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Engineering dicamba selectivity in crops: a search for appropriate degradative enzyme(s)

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The biotechnologial approaches to conferring crop selectivity to herbicides have been demonstrated for a number of compounds such as glyphosate, glufosinate, imidazolinones and cyclohexanediones. Imidazolinone-resistant and cyclohexanedione-resistant maize lines are already in the market. There are several other effective and environmentally benign herbicides such as dicamba, for which engineering crop selectivity is desirable, to broaden the product utility in different crops and provide new solutions for weed control. One of the most effective approaches to conferring dicamba selectivity in crops is to incorporate a gene for its rapid metabolism. It is advantageous to have different dicamba-metabolizing enzymes in order to maximize the chances of at least one functioning optimally in a plant environment. Three different metabolizing enzymes are currently available to engineer crop selectivity. The first one is the folate-dependent O-demethylase from *Clostridium thermoaceticum*, that converts dicamba to herbicidally inactive 3,6-dichlorosalicylate. The second enzyme is the NADH-dependent, multi-component monooxygenase from *Pseudomonas maltophilia* DI-6 that also converts dicamba to 3,6-dichlorosalicylate. The third enzyme is from corn endosperm cultures that catalyzes the 5-hydroxylation of dicamba. The merits of these three enzymes are discussed with respect to conferring crop selectivity to dicamba. In addition, a rapid microbial screen was conceived for discovery of new dicamba-degrading bacteria, which resulted in identification of *Pseudomonas orvilla*. This bacteria degraded dicamba by the same pathway, perhaps using a similar enzyme system as *Pseudomonas maltophilia* DI-6. However, the microbial screen has the potential to identify novel bacteria that degrade dicamba by a different pathway, providing more options for metabolizing enzymes to confer herbicide selectivity in crops.

Keywords: dicamba; dicamba-metabolizing enzymes; engineering crop selectivity; screen for dicamba-metabolizing enzymes

Introduction

Over the last 50 years, the use of chemical herbicides in crops has resulted in an enormous increase in agricultural productivity [17]. The fact that such herbicides are used in crops automatically implies that they are completely safe (selective) to crops, while providing total or acceptable control of economically important weeds. The basis for herbicide selectivity in most cases is the inherent ability of the crops to rapidly metabolize the herbicides prior to the toxophore reaching the cellular target site [14,15]. The discovery of crop-selective herbicides is an arduous and expensive process [17]. Initially thousands of chemicals are screened on key weeds. Subsequently, select, active compounds are thoroughly explored synthetically in terms of improving the toxophore moiety as well as incorporating chemical handles for rapid metabolism by crops. Building crop selectivity into a toxophore is purely a 'trial and error' synthetic effort, although the knowledge of the inherent metabolic machinery of the crops has been useful in designing metabolic handles in the molecule [14,15]. It is estimated that discovery of one crop-selective herbicide involves screening more than 30 000 compounds [17,18].

Recent developments in the area of plant biotechnology, notably the ability to stably integrate foreign genes into crops, has opened up an alternative approach to engineering herbicide selectivity [7,10]. In the last 10 years, several crops have been engineered or selected in tissue culture, to be selective to specific herbicides. For example, glyphosate selective soybeans were genetically engineered by incorporating a gene that codes for a less sensitive form of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) [16]. The herbicidal activity of glyphosate is due to inhibition of the wild-type EPSP synthase [16]. Similarly, glufosinate selectivity was engineered into maize and other crops by incorporating a bacterial gene that codes for an acetyl transferase [24]. This results in rapid metabolism of the herbicide in the transgenic crop, imparting selectivity. Examples of crops that have been engineered to be selective to different herbicides [7], source of the gene and the mechanism of selectivity are shown in Table 1. It is important to note that several enzymes listed in Table 1 are metabolizing enzymes from microorganisms.

The biotechnological approaches to impart crop selectivity to herbicides have several distinct advantages [10,18]: (a) The cost associated with discovery of crop-selective herbicides *via* screening thousands of chemicals is expected to escalate [17]. Thus, it might be prudent to impart desired crop-selectivity to some of the existing herbicides and find new applications in different cropping systems. (b) There is also the challenge of meeting the increasingly stringent regulatory hurdles to register new crop-selective molecules. Finding new uses for some of the already registered herbicides is a solution offered by plant biotechnology. (c) One

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Table 1 Examples of crops engineered to be selective to different herbicides

Crops	Herbicide	Selectivity mechanism	Enzyme source
Soybeans	Glyphosate	Altered target enzyme EPSP synthase	*Agrobacterium* sp
		Metabolism by glyphosate oxidoreductase	*Achromobacter* sp
Several	Phosphinothricin	Metabolism by phosphinothricin-*N*-acetyl transferase	*Streptomyces hydroscopicus*
Cotton	Bromoxynil	Metabolism by nitrilase	*Klebsiella ozaenae*
Maize	Imidazolinone	Altered target enzyme acetolactate synthase	Host plant[a]
Cotton	2,4-D	Metabolism by 2,4-D dioxygenase	*Alcaligenes eutrophus*
Maize	Cyclohexanedione	Altered target enzyme acetyl-coenzyme A carboxylase	Host plant[a]

[a]Herbicide-resistant trait was selected in tissue culture.

of the most difficult tasks in weed control is to selectively eliminate a weed that is closely related to a crop; biotechnology provides a unique solution to this problem because crops can be specifically engineered to be selective to herbicides [18]. (d) There are several environmentally acceptable, highly active herbicides for which new applications can be conceived *via* engineering crop selectivity [18].

Dicamba

Dicamba, 2-methoxy-3,6-dichlorobenzoate, is a well-established, environmentally benign product that has been used as a corn and wheat-selective postemergence herbicide for control of annual and perennial dicot weeds [23]. Dicamba is a synthetic mimic of the natural plant hormone, indole-3-acetic acid, which has profound effects on plant growth and development [6]. The inherent ability of corn and wheat to rapidly metabolize dicamba to 5-hydroxy-2-methoxy-3,6-dichlorobenzoic acid and its polar conjugate, both of which are inactive as herbicides, accounts for the selectivity [9]. The technology to genetically engineer crops for herbicide resistance has opened up new applications for this molecule. For example, there is greater opportunity for pre-emergence application of dicamba in corn and wheat, which has been limited by excessive crop injury. It is also possible to enhance the weed spectrum of dicamba by increasing the application rate, if the selectivity margin is enhanced in corn and wheat. The very high intrinsic activity of dicamba on soybeans [23] creates a risk of injury where they are planted near corn. Engineering dicamba selectivity to crops such as soybeans can not only provide a solution to this problem, but may also open up opportunities for weed control in soybeans.

Approaches for engineering dicamba selectivity

It is clear from Table 1 that most approaches to engineering herbicide selectivity in crops have been *via* rapid metabolism of the parent compound by an appropriate microbial enzyme. This approach does not alter any of the plant genes, but integrates into the host genome, a foreign gene for the metabolism of the herbicide [16,24]. Metabolism as a means of achieving crop selectivity also ensures that the parent molecule is degraded into an inactive ingredient, reducing or eliminating any residue of the parent toxophore in the plant. With respect to dicamba, engineering crop selectivity *via* rapid metabolism is the only option. There are several metabolizing enzymes available and their merits are discussed below. The second option of modifying the target site (binding site) for dicamba, to render crops insensitive or less sensitive to this herbicide is not discussed since the target site is unknown [6].

Dicamba-metabolizing enzymes: I. O-demethylase from *Clostridium thermoaceticum*

Acetogenic bacteria such as *Clostridium thermoaceticum* can utilize the methyl group of aromatic methyl ethers such as syringic acid, as a carbon and energy source [8]. It has been established that assimilation of the methyl carbon in this bacteria occurs *via* transfer to tetrahydrofolate, prior to its incorporation in the acetogenic pathway of energy metabolism [8]. The first enzyme of the pathway O-demethylase (ODM), inducible upon growth on syringic acid, catalyzes the conversion of the growth substrate to 5-hydroxyvanillate and then to gallate [8]. Besides syringic acid, several other methoxylated aromatic compounds are also demethylated by ODM, including vanillate, methoxybenzenes, methoxyphenol and dicamba [8]. The reaction catalyzed by ODM and the products derived from syringic acid and dicamba are shown in Figure 1. Initial analyses indicate that dicamba is as good a substrate for ODM, if not better, than syringic acid [S Ragsdale, University of Nebraska, personal communication]. The gene coding for ODM is an ideal candidate for incorporating into crops for rapid metabolism of dicamba, to achieve herbicide selectivity. The enzyme has a subunit molecular weight of 21 000 Da, is thermostable, and is insensitive to oxygen and propyliodide [8; S Ragsdale, University of Nebraska, personal communication]. It does not have any bound metals as cofactors nor is a reductant required for the reaction. The enzyme, however, uses stoichiometric amounts of folate for the demethylase reaction (Figure 1). Thus, it is important to make sure that the host plant in which the gene is to be incorporated can support the reaction by providing adequate amounts of folate. This can be verified *via* creating transgenic crops containing the gene for ODM and testing its ability to metabolize dicamba *in vivo*. ODM has been cloned into *E. coli* and the activity of the enzyme has been demonstrated *vs* both syringic acid and dicamba [S Ragsdale, University of Nebraska, personal communication].

Dicamba-metabolizing enzymes: II. O-demethylase from *Pseudomonas maltophilia* DI-6

Microorganisms capable of mineralization of methoxybenzoates have been well documented [1]. Dicamba, a chlori-

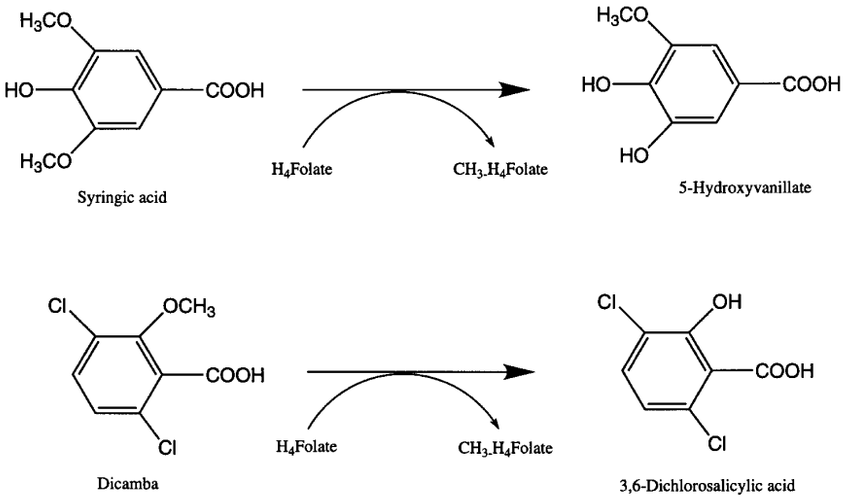

Figure 1 Reaction catalyzed by O-demethylase from *Clostridium thermoaceticum*.

nated methoxybenzoate, is a stable molecule; however in soil, the herbicidal activity of this molecule is not persistent, which indicates degradation by soil microflora [9]. One particular soil bacterium, *Pseudomonas maltophilia*, strain DI-6, has been shown to utilize dicamba as a sole source of carbon and energy [12]. The proposed pathway for metabolism shows 3,6-dichlorosalicylate as the initial product from dicamba [3,28]. Subsequent degradation appears to be *via* 3,6-dichlorogentisic acid based on oxygen uptake studies (Figure 2). Degradation of dicamba by *P. maltophilia* DI-6 *via* the corresponding gentisate has also been proposed based on metabolite analyses of growth media [DP Weeks, University of Nebraska, personal communication]. Incorporation of the gene(s) coding for the conversion of dicamba to 3,6-dichlorosalicylate (dicamba O-demethylase, DODM) into crops should help achieve selectivity *via* rapid metabolism of the herbicide. 3,6-Dichlorosalicylate has been determined to be herbicidally inactive. DODM is a multi-component enzyme system consisting of a reductase, a ferredoxin and an oxygenase that catalyzes an NADH-dependent oxidation [25]. The organization of the electron transport system in DODM [25] appears to be similar to several other well-characterized two and three component xenobiotic-degrading bacteria [1,21]. Two of the three genes representing the ferredoxin and oxygenase components of DODM have been cloned in *E. coli*; but oxidation of dicamba has not been demonstrated [DP Weeks, University of Nebraska, personal communication].

Based on the rate of dicamba-dependent oxygen consumption (Figure 2), it is reasonable to conclude that DODM may be able to rapidly metabolize dicamba in transgenic crops. However, the complexity of the enzyme system and its ability to function optimally in plants is an issue. It is clear that in order to achieve rapid dicamba degradation in crops, the genes coding for all the three components of DODM have to be incorporated, with the implicit assumption that the components will assemble spontaneously to form an active catalytic core. Conferring herbicide selectivity in transgenic crops, based on metabolism of the toxophore by multi-genes, has not been demonstrated. Alternately, the gene for the oxygenase component alone or in combination with the gene for the ferredoxin component of ODM could be cloned in plants. This strategy will help determine if the plant can effectively couple to the oxygenase, one or more endogenous electron transfer functions (either the reductase or ferredoxin or both). It is well known that in multi-component oxygenase

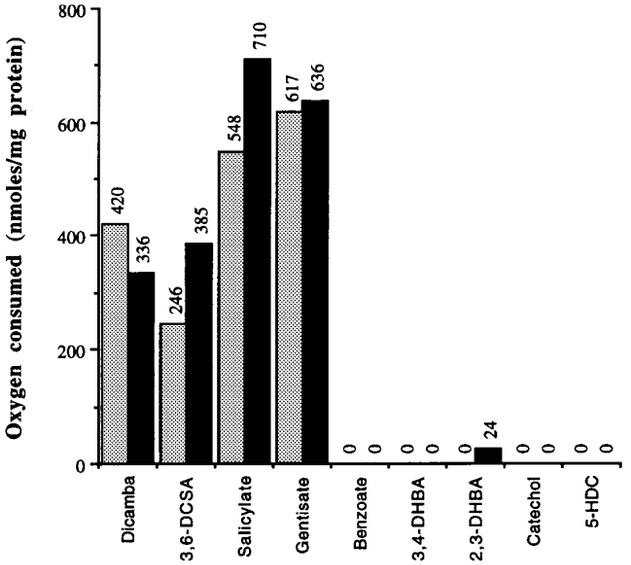

Figure 2 Oxidation of dicamba and other related substrates by *Pseudomonas arvilla* (▨) *and Pseudomonas maltophilia* DI-6 (■). Both *P. arvilla* and *P. maltophilia* DI-6 were grown at 30°C on M9 minimal medium [13] supplemented with 0.2% (w/v) dicamba and adjusted to pH 6.35. Cells were harvested at $A_{600} = 0.65–1.0$ by centrifugation at $6000 \times g$ for 10 min, washed once with 50 mM potassium phosphate buffer pH 7.0 (KP buffer) and resuspended in the same buffer to a final $A_{600} = 6–9$. Oxygen uptake was measured polarographically using a Gilson Model 5/6 oxygraph (Gilson Medical Electronic Inc, Middleton, WI, USA) calibrated with sodium dithionite. The reaction mixture of 1.6 ml air-saturated KP buffer consisted of 20–50 μl of cell suspension and a final concentration of 2 mM substrate (prepared as a 1-M stock solution in DMSO). Basal rate of oxygen consumption by cells alone was determined prior to addition of the substrate and subtracted to calculate the actual substrate-dependent rate of oxygen consumption. DCSA, dichlorosalicylic acid; DHBA, dihydroxybenzoic acid; and HDC, hydroxydicamba.

systems, reductase and/or ferredoxins can be substituted by functional equivalents from other species including plant reductases [20].

Dicamba-metabolizing enzymes: III. Enzyme system from maize

Dicamba has been used as a maize and wheat-selective herbicide for more than 30 years [9]. It is well known that maize and wheat rapidly oxidize dicamba to 5-hydroxydicamba and its glucose conjugate [9]. Thus, amplifying the dicamba-metabolizing gene in maize to higher levels may provide additional margins of selectivity for this herbicide. In theory, the same gene could also be used to confer dicamba selectivity in crops such as soybeans. However, dicamba oxidation in a cell-free preparation from plants has not been demonstrated. In experiments conducted in the author's laboratory using corn endosperm cell cultures treated with 50 μM ^{14}C-dicamba in the growth media, 48% of the parent compound was found to be converted in 24 h to a polar conjugate of 5-hydroxydicamba at a rate of 7.3 nmol h^{-1} g^{-1} fresh weight (unpublished data). This conversion was inhibited by the addition of cytochrome P450 inhibitors such as metyrapone (80% inhibition of 5 mM) and aminobenzotriazole (65% inhibition at 1 mM) to the growth media (unpublished data). Several attempts to obtain *in vitro* activity using both membranes and soluble fractions of the cell culture have been unsuccessful. The involvement of P450 in the conversion of dicamba to 5-hydroxydicamba in corn cultures remains to be established in an *in vitro* system. The low level of metabolism of dicamba by corn endosperm cell cultures and the inability to isolate a stable preparation of the enzyme for further characterization has created little interest in using this gene for conferring dicamba selectivity in crops. However, in recent years, the progress in cloning and expression of plant cytochrome P450 in heterologous systems such as yeast [26], offers renewed interest in using dicamba-metabolizing enzymes from plants for crop selectivity. For example, it should be possible to screen diverse sources of cloned cytochrome P450 for activity *vs* dicamba and determine if they are useful with respect to engineering crop selectivity.

Dicamba-metabolizing enzymes: IV. Screening for new dicamba degraders

Two different approaches could be employed for discovery of novel microbial enzyme activity on dicamba: (a) Soil enrichment techniques [19] for isolation of different dicamba-degrading bacteria and determining the catabolic pathway of the herbicide. This approach was not tried due to the long-term nature of the task. (b) Screening microorganisms that have been well characterized with respect to degradation of molecules related to dicamba, such as benzoate, methoxybenzoate, chlorobenzoate, salicylate etc, and examining their potential to grow and/or oxidize dicamba. Well-characterized microorganisms could be purchased from ATCC as well as obtained from different laboratories involved in the area of microbial degradative pathways. The cultures can be tested for oxidation of dicamba after growth on the different aromatic compounds and directly examined for growth and oxidation of dicamba. To verify the proposed screen, twelve different *Pseudomonas* species were examined. These include *Pseudomonas orvilla* ATCC 15524, *P. cepacia* ATCC 17616, *P. putida* ATCC 11250, 12633, 17426, 17483 and 39213, *P. fluorescens* ATCC 11172, *Pseudomonas* sp ATCC 19151, *Pseudomonas* sp strain JS150 [11], *P. pseudoalcaligenes* POB310 [5] and *P. mendocina* KR1 [27]. All these cultures were grown on M9 minimal media [13] containing 0.1% (w/v) of benzoate, salicylate, 3-chlorobenzoate, *o*-anisic acid or dicamba. M9 media was chosen for growth of all bacteria since this media supported growth as well as other described media for *Pseudomonas* [11,25], which are more difficult to prepare. Also, oxidation of the growth substrate by whole cells grown in M9 media (an example shown in Figure 2) was equal to or better than the cells grown in other media [11,25].

All of the above mentioned cultures grew on benzoate and/or salicylate, but these cells showed no activity on dicamba. The vanillate (3-methoxy-4-hydroxybenzoate) utilizing *Pseudomonas* sp [4] did not utilize dicamba as a growth substrate, neither did the vanillate-grown cells oxidize dicamba. One bacterium, *P. orvilla*, grew profusely on dicamba and the cells showed high activity on dicamba as measured by oxygen uptake studies. The oxygen uptake profile of dicamba-grown *P. orvilla* and *P. maltophilia* DI-6 on various compounds is shown in Figure 2. Gentisate was used in these experiments due to non-availability of dichlorogentisate. Based on Figure 2, it can be proposed that the degradative pathway for dicamba in *P. orvilla*, like *P. maltophilia* DI-6, is *via* the corresponding gentisic acid. Oxidation of dicamba by whole cells and crude extracts of *P. orvilla* was also assayed by following dicamba disappearance and the results are shown in Figure 3. Almost complete disappearance of the substrate was observed in about 5 h by whole cells (Figure 3a). The only metabolite detected was a small amount of a fraction whose retention time corresponded to gentisic acid (Figure 3a). In the reaction involving the crude extract of *P. orvilla*, about 38% disappearance of dicamba was noticed with concomitant appearance of radioactivity in the fraction that corresponded to gentisic acid, which is very likely 3,6-dichlorogentisic acid (Figure 3b). The proposed initial metabolite of dicamba based on oxygen uptake studies with *P. orvilla* and *P. maltophilia* DI-6 (Figure 2), 3,6-dichlorosalicylate, was not detected in either of the assays. Dicamba oxidation in crude cell extract was dependent on NADH and Fe^{2+} (data not shown), indicating that the enzyme system for conversion of dicamba to 3,6-dichlorosalicylate may be similar to the multicomponent systems of *P. maltophilia* DI-6 [25] and other monooxygenases and dioxygenases involved in xenobiotic degradation [1,21]. The above results demonstrate that a screen involving already characterized microorganisms could expedite the discovery of a new dicamba-degrading bacteria. However, any bacteria discovered using such a screen or by soil enrichment techniques [19], may not necessarily elaborate a new pathway. In addition to the above bacterial screen, one could also screen for different oxidative enzymes such as dehaloperoxidase [2], to discover new dicamba-metabolizing enzymes. Also, a screen along the concept described above using

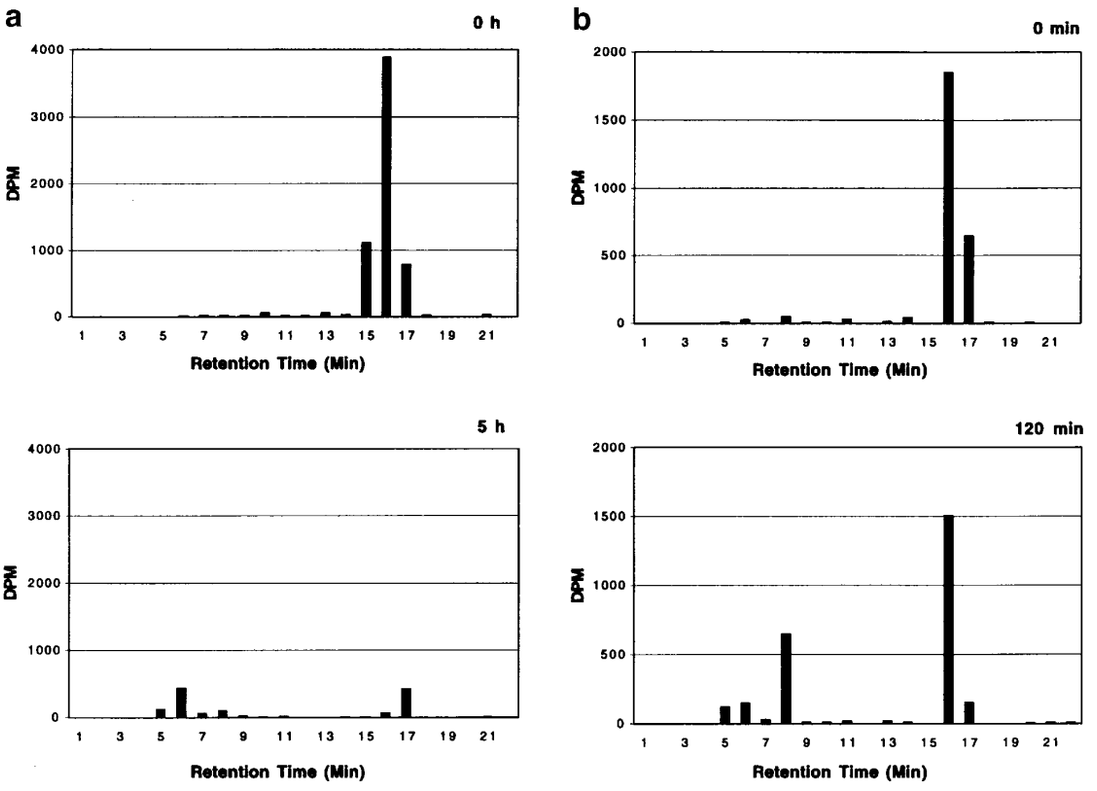

Figure 3 Oxidation of dicamba by whole cells (a) and crude cell extracts (b) of *P. arvilla*. The cells were grown to $A_{600} = 0.65–1.0$ and pelleted by centrifugation, washed once with KP buffer and resuspended in KP buffer (whole cells) or KP buffer containing 1 mM l-cysteine and 10 μM $FeSO_4$, to a final A_{600} of 6–9 (whole cells), or 70–90 to prepare cell extracts. Cells were broken using French Press at 20000 p.s.i. followed by addition of 1 μl ml^{-1} of a 1 mg ml^{-1} solution of deoxyribonuclease I. The broken cells were centrifuged at 100 000 $\times$ *g* for 20 min and the supernatant (12–17 mg ml^{-1} protein) was used for assays. Whole cell assay mixtures consisted of 0.5 ml of cells in a final volume of 1.5 ml of M9 minimal media [13] containing 20 000 dpm of ^{14}C dicamba (42 mCi mmol^{-1}) and 0.67 mM cold dicamba. The crude extract assay mixtures consisted of 0.5 ml of cell extract in 1.5 ml of KP buffer containing a mixture of hot and cold dicamba as noted above. The reaction was started by the addition of 30 μl of 100 mM NADH. Both whole cell and crude extract assays were conducted at 30°C, in 10-ml Erlenmeyer flasks, in a shaker. At various time points, reaction was terminated by the addition of 30 μl of 100% TFA. The reaction suspension was clarified in a microcentrifuge at 13000 rpm and supernatant analyzed by HPLC using Phenomenex Ultramex 5 C18 reverse phase column of 250 $\times$ 4.6 mm (Phenomenex Inc, Torrance, CA, USA). Separation was accomplished using acetonitrile containing 7.5 mM TFA and the following step gradient: 0–15 min, 35–55%, 15–20 min, 55–85% and 20–22 min, 85% acetonitrile at a flow rate of 0.6 ml min^{-1}. Under these conditions, standards of gentisic acid, DCSA and dicamba eluted at 6.8, 13.8 and 16.8 min, as detected at 274 nm. Reaction mixtures were separated and fractions of 0.6 ml were collected and counted for radioactivity in a Packard 1900TR liquid scintillation analyzer (Packard Instrument Co, Meriden, CT, USA). The radioactive peaks were also identified by spiking cold standards. Since 3,6-dichlorogentisic acid was not available, gentisic acid was used as the standard. Figure 3a shows the profiles of radioactive fractions from reactions using whole cells at 0 and 5 h. Figure 3b shows the profiles of radioactive fractions from reactions using crude extracts at 0 and 120 min. The total recovery of radioactivity from the 0 time reaction mixtures was >90%.

anaerobes could help discover organisms capable of dehalogenation and decarboxylation [22] of dicamba.

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