoded by a large *K. ozaenae* plasmid element maintained in *E. coli*. (Gel A) A 0.4% agarose gel containing undigested supercoiled plasmid DNAs. The DNAs were prepared by the clone analysis procedure from 10-ml L-broth cultures of *E. coli* MM294 carrying the large *K. ozaenae* plasmid and grown under various selective conditions. Approximately 0.5 μg of DNA was applied to each lane. Lane 1, plasmid DNA prepared from MM294 grown in the presence of 0.05% bromoxynil; lane 2, plasmid DNA prepared from MM294 colonies picked from bromoxynil-selective media and grown in the absence of bromoxynil; lane 3, plasmid DNA prepared from a MM294 variant colony maintained on nonselective media and grown in the absence of bromoxynil. Positions of supercoiled marker plasmids F (94 kb), R485 (56 kb), and R6K (38 kb) are indicated by arrows. The large plasmid in lane 1 and the smaller plasmid form in lane 3 are designated pBrx1 and pBrx1 Δ , respectively. (Gel B) Restriction enzyme digests of plasmids pBrx1 Δ and pBrx1 analyzed on a 0.7% agarose gel. Lanes 1 and 2, *Bam*HI digests; lanes 3 and 4, *Bgl*II digests; lanes 5 and 6, *Eco*RI digests; lanes 7 and 8, *Hind*III digests; lanes 9 and 10, *Pst*I digests. Standards (Std) are lambda *Hind*III fragments of 23, 9.4, 6.6, 4.4, 2.3, and 2.0 kb. The arrow indicates the cloned 2.6-kb *Pst*I DNA fragment containing the *bxn* gene locus. This band is absent in the *Pst*I digest of pBrx1 Δ (lane 9).

cultured in defined liquid medium utilized bromoxynil as the sole nitrogen source. Cell extracts prepared from logarithmically growing cultures exhibited a nitrilase specific activity of 0.34 μmol of NH_3 released/min per mg of protein. Plasmid DNA prepared from these *E. coli* transformants revealed the presence of a large plasmid element that was similar in size and relative copy number to the plasmid found in *K. ozaenae* (data not shown). This observation suggested the involvement of a large *K. ozaenae* plasmid in expression in *E. coli* of a bromoxynil-specific nitrilase.

E. coli MM294 harboring the *K. ozaenae* plasmid was routinely maintained on rich medium (L agar) in the absence of bromoxynil selection. During this maintenance variant *E. coli* colonies were obtained that no longer utilized bromoxynil or expressed any detectable nitrilase activity. To investigate whether plasmid loss or DNA rearrangements were responsible for the loss of bromoxynil utilization, these strains were screened for plasmid DNA (Fig. 1, gel A). Plasmid DNA prepared from *E. coli* cells grown in L broth supplemented with 0.05% bromoxynil indicated the presence of a large *K. ozaenae* plasmid that was approximately 82 kb (Fig. 1, gel A, lane 1). DNA prepared from an *E. coli* colony

FIG. 2. Stability in *E. coli* of a DNA segment carrying the bromoxynil-specific nitrilase gene is *recA* dependent. The construction of plasmid pBrx2 is described in the text. pBrx2 was transformed to *E. coli* MM294 and DH1, and single colonies were grown in 10-ml cultures under antibiotic selection (chloramphenicol) for the number of generations indicated. Plasmid DNAs were prepared by clone analysis, digested with *Bam*HI, and analyzed on a 0.7% agarose gel. Fragment sizes are indicated. The 4-kb DNA fragment corresponds to the linear fragment of the plasmid vector pACYC184. Lane a, pBrx2 control DNA; lanes b, c, and d, pBrx2 maintained in *E. coli* MM294 for 100, 300, and 500 generations, respectively; lanes e, f, and g, pBrx2 in *E. coli* DH1 maintained for 100, 300, and 500 generations, respectively.

picked from a bromoxynil selective plate, restreaked twice on rich medium without bromoxynil, and grown in the absence of bromoxynil showed two plasmid bands of approximately 82 and 68 kb, respectively (Fig. 1, gel A, lane 2). DNA prepared from a variant MM294 colony that expressed no nitrilase activity is shown in lane 3 (Fig. 1, gel A), and only the smaller 68-kb plasmid species was detected. These results suggest that the irreversible loss of the ability to synthesize a bromoxynil-specific nitrilase is coupled to conversion of the 82-kb *K. ozaenae* plasmid to a 68-kb species. This conversion of the 82-kb plasmid species to one of 68 kb occurred in the absence of bromoxynil selection and could also be demonstrated in the original *K. ozaenae* isolate (data not shown). The 82- and 68-kb plasmid elements were designated pBrx1 and pBrx1Δ, respectively.

To demonstrate the relatedness of plasmids pBrx1 and pBrx1Δ, each plasmid DNA was purified and restriction digests were analyzed by using the enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, and *Pst*I (Fig. 1, gel B). In each digest, common restriction fragments were visualized between the two plasmids, and additional fragments were detected (with the exception of the *Bam*HI digest) in pBrx1 digests (Fig. 1, gel B, lanes 4, 6, 8, and 10). Determination of fragment sizes and the addition of fragments indicated that plasmid pBrx1 was 82 kb and that pBrx1Δ was 68 kb. In addition, restriction digests of plasmid pBrx1Δ with *Bam*HI, *Eco*RI, and *Hind*III (Fig. 1, gel B, lanes 1, 5, and 7, respectively) indicated new unique plasmid bands of 13, 10.5, and 8 kb, respectively. These data suggest that a precise 14-kb excision event occurs in the absence of bromoxynil selection and that the gene encoding a bromoxynil-specific nitrilase is located within this unstable 14-kb DNA segment.

Plasmid conversion in the absence of bromoxynil selection is host *recA* dependent. It was noted in the *Bam*HI digests of pBrx1Δ and pBrx1 (Fig. 1, gel B, lanes 1 and 2) that each

plasmid contained two *Bam*HI sites. This indicates that the excised 14-kb segment is internal to one of the *Bam*HI fragments found in a *Bam*HI digest of pBrx1 (Fig. 1, gel B, lane 2). A 27-kb *Bam*HI fragment from pBrx1 was cloned into the *E. coli* plasmid vector pACYC184 (8) at the unique *Bam*HI site to create the 31-kb chloramphenicol-resistant plasmid pBrx2. Plasmid DNA was purified from cells grown under bromoxynil selection and digested with *Bam*HI to generate fragments of 27 and 4 kb (Fig. 2, lane a). Plasmid DNA was then transformed into isogenic *E. coli* MM294 and DH1, which differed only in the presence of the *recA* allele (strain DH1). Cultures were grown with chloramphenicol in the absence of bromoxynil selection for the number of generations indicated, plasmid DNA was prepared and digested with *Bam*HI, and the fragments were visualized (Fig. 2). Plasmid pBrx2, which was maintained in MM294 and grown for 100, 300, and 500 generations (Fig. 2, lanes b to d, respectively), demonstrated a progressive loss of the 27-kb fragment and the corresponding appearance of a fragment of 13 kb. In 500 generations the resulting conversion from a 27-kb *K. ozaenae* fragment to one of 13 kb was complete and was associated with the corresponding loss of ability of cells harboring pBrx2 to express nitrilase. Restriction enzyme digests of plasmid pBrx2 grown for 500 generations in MM294 indicated that the identical DNA fragments were lost from this plasmid when compared with pBrx1Δ digests (data not shown). Plasmid pBrx2 maintained in strain DH1 (*recA*) demonstrated complete stability over the number of generations tested and expressed the bromoxynil-specific nitrilase. These data suggest that the 14-kb excision event is dependent on a functional host *recA* gene product.

The bromoxynil-specific nitrilase is constitutively expressed. To determine whether any growth conditions existed that affected expression of the bromoxynil-specific nitrilase, *E. coli* DH1(pBrx2) was grown in different media (Table 1), and the specific activity of the different enzyme preparations prepared from these cultures was measured. Under the growth conditions described, expression of enzyme activity was relatively constant. The addition of bromoxynil was not required for induction of bromoxynil-specific nitrilase activity, nor did the addition of bromoxynil result in higher enzyme levels in either rich or minimal media. Alternatively, nutrients available in rich medium did not significantly repress expression of the enzyme, although specific activity measurements in rich medium were slightly lower. These data indicate the constitutive expression of the bromoxynil-specific nitrilase in *E. coli*. The situation is similar in the original isolate of *K. ozaenae* containing the plasmid pBrx1 (data not shown). An interesting observation was that whole cells could be reliably assayed for nitrilase activity, with

TABLE 1. Nitrilase activities in various growth media and under various assay conditions

Growth conditions ^a	Assay fraction ^b	Sp act ^c
M9-glucose (Brx1)	Crude extract	0.30
M9-glucose	Crude extract	0.34
M9-glucose	Whole cells	0.42
L broth (Brx1)	Crude extract	0.22
L broth	Crude extract	0.24
L broth	Whole cells	0.37

^a Cultures were grown as described in the text. M9-glucose media is the same as described in the text, except that ammonium chloride was added to 1 g/liter. Bromoxynil (Brx1) was added at a concentration of 0.05%, where indicated.

^b Preparation of fractions was as described in the text.

^c Micromoles of NH₃ released per minute per milligram of protein.

FIG. 3. Location and transcriptional polarity of bromoxynil-specific nitrlase gene. Plasmid constructions are described in the text. The solid bold lines indicate the cloned *K. ozaenae* 2.6-kb *Pst*I DNA fragment. Relevant restriction sites are indicated, and Ap^r refers to the positions of the ampicillin resistance determinant. The open arrow indicates the position and direction of transcription of the *lac* promoter located in pUC18. The dashed arrow indicates the position and polarity of the nitrlase gene within the 2.6-kb *Pst*I fragment. L-broth cultures (2 ml) of *E. coli* 71-18 containing plasmids pBrx9 and pBrx10 were grown to a density of 10^9 cells per ml. Isopropyl- β -D-thiogalactopyranoside (IPTG) was included in the cultures as indicated at a concentration of 2.5×10^{-4} M. A total of 1 ml of each culture was assayed for nitrlase activity by the whole cell enzyme assay described in the text. Specific activities for pBrx9 with and without IPTG were 0.59 and 0.21 μ mol of NH₃ released/min per mg of protein, respectively. Specific activities for pBrx10 with and without IPTG were 0.01 and 0.02 μ mol of NH₃ released/min per mg of protein, respectively.

specific activities being slightly higher than those obtained in crude extract preparations. This phenomenon facilitated the subcloning of the gene encoding the bromoxynil-specific nitrlase in that individual colonies containing plasmid constructs and expressing nitrlase could be rapidly identified.

Subcloning of the gene encoding a bromoxynil-specific nitrlase. Subcloning of the gene encoding the bromoxynil-specific nitrlase from plasmid pBrx1 was performed as follows. Plasmid pBrx1 was digested with the restriction enzyme *Eco*RI, and the fragments were ligated into the unique *Eco*RI site of the *E. coli* vector pACYC184. Tetracycline-resistant, chloramphenicol-sensitive transformants of *E. coli* MM294 were screened on L agar containing 0.05% bromoxynil. Ten colonies were found to exhibit nitrlase activity, as determined by the whole cell nitrlase assay described above. Plasmid DNA isolated from all 10 clones indicated the presence of a common 18-kb *Eco*RI fragment in each of the plasmid clones. One clone, designated plasmid pBrx5, contained only the 18-kb *Eco*RI fragment cloned into the *Eco*RI site of the plasmid vector pACYC184. Plasmid pBrx5 was mapped with a number of restriction enzymes and found to contain three *Bgl*II restriction sites within the cloned *K. ozaenae* 18-kb *Eco*RI fragment. Plasmid pBrx5 was digested to completion with *Bgl*II and religated in vitro, and the DNA was transformed to competent MM294 cells. Tetracycline-resistant clones encoding nitrlase activity were analyzed and found to contain a single *Bgl*II restriction site, and approximately 11-kb of *K. ozaenae* plasmid DNA was deleted. This 10.6-kb plasmid contained a 6.6-kb *K. ozaenae* DNA insert and was designated pBrx7. This insert was subsequently mapped with the restriction enzymes *Pst*I, *Sal*I, and *Sma*I. A 3.9-kb *Sma*I-*Bgl*II fragment was excised and cloned into the unique *Sma*I-*Bam*HI sites of the plasmid vector pACYC177 (8) to construct plasmid pBrx8. Plasmid pBrx8 was mapped with the restriction enzymes *Pst*I and *Sal*I. A unique *Sal*I site was located within the cloned 3.9-kb

K. ozaenae DNA segment. Two *Pst*I fragments of 2.6 and 1.0 kb were also localized within the 3.9-kb DNA segment.

The DNA segment containing the nitrlase gene was subcloned further by digesting plasmid pBrx8 with the restriction enzyme *Pst*I, the fragments were ligated into the *Pst*I cut plasmid vector pUC18 (29), the DNA was transformed to *E. coli* 71-18, and ampicillin-resistant colonies were screened for the presence of nitrlase activity. Two types of transformants synthesizing nitrlase activity were observed; one type exhibited high levels of activity and the other type exhibited extremely low levels of enzyme activity. Plasmids were isolated from these two different nitrlase-bearing colonies, and all plasmid clones were found to contain a single 2.6-kb *Pst*I insert of *K. ozaenae* DNA in the unique *Pst*I site of the pUC18 vector. The orientation of the 2.6-kb *Pst*I fragment with respect to the vector was determined with the enzyme *Sal*I. Both orientations were confirmed and the plasmids (Fig. 3) were designated pBrx9 and pBrx10, respectively. Plasmid pBrx9 exhibited high levels of nitrlase activity, while pBrx10 encoded low levels of enzyme, as measured by the whole cell assay (Fig. 3). This indicates deletion or interruption of the endogenous *bxn* gene promoter during construction of the plasmids pBrx9 and pBrx10 and polarity of transcription of the *bxn* gene. Restriction mapping revealed unique *Cla*I, *Sca*I, and *Sal*I restriction sites within the 2.6-kb *Pst*I fragment.

The location of the gene within the cloned 2.6-kb *Pst*I fragment was further delineated by restriction mapping and subcloning of the DNA segments (data not shown). The cloned 2.2-kb *Cla*I-*Pst*I fragment and the cloned 0.7- and 1.9-kb *Pst*I-*Sal*I DNA fragments did not encode functional nitrlase. This information localized the gene within the left-hand portion of the 2.6-kb *Pst*I fragment (Fig. 3) and suggests that the *Cla*I and *Sal*I restriction sites interrupt the nitrlase structural gene-coding region.

DISCUSSION

Cloning and expression in *E. coli* of a gene encoding a bromoxynil-specific nitrlase has been described, and the genetic locus for the bromoxynil-specific nitrlase was designated *bxn*. The enzyme rapidly converted the herbicide bromoxynil to its primary metabolite 3,5-dibromo-4-hydroxybenzoic acid (23) and was shown to be plasmid encoded in the natural soil isolate *K. ozaenae*. Transfer to and replication in *E. coli* of the large 82-kb *K. ozaenae* plasmid (pBrx1) and expression of the bromoxynil-specific nitrlase was not surprising given the close phylogenetic relationship between *K. ozaenae* and *E. coli*. The ability to synthesize the bromoxynil-specific nitrlase could be irreversibly lost in both *K. ozaenae* and *E. coli* by growth in the absence of bromoxynil selection. This loss was associated with a precise 14-kb excision event from pBrx1, implying that bromoxynil selection is required for maintenance of plasmid pBrx1 in relation to the smaller, related 68-kb plasmid pBrx1 Δ . The cloned 2.6-kb *Pst*I fragment that encoded the bromoxynil-specific nitrlase was shown to be internal to the 14-kb excision fragment by the fact that it was absent in a *Pst*I digest of pBrx1 Δ (Fig. 1, gel B, lanes 9 and 10). The precise excision event was dependent, at least in *E. coli*, on a functional host *recA* gene product, suggesting that this 14-kb segment is bounded by nucleotide sequences that facilitate homologous recombination (1, 26). This excision event is similar to a 40-kb *recA*⁺-dependent excision event that has been described for the TOL plasmid (5, 13, 24) and a 20-kb spontaneous deletion from *P. putida* (pRE4), resulting in the loss of isopropylbenzene metabolism (11).

Growth of *K. ozaenae* and *E. coli* in either rich or minimal medium resulted in similar specific activity measurements for the nitrilase. Also, the addition of bromoxynil to either medium was not required for enzyme induction, nor did nitrogen or carbon sources repress expression of the enzyme. Although a degree of fine-tuning for *bxn* gene expression may be present, as suggested by slightly lower enzyme levels from cells grown in rich medium, coarse metabolic controls for the *bxn* gene are not in evidence. Because the bromoxynil-specific nitrilase is most likely an initial enzymatic step in bromoxynil metabolism, constitutive expression of this enzyme is analogous to that of 2,4,5-trichlorophenoxyacetic acid in which the initial reaction in the metabolism of this compound is also constitutively expressed (20). The observation that whole bacterial cells synthesizing the enzyme can be reproducibly assayed for nitrilase expression raises the possibility that the bromoxynil-specific nitrilase is a periplasmic or membrane-associated protein in *K. ozaenae* and *E. coli*. Alternatively, the rapid exchange of bromoxynil and its reaction products across the cell membrane may account for this phenomenon.

The *bxn* gene was subsequently cloned as a 2.6-kb *Pst*I fragment in both orientations in the plasmid vector pUC18 (plasmids pBrx9 and pBrx10). Induction experiments with isopropyl- β -D-thiogalactopyranoside and further characterization of the 2.6-kb *Pst*I fragment by subcloning internal fragments positioned the *bxn* gene and indicated the direction of gene transcription. The low level of induction with isopropyl- β -D-thiogalactopyranoside in these experiments probably reflects the high copy number of the vector plasmid and has been reported for other plasmid-borne *lac* promoter-gene fusions (3). Results of recent experiments indicate that *E. coli* minicells containing plasmid pBrx9 synthesize a polypeptide with a molecular weight of approximately 37,000 (unpublished data). This value is also in good agreement with the size obtained for the purified bromoxynil-specific nitrilase (D. M. Stalker and L. D. Malyj, manuscript in preparation).

Cloning of the gene locus (*bxn*) encoding the bromoxynil-specific nitrilase will facilitate comparison at the gene level of this enzyme to nitrilases identified in other organisms (4, 17, 18). The *bxn* gene is also being tailored for expression in plants to confer a bromoxynil-resistant phenotype to bromoxynil-sensitive crops.

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