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Biodegradation and Mineralization of Metolachlor and Alachlor by *Candida xestobii*

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Metolachlor (2-chloro-6′-ethyl-*N*-(2-methoxy-1-methylethyl)aceto-*o*-toluidide) is a pre-emergent chloroacetanilide herbicide used to control broadleaf and annual grassy weeds in a variety of crops. The *S* enantiomer, *S*-metolachlor, is the most effective form for weed control. Although the degradation of metolachlor in soils is thought to occur primarily by microbial activity, little is known about the microorganisms that carry out this process and the mechanisms by which this occurs. This study examined a silty-clay soil (a Luvisol) from Spain, with 10 and 2 year histories of metolachlor and *S*-metolachlor applications, respectively, for microorganisms that had the ability to degrade this herbicide. Tis paper reports the isolation and characterization of pure cultures of *Candida xestobii* and *Bacillus simplex* that have the ability to use metolachlor as a sole source of carbon for growth. Species assignment was confirmed by morphological and biochemical criteria and by sequence analysis of 18S and 16S rRNA, respectively. High-performance liquid chromatography (HPLC) and liquid chromatography−mass spectrometry (LC-MS) analyses indicated that *C. xestobii* degraded 60% of the added metolachlor after 4 days of growth and converted up to 25% of the compound into CO_2 after 10 days. In contrast, *B. simplex* biodegraded 30% of metolachlor following 5 days of growth in minimal medium. In contrast, moreover, the yeast degraded other acetanilide compounds and 80% of acetochlor (2-chloro-*N*-ethoxymethyl-6′-ethylaceto-*o*-toluidide) and alachlor (2-chloro-2′,6′-diethyl-*N*-methoxymethylacetanilide) were degraded after 15 and 41 h of growth, respectively. The results of these studies indicate that microorganisms comprising two main branches of the tree of life have acquired the ability to degrade the same novel chlorinated herbicide that has been recently added to the biosphere.

KEYWORDS: Metolachlor; catabolism; bacteria; yeast; soil; mineralization

INTRODUCTION

Metolachlor (2-chloro-6′-ethyl-*N*-(2-methoxy-1-methylethyl)-aceto-*o*-toluidide) (**Figure 1A**) is one of the most extensively used chloroacetamide herbicides and was first registered for use with the U.S. Environmental Protection Agency (USEPA) in 1976 (*1*, *2*). Metolachlor is commonly used as a pre-emergence herbicide for the control of annual grasses and some broad-leaved weeds in a variety of crops, including maize, sorghum, cotton, sugar cane, sugar beet, potato, peanuts, soybean, sunflower, safflower, and some vegetables. This chemical acts by inhibiting elongases and the biosynthesis of gibberellic acid (*3*), resulting in plant death when absorbed through the roots and shoots just above the seed of the target plants.

The USEPA estimated that 59−64 million pounds of metolachlor was applied in 1995 (*4*), and its use has been steadily declining during recent years. Recommended application levels of the chemical were 1.2−5 lb/acre in 1995 (*5*). In 1999, however, Syngenta Crop Protection, one of the main manufacturers of this herbicide, discontinued sales of metolachlor and replaced it with the reduced-risk compound *S*-metolachlor. This enantiomer is more effective in weed control than racemic metolachlor, providing the same weed control but requiring 35% less applied chemical (*6*). Metolachlor use in the United States was subsequently reduced by 15−24 million pounds in 2001, as herbicides containing this chemical were replaced with *S*-metolachlor, of which 20−24 million pounds was applied during that year. This is the largest reduction of pesticide use in the United States to date (*7*). Since atrazine was banned in Europe in 2003, there had been increasing use of metolachlor combined with postemergence herbicides until *S*-metolachlor was substituted for use of the mixed enantiomer. The European Union presently allows application of only *S*-metolachlor for weed control. In Spain, it has been estimated that 5000 t of *S*-metolachlor is applied on 1.3 million hectares (3.75 kg/ha) per year (*8*)

Metolachlor is slightly soluble in water (488 mg/L at 20 °C) and is moderately sorbed by most soils (sorption range = 99−307 mL/mg) (*9*, *10*), with greater sorption occurring on soils having greater organic matter and clay contents. Extensive leaching of

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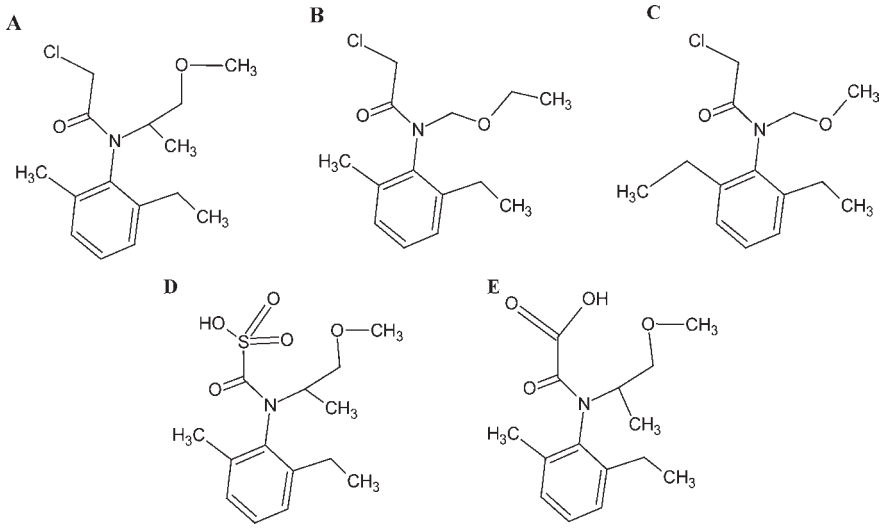

Figure 1. Structures of (**A**) metolachlor (2-chloro-6′-ethyl-*N*-(2-methoxy-1-methylethyl)aceto-*o*-toluidide), (**B**) acetochlor (2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide), (**C**) alachlor (2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide), (**D**) metolachlor ethanesulfonic acid (MESA), and (**E**) metolachlor oxanilic acid (MOA).

metolachlor is reported to occur, especially in soils with low organic content (*11*). Metolachlor is relatively more persistent in soils as compared to other widely used chloroacetanilide herbicides, such as alachlor and propachlor. Metolachlor half-lives ranging from 15 to 70 days have been observed in different soils (*11*, *9*). The herbicide is highly persistent in water, over a wide range of pH values, with reported half-life values of ≥200 and 97 days in highly acid and basic conditions, respectively (*12*). Metolachlor is also relatively stable in water, and under natural sunlight, only about 6.6% was degraded in 30 days (*12*). Because very little metolachlor volatilizes from soil ($K_h = 2.44 \times 10^{-8}$ atm m^3/mol^{-1} at 25 °C), photodegradation is thought to be a pathway for loss, but only in the top few centimeters of soil.

On the basis of these observations, it has been postulated that metolachlor dissipation in soil mainly occurs via biological degradation, rather than chemical processes (*13*, *14*). The degradation of metolachlor in soils has been proposed to occur via co-metabolic processes that are affected by soil texture, microbial activity, and bioavailability (*15*). The limited number of reports on the microbial degradation of metolachlor, and its long half-life, led to contrasting hypotheses that microbial consortia are likely needed for metolachlor catabolism in soils or that metolachlor is not readily metabolized by soil microorganisms (*6*, *14*, *16*−*18*). Moreover, previous attempts to enhance metolachlor degradation in natural fields have generally not been successful (*19*). This was, in part, attributed to the low bioavailability of this herbicide to microorganisms. However, the half-life of metolachlor in sterile soil was reduced from 97 to 12 days after the addition of an active microbial community (*20*), indicating that other biotic factors influence metolachlor degradation in soils. Whereas pure cultures of an actinomycete (*21*), a streptomycete (*22*), and a fungus (*23*) capable of metabolizing metolachlor have been reported, degradation times were long (16 days), and only small amounts of the herbicide were degraded or mineralized (40 and 20%, respectively).

Similarly, low rates of mineralization of the chloroacetanilide herbicide alachlor (2-chloro-2′,6′-diethyl-*N*-methoxymethylacetanilide) have also been reported, only 3−14% of the herbicide was mineralized after 30−122 days (*13*, *24*−*26*). Pure microbial cultures have also been reported to be relatively ineffective in mineralizing acetochlor (2-chloro-*N*-ethoxymethyl-6′-ethylaceto-*o*-toluidide), a related herbicide, with maximum rates of 24% (*27*, *28*). Recently, Xu et al. (*29*) reported that 89, 63, and 39% of the chloroacetanilide herbicides propachlor, alachlor, and metolachlor were degraded, respectively, after 21 days of incubation. The major dissipation routes for both alachlor and acetochlor appear to be due to microbiologically mediated degradation, runoff, and leaching (*30*). Most chloroacetanilide-degrading microorganisms reported to date are fungi (*31*), and metolachlor is thought to be more persistent and recalcitrant to degradation than the other chloroacetanilide herbicides in soils and water (*32*).

In this study, we examined Spanish soils with a history of metolachlor application for the presence of pure microbial cultures capable of catabolizing this herbicide. Here we report the isolation and characterization of a pure culture of a yeast, *Candida xestobii*, and a bacterium, *Bacillus simplex*, that have the ability of catabolize metolachlor and use this herbicide as a sole source of carbon for growth. We also report that the yeast is also capable of rapidly catabolizing other chloroacetanilide herbicides, such as acetochlor and alachlor. These newly isolated organisms will allow us to obtain a better understanding of the biochemistry and genetics of acetanilide herbicide catabolism by microorganism and will provide new tools for the bioremediation of environments affected by these herbicides.

MATERIALS AND METHODS

Chemicals. Metolachlor (99% chemical purity) was a gift from Syngenta Crop Protection, Greensboro, NC. Uniformly ring-labeled [^{14}C]metolachlor (specific activity = 54.6 μCi/mg, 99.2% radiochemical purity) was graciously supplied by Syngenta Crop Protection. Acetochlor (99% chemical purity) and alachlor (99% chemical purity) were purchased from Chem Service (West Chester, PA). Uniformly ring-labeled [^{14}C]alachlor (specific activity = 23.6 μCi/mg, 99% radiochemical purity) was graciously supplied by Monsanto Corp. The metolachlor standard for LC-MS analysis was obtained from Chem Service.

Stock solutions of metolachlor, acetochlor, and alachlor (100 ppm) were prepared in water and stored at 4 °C until used. All other chemicals were obtained from Fischer Scientific, Pittsburgh, PA.

Growth Conditions and Isolation of Microorganisms. Silty-clay soils from Spain (a Luvisol), which had 10 and 2 year histories of metolachlor and *S*-metolachlor application, respectively, were used in this study. These soils received 3.75 kg/ha of metolachlor once per year (in May). Microorganisms were obtained from the soil following enrichment for 5 days in minimal medium using metolachlor as the sole source of C for growth. The liquid mineral medium (MM) used in this study contained KH_2PO_4 (27.2 g L^{-1}), K_2HPO_4 (45.6 g L^{-1}), $MgSO_4$ (0.5 g L^{-1}), KCl

(0.5 g L^{-1}), $NaNO_3$ (3 g L^{-1}), $FeSO_4$ (0.01 g L^{-1}), $CaCl_2$ (0.04 g L^{-1}), $MnSO_4$ (0.001 g L^{-1}), and metolachlor as the sole source of carbon at a final concentration of 50 μg mL^{-1}. The metolachlor was added after autoclaving, and the pH was adjusted to 7.0. The same procedure was used for media containing acetochlor and alachlor. All of the experiments were conducted at 30 °C, because the isolated yeast had difficulties growing at lower temperatures (*33*). Cultures were incubated for up to 3 days, and microorganisms were isolated using a dilution plating technique and by picking isolate colonies. Presumptive metolachlor-degrading microorganisms were restreaked for purity, several times, on the same medium and examined microscopically following gram staining. The MM was amended with 0.04% (w/v) yeast extract, 0.05% (w/v) sucrose, or both to enhance the growth of microorganisms at the beginning of the exponential phase of growth.

Microbial Identification. DNA was extracted from bacterial and yeast cells by using a freeze−thaw and sonication technique. For the bacteria, the 16S rRNA gene was amplified by PCR using universal bacterial primers 8F 5-GAGTTTGATCCTGGCTCAG-3 and 1492R 5-TACCTTGTTACGACTT-3 as described by Polz and Cavanaugh (*34*). These primers were also used for sequencing. For the yeast, three different regions of 18S rRNA were amplified and sequenced. The universal fungal primers 1F 5-AACCTGGTT GATCCTGCCAGT-3 and 1772R 5-TGATCCTTCTG CAGGTTCACCTAC-3 were used for the amplification and sequencing of the 18S rRNA gene (*35*). The sequences of the ITS1-5.8S-ITS2 regions were determined using primers ITS1 and ITS4 (TCCGTAGGTGAACCTGCGG/GCATATCAATAA GCGGAGGA). Primers NL1 5-CATATCAATAAGCGGAGGAAAAG-3 and NL4 5-GTCCGTGTTTCAAGACGG-3 (*35*) were used for amplification of the D1/D2 segment of 26S rDNA (*36*). PCR reactions were carried out using an iCycler thermocycler (Bio-Rad), using different protocols depending on the primers used. For the 16S amplification, an initial denaturation step of 3 min at 94 °C was followed by 35 cycles of amplification consisting of 1 min at 94 °C, 1 min at 50 °C, and 2 min at 72 °C. For amplification of 18S rRNA gene samples were denatured for 10 min at 95 °C, followed by 30 cycles of denaturation at 95 °C for 15 s, 15 s at 50 °C, and elongation at 72 °C for 2 min, with a final extension step of 10 min at 72 °C. The ITS region was amplified using ITS1/ITS2 primers and an initial denaturation step of 10 min at 95 °C. This was followed by 30 cycles of denaturation at 94 °C for 30 s, 30 s at 58 °C, elongation at 72 °C for 30 s, and a final extension step of 10 min at 72 °C. For amplification of the 26S rRNA gene with NL1/NL4 primers, the reaction was initiated with an initial denaturation at 94 °C for 10 min. This was followed with 36 cycles of 30 s at 94 °C, 30 s at 52 °C, and 1 min at 72 °C, with a final extension at 72 °C for 5 min.

DNA sequencing was done at the University of Minnesota BioMedical Genomics Center. All PCR products were purified by using a QIAquick Purification Kit (Qiagen, Inc., Valencia, CA) prior to sequencing. Sequences were analyzed with Applied Biosystems Sequence Scanner software v1.0 (Carlsbad, CA) and were assembled by using Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Sequence identity was determined by using BLAST (*37*). Species identification was obtained by using BLAST, sequence match software of the Ribosomal Database Project − RDP II (http://rdp.cme.msu.edu/) and the CBS Yeast Database (http://www.cbs.knaw.nl/yeast/BioloMICSID.aspx).

Additional biochemical tests were performed to more accurately assign species status to the isolated yeast. The yeast was grown in the presence of a discriminatory carbon source (*38*), in MM containing glucose, sucrose, D-xylose, trehalose, maltose, starch, rhamnose, galactose, inositol, lactose, D-arabinose, or D-mannitol. Plates were incubated at 30 °C in the dark, and growth was recorded 24−96 h after inoculation.

Microbial Growth. The influence of metolachlor on the growth kinetics of the isolated yeast and bacterium was determined. Cells were grown at 30 °C in 250 mL flasks containing 100 mL of MM medium and 50 μg mL^{-1} metolachlor, pH 7.0, with or without 0.05% sucrose, 0.04% yeast extract, or both. The initial culture had an OD_{600} of 0.10 ± 0.02, and growth was measured at 600 nm by using a DU-70 spectrophotometer (Beckman Instruments, Fullerton, CA). Data reported are mean values of two independent growth experiments carried out under identical conditions. For the experiments with acetochlor and alachlor, MM medium plus 0.04% yeast extract was exclusively used.

Herbicide Degradation. Exponential-phase yeast cells grown in MM containing 50 μg mL^{-1} herbicide were harvested by centrifugation at 10000*g* for 15 min, washed twice with 50 mM phosphate buffer (pH 7.2), and resuspended in fresh buffer to an OD_{600} of 1.0. Metolachlor disappearance and metabolite formation were determined by HPLC analysis. Analyses were performed using a Waters high-performing liquid chromatograph equipped with a C_{18} 5 μm column (ODS II, 250 × 4.6 mm) and a UV photodioarray detector. For the detection of the metolachlor, the isocratic mobile phase consisted of water and acetonitrile (50:50). The flow rate was 1.0 mL min^{-1}, the column was operated at room temperature, and the injection volume was 50 μL. Metolachlor was detected at 210 nm after approximately 5.8 min. For the detection of the acetochlor, the isocratic mobile phase consisted of water and methanol (25:75) at a flow rate of 1.0 mL min^{-1}, the column was operated at room temperature, and the injection volume was 25 μL. Acetochlor was detected at 200 nm after approximately 4.8 min. For the detection of the alachlor, the isocratic mobile phase consisted of water and acetonitrile (40:60) at a flow rate of 1.0 mL min^{-1}, the column was operated at room temperature, and the injection volume was 25 μL. Alachlor was detected at 205 nm after approximately 6.5 min.

Mineralization of the metolachlor and alachlor ring structures was determined in 250 mL biometer flasks containing 10 mL of 25 mM phosphate buffer (pH 7.0). Bacteria and yeast cells obtained from cultures grown on metolachlor or alachlor were added to final concentrations of 10^9 or 10^7 cells mL^{-1}, respectively. Metolachlor or alachlor was added to flasks to a final concentration of 50 μg mL^{-1} and [^{14}C]metolachlor or [^{14}C]alachlor was added to a final concentration of 3000 dpm mL^{-1}. A 7 mL vial containing 5 mL of 0.5 N NaOH was placed into the biometer flasks to quantify $^{14}CO_2$ released. The vials containing NaOH were removed and replaced at selected times during the incubation period. To determine $^{14}CO_2$, a 1 mL aliquot of the NaOH solution was mixed with 6 mL of Ecolite cocktail and radioactivity was quantified by using a Beckman model LS 6800 scintillation counter (Beckman Instruments, Irvine, CA). Samples were held in the dark for 24−48 h prior to counting and were corrected for quenching. No chemiluminescence was observed. The buffer medium was analyzed for the presence of metolachlor or alachlor and potential metabolites by HPLC as described below.

Mass Balance Determination. After the final sampling period, the solution in biometer flasks was dried to a constant weight at 80 °C for 24 h. Duplicate aliquots of the dried samples were weighed and mixed with an equal volume of powdered micro crystalline cellulose powder CF-11 (Whatman catalog no. 4021-500, Piscataway, NJ), and samples were oxidized for 1.4 min using a model 306 sample oxidizer (Packard Instruments, Downers Grove, IL). The [^{14}C]CO_2 evolved during combustion process was trapped in Carbosorb solvent (Packard Instruments), mixed with Permafluor (Packard Instruments) in a liquid scintillation vial, and quantified by using a Beckman model LS 6800 scintillation counter. The instrument combustion efficiency was determined before and after the combustion of each set of test samples. The efficiency of the oxidizer was calculated on the basis of the recovery of radioactivity from cellulose containing a known quantity of [^{14}C]metolachlor or [^{14}C]alachlor, and averaged 97.0% during the course of the study.

LC-MS Analysis. The concentration of metolachlor and its metabolites in growth medium was determined by using HPLC and LC-MS analyses. The HPLC analyses were done as described above. The LC-MS analysis for loss of parent compound metolachlor was done using a Waters Alliance 2695 high-performance liquid chromatograph (Milford, MA), coupled to an Applied Biosystems (Carlsbad, CA) API 3200 LC-MS-MS. A Zorbax RX-C8 column (2.1 mm i.d. × 150 mm long × 5 μm film thicknesses) (Agilent Technology, Santa Clara, CA) was used for separation. The column temperature was maintained at 40 °C, and the mobile phase was a gradient starting with 95% water (0.1% formic acid) (A)/5% acetonitrile (B); 95% A at 0 min; 95% A at 5 min; 50% A at 10 min; 3% A at 15 min; 3% A at 20 min; 95% A at 25 min; and 95% A at 30 min. The mobile phase flow rate was 0.2 mL min^{-1}, and the sample injection volume was 50 μL. Samples were maintained at 10 °C in the autosampler to minimize decomposition. Tuning parameters were optimized by direct infusion. All compounds were detected using LC-DAD (Waters 2996 photodiode array detector), and positive ionization or thermospray ESI+ multiple reaction monitoring (MRM) mode with the following mass spectrometer conditions: curtain gas interface, 30 psi; IS voltage, −4000 V; gas 1, 30 psi; gas 2, 30 psi; ion source temperature, 300 °C; collision gas, medium; dwell time, 200 ms. DAD monitoring was done at 210−400 nm.

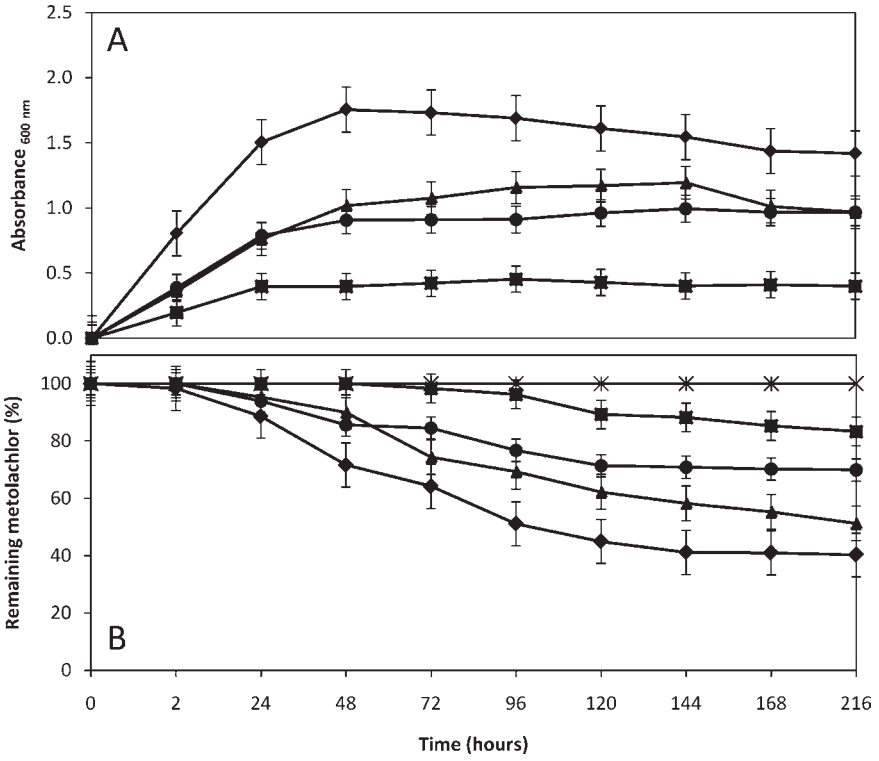

Figure 2. (**A**) Growth of *Candida xestobii* in minimal medium (MM) in the presence of 50 μg mL^{-1} metolachlor (■) and in MM with metolachlor plus sucrose (▲), yeast extract (●), or sucrose plus yeast extract (◆). (**B**) Metolachlor degradation by *Candida xestobii* in MM in the presence of 50 μg mL^{-1} metolachlor (■), in MM plus sucrose (▲), yeast extract (●), and sucrose plus yeast extract (◆), and in control medium containing metolachlor but without added inoculum (∗).

Metolachlor MRM transitions were as follows: 283.8 M + H 284.2 > 252.2 and 284.2 > 176.2. Minimal matrix effects (e.g., signal enhancement or suppression) were observed.

RESULTS AND DISCUSSION

Metolachlor, a member of the chloroacetanilide class of herbicides, contains 15 carbon atoms and one nitrogen atom per molecule (**Figure 1A**) and, thus, can potentially serve as a nutrient source for microbial growth. However, despite its use over the past 30 years, only a relatively few microorganisms that can incompletely transform metolachlor have been identified (*21*−*23*). This was thought to be due, in part, to its sorptive behavior, lack of bioavailability, and requirements for co-metabolism in the presence of microbial consortia.

In the study reported here, we describe the isolation and identification of two microorganisms that were capable of using metolachlor as the sole source of C for growth. Both microbes were isolated, via enrichment, from the same Spanish soil with a history of metolachlor application. Microscopic and molecular analyses showed that the isolated organisms were a bacterium and a yeast. The bacterium was a Gram-positive, spore-forming, microorganism, and 16S rRNA sequence analysis confirmed the isolate was *B. simplex* (GenBank accession no. DQ159336), with 99% nucleotide sequence similarity.

The identification of the yeast was much more difficult, in part due to incomplete and complicated taxonomy of yeasts isolated from natural substrates, such as soil. Consequently, they are extremely difficult to differentiate phenotypically and are very often misidentified (*38*). Sequence analysis of 18S and 26S rDNA and the ITS region led to the conclusion that the isolated yeast was *C. xestobii* (GenBank accession no. AM160626.1), with 99% nucleotide similarity in the GenBank CBS Yeast databases. Because only 2 bp differentiate *C. xestobii* and *Pichia guilliermondii* in the D1/D2 and ITS regions (*39*), species identity was confirmed by using biochemical analyses. The isolated yeast grew in MM containing glucose, sucrose, D-xylose, trehalose, maltose, starch, and galactose, but failed to grow on rhamnose, inositol, lactose, D-mannitol, and D-arabinose. Results of these analyses were consistent with taxonomic assignment of the yeast to *C. xestobii* (*40*).

Growth and Degradation of Metolachlor by *C. xestobii* and *B. simplex*. The influence of culture media and carbon sources on the degradation of 50 μg mL^{-1} metolachlor was examined. The disappearance of metolachlor was determined to be due to microbial metabolism. Results in **Figure 2A** show that as *C. xestobii* grew in MM amended with metolachlor, with or without other added amendments, the concentration of metolachlor decreased to 40% of the initial concentration after 6 days of incubation. No further degradation of metolachlor was observed after this time. Control media, which were not inoculated, did not exhibit metolachlor disappearance (**Figures 2B**, **3** and **4**), in agreement with previous reports that metolachlor degradation is mainly due to biological rather than chemical processes (*14*, *18*, *41*).

The greatest amount (60%) and fastest rate of metolachlor degradation were observed in metolachlor medium amended with 0.04% of yeast extract. In contrast, whereas growth of the yeast was faster and greatest in metolachlor medium amended with sucrose and yeast extract (**Figure 2A**), only about 20% of metolachlor was degraded after 9 days of incubation (**Figure 2B**). Taken together, these results indicated that the yeast has the ability to catabolize metolachlor as a sole source of nutrients for growth, but preferred other nutrient sources, such as yeast extract and sucrose, which are probably easier to metabolize. Because the yeast also grew in MM amended only with metolachlor, data presented in **Figure 2** also show that *C. xestobii* uses metolachlor as a sole C source for growth.

To our knowledge, this is the first reported yeast that has the ability to catabolize metolachlor and use this compound as sole C

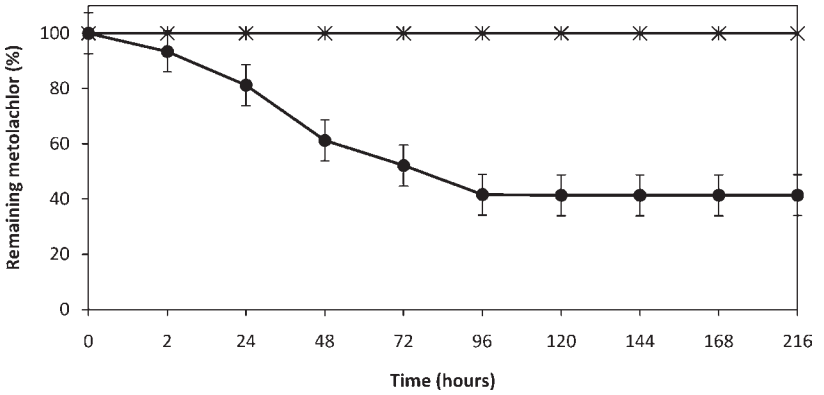

Figure 3. Metolachlor degradation by resting cells of *Candida xestobii* (●) and in control medium containing metolachlor but without added inoculum (∗).

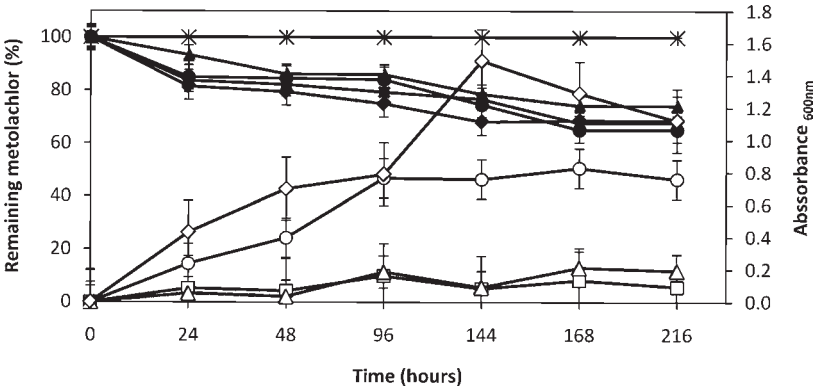

Figure 4. Metolachlor degradation (solid symbols) and growth (open symbols) of *Bacillus simplex* in MM in the presence of 50 μg mL^{-1} metolachlor (■), in MM with sucrose (▲), yeast extract (●), and sucrose plus yeast extract (◆), and in control medium containing metolachlor but without added inoculum (∗).

and energy sources for growth. Degradation of metolachlor, however, has been previously reported for the filamentous fungi *Chaetomium globosum*, which can also use this compound as a sole source of C and energy (*23*), and *Aspergillus flavus* and *Aspergillus terrícola* (*42*). Moreover, certain fungicides have been shown to significantly reduce metolachlor dissipation in soils, as compared to nontreated controls (*31*). On the basis of our results and those of others, metolachlor degradation by fungi appears to be a widespread phenomenon.

Further evidence that *C. xestobii* was capable of catabolizing metolachlor was obtained by examining stationary phase, resting cell, cultures that were pregrown on 50 μg mL^{-1} metolachlor. Following the further addition of 50 μg mL^{-1} metolachlor, these cultures demonstrated a faster rate of degradation (60% after 4 days) than that seen with the initial degradation of the compound (**Figure 3**). This indicated that *C. xestobii* more actively degraded metolachlor following initial growth on this substrate, perhaps due to either the presence of more cells or the induction of enzymes required for metolachlor degradation.

Results in **Figure 4** show that *B. simplex* also grew in metolachlor medium, with or without added amendments. The initial concentration of metolachlor decreased 65% after 6 days of incubation, after which time no further degradation of the compound was observed. The degradation of metolachlor by *B. simplex* was approximately 25% less than that observed with the yeast under the same conditions. The degradation rate of metolachlor was similar in the different culture media used, despite the greater growth observed when the growth medium containing metolachlor was amended with yeast extract or with sucrose plus yeast extract. Similar to what was found with *C. xestobii*, our studies also indicate that *B. simplex* uses metolachlor as a sole source of C and energy for growth. However, neither microorganism had the ability to degrade some of the proposed main metabolites of metolachlor, MESA (**Figure 1D**) or MOA (**Figure 1E**).

Under aerobic conditions, only partial biodegradation of metolachlor by bacteria was previously reported, and it has been proposed that degradation proceeds through a co-metabolic process in the presence of other C sources (*18*, *21*, *22*, *32*). However, the catabolism of metolachlor by *B.simplex* does not appear to be due to a co-metabolic process, because it occurred in MM without other added carbon substrates and with only a single microorganism present. Despite this, the transformation of metolachlor by *B. simplex* was not complete, and this may be related, in part, to the apparent persistence of metolachlor in soils. For example, in laboratory incubation experiments Konopka and Turco (*43*) reported that metolachlor (0.58−9.1 μg mL^{-1}) was not degraded over a period of 128 days in vadose zone samples obtained from an agricultural field. Nevertheless, our data indicate that partial transformation of this herbicide was still sufficient to supply this bacterium with sufficient C and energy for growth.

Degradation of Acetochlor and Alachlor by *C. xestobii*. The degradation of relatively high concentrations (50 μg mL^{-1}) of acetochlor and alachlor by *C. xestobii* was also examined, and the disappearance of both of these substrates was also determined to be due to the result of microbial metabolism. Results in **Figure 5** show that > 50% of the added acetochlor was degraded by *C. xestobii* in the first 15 h of growth, and the concentration decreased by 60% after 312 h. In the resting cell assays, however, about 80% of the acetochlor was degraded in 15 h, but the degradation was also incomplete, and there was no degradation after that time. Whereas acetochlor was previously shown to be completely degraded by a consortium of eight microorganisms after 4 days (*29*), no single isolate was able to degrade acetochlor efficiently.

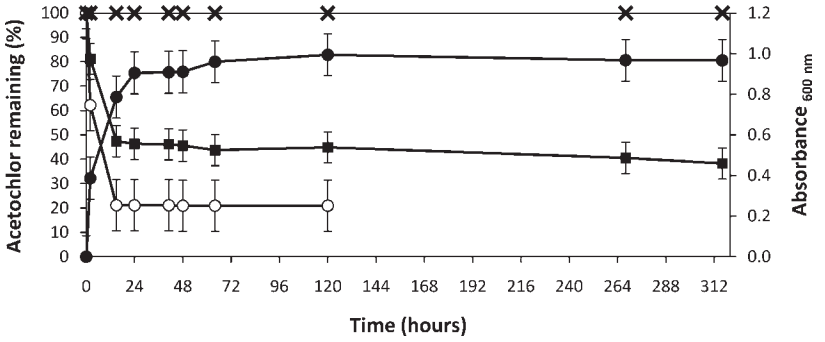

Figure 5. Acetochlor degradation (solid symbols) and growth of *Candida xestobii* (open symbols) in MM in the presence of 50 μg mL^{-1} acetochlor with yeast extract (■), in resting cell assays (●), and in control medium containing acetochlor but without added inoculum (∗).

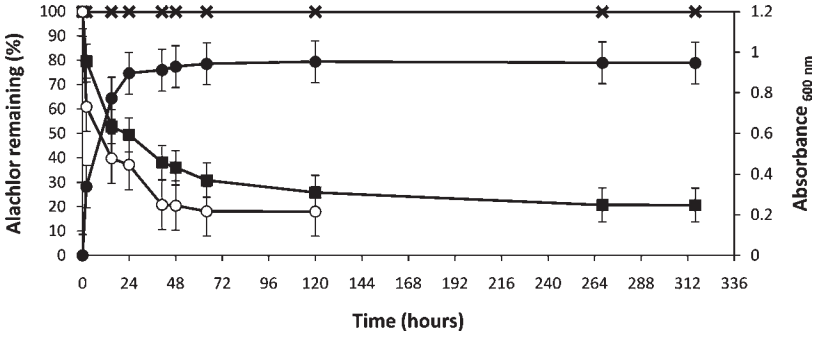

Figure 6. Alachlor degradation (solid symbols) and growth of *Candida xestobii* (open symbols) in MM in the presence of 50 μg mL^{-1} alachlor and yeast extract (■), in resting cell assays (●), and in control medium containing alachlor but without added inoculum (∗).

Table 1. Summary of Degradation of Acetanilide Herbicides by *Candida xestobii* and *Bacillus simplex* in Different Culture Media and under Resting Cell Conditions

microorganism	medium	herbicide	maximum degradation[a] (%)	time (h)
Candida xestobii	MM + sucrose and yeast extract	metolachlor	59.7	144
	MM + sucrose		48.7	96
	MM + yeast extract		30.1	120
	MM no amendments		16.7	144
	resting cells		58.4	96
Bacillus simplex	MM + sucrose and yeast extract		31.9	144
	MM + sucrose		25.9	168
	MM + yeast extract		35.0	168
	MM no amendments		32.4	168
Candida xestobii	MM + yeast extract	acetochlor	78.9	15
	resting cells		56.2	64
	MM + yeast extract	alachlor	79.3	268
	resting cells		81.9	64

[a] Values are means of three replicates.

Results in **Figure 6** show that *C. xestobii* also transformed ~70% of the initial concentration of alachlor after 3 days of growth, after which time degradation was much slower. In the resting cell assays, however, degradation proceeded more quickly, and ~80% was transformed after 2 days. Whereas Xu et al. (*29*) reported that 63 and 39% of alachlor and metolachlor, respectively, were degraded by mixed microbial consortia after 21 days of incubation, *C. xestobii* surpassed those degradation amounts in shorter incubation periods. Control media, which were not inoculated, did not exhibit acetochlor or alachlor disappearance (**Figures 5** and **6**). A summary of the degradation of acetanilide herbicides by the isolated microorganisms is shown in **Table 1**.

Mineralization of Metolachlor and Alachlor by *C. xestobii* and *B. simplex*. Growth of *C. xestobii* in the presence of [^{14}C]metolachlor showed that up to 25% of the ring-labeled compound was converted into $^{14}CO_2$ after 10 days of growth (**Figure 7**). Like catabolism, the mineralization of metolachlor by *C. xestobii* was not complete, and no further mineralization occurred even after 360 h of incubation. Interestingly, mineralization of metolachlor in MM amended with yeast extract was greater than that seen in MM containing only metolachlor. In the former case, mineralization started after 144 days of incubation and reached only 6% after 240 days of incubation, whereas mineralization started 24 h earlier in resting cells assays, indicating a direct relationship between cell numbers and mineralization rate.

Growth of *C. xestobii* in the presence of [^{14}C]alachlor showed that up to 20% of the ring-labeled compound was mineralized to $^{14}CO_2$ after 48 h. After that time, mineralization proceeded much more slowly, and 40% was transformed after 336 h of incubation (**Figure 8**). Whereas white rot fungi were previously reported to

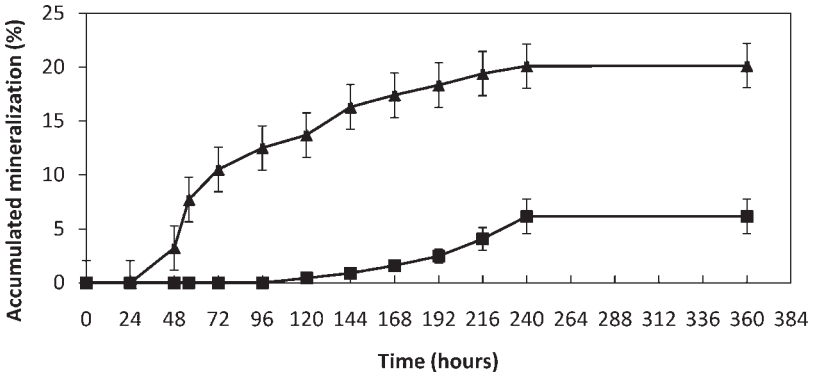

Figure 7. Mineralization of [^{14}C]metolachlor by *Candida xestobii* in MM with yeast extract (●) and in resting cell assays (▲).

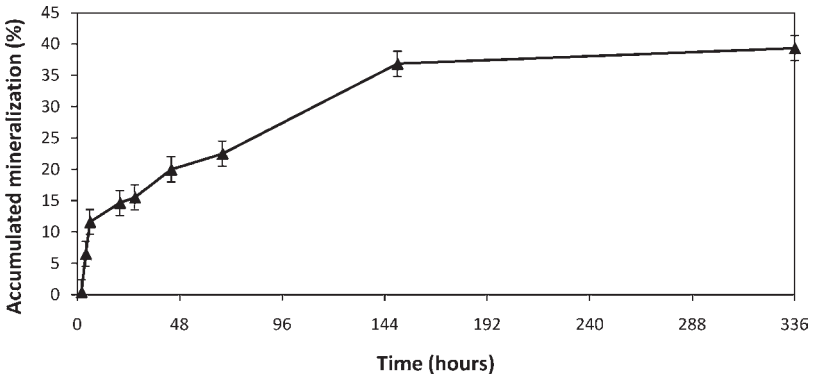

Figure 8. Mineralization of [^{14}C]alachlor by *Candida xestobii* in resting cell assays (▲).

mineralize the aromatic ring carbon of alachlor to CO_2, only 14% was converted to CO_2 after 122 days (*24*).

Although chloroacetanilide herbicides are nonionizable and have moderate to low volatilities (*44*), a volatile transparent yellow metabolite was observed in the NaOH vial used to trap $^{14}CO_2$ during the first 3 days of the alachlor mineralization experiment. The colored product was not seen in NaOH vials in control uninoculated biometer flasks containing alachlor or metolachlor mineralization studies.

Whereas *B. simplex* has the ability to use metolachlor as the sole C and energy sources for growth, the bacterium failed to mineralize this herbicide, at least the ^{14}C-ring labeled atoms. This indicated that *B. simplex* likely uses a different degradation pathway for metolachlor than does *C. xestobii*. In some ways, this result is similar to those reported by Saxena et al. (*18*), who failed to isolate bacteria that could mineralize metolachlor. However, these authors did report that strains of *Bacillus circulans*, *Bacillus megaterium*, and an *actinomycete* were able to transform metolachlor into several metabolites.

Although Stamper and Tuovinen (*32*) postulated that mineralization of metolachlor may not be the major route for its dissipation in natural systems, results are currently contradictory. For example, Staddon et al. (*45*) reported that $<4\%$ of metolachlor was mineralized after 46 days, but Krutz et al. (*46*) reported that 40% of metolachlor was mineralized after 63 days in a soil. Similarly, *C. xestobii* was also shown here to rapidly mineralize up to 25% of metolachlor after 10 days of growth. Because differences in mineralization rates among microorganisms in soils are likely due to both biotic and abiotic factors, more studies are needed to assess the contribution of mineralization to loss of this herbicide in soils.

Results of mass balance analyses indicated that $<5\%$ of metolachlor in the culture medium was present in *C. xestobii* and *B. simplex* cells following incubation with [^{14}C]metolachlor (data not shown). This result indicated that metolachlor was not significantly incorporated into biomass and, thus, metabolites that were not mineralized were likely released into the growth medium. Our results are in contrast to those reported in ref *17*, which reported that 80% of ring-labeled [U-^{14}C]metolachlor added to a microbial community was removed from the medium and accumulated inside cells.

Mechanism of Degradation. The mechanism by which metolachlor is transformed by *C. xestobii* is not clear. Because analytical standards of possible metolachlor metabolites were not available, we used the University of Minnesota Biocatalysis/Biodegradation Database Pathway Prediction System (PPS) to predict plausible pathways for the microbial degradation of metolachlor (*47*) (http://umbbd.msi.umn.edu/predict/). The PPS identified 22 possible molecules with molecular ions (M^+, M^-) > 190. Comparison of the possible molecular ions from the total ion current plot of culture medium obtained following growth of *C. xestobii* on metolachlor resulted in no positive matches. Also, HPLC fractionation of the spent medium following growth of *C. xestobii* in uniformly ring-labeled [^{14}C]metolachlor did not result in any peaks that had $>2\%$ of the applied ^{14}C, other than the metolachlor peak, leading to difficulty in extrapolating a degradation pathway. Although it is tempting to speculate that dechlorination was not a major mechanism for the degradation of metolachlor by the isolated yeast, too few data are available to accurately determine this. Consequently, the pathway by which metolachlor is transformed by *C. xestobii* is currently unknown and awaits further analyses.

In summary, in this study we report on the isolation of a bacterium and yeast that have the ability to catabolize metolachlor (**Table 1**). We also show that the yeast *C. xestobii* uses metolachlor as a sole C and energy source for growth and is able to mineralize

this compound under controlled laboratory conditions. Although other fungal and bacterial strains have been isolated that are able to partially transform metolachlor, most attempts to isolate pure or mixed microbial cultures capable of mineralizing metolachlor have been unsuccessful (*32*, *48*). Whereas the degradation of metolachlor has been previously studied with a pure culture of the fungus *Ch. globosum*, which also used this herbicide as a sole source of C and energy, gas−liquid chromatographic analysis of the concentrated extract from resting cell experiments with this fungus showed that at least eight extractable products were produced from the original compound (*23*). Tiedje and Hagedorn (*49*) reported that the major product of alachlor degradation by this fungus was likely 2,6-diethyl-*N*-(methoxymethyl)aniline, and McGahen and Tiedje (*23*) reported that the co-metabolism of metolachlor by *Ch. globosum* is thought to occur by removal of one or both R groups from the nitrogen atom and subsequent dehydrogenation of the ethyl substituent. These authors also postulated that the fungus may eventually remove the chloro, methoxy, or ethoxy substituent from the R groups. In addition to fungi, bacteria have also been reported to transform alachlor. For example, Sette et al. (*50*) reported that a *Streptomycetes* sp. strain degraded ∼60−75% of the alachlor within 14 days to produce indole and quinoline derivatives, and Villareal et al. (*51*) reported that *Moraxella* sp. strain DAK3 respired and grew on N-substituted acylanilides containing methyl, ethyl, or isopropyl substitutions, but failed to grow on alachlor and metolachlor.

In contrast to previous studies with fungi, the isolated *C. xestobii* strain degraded 50% of metolachlor after 4 days of growth, and no metabolites, such as the ethanesulfonic acid (**Figure 1D**) and oxanilic acid (**Figure 1E**), were detected in the growth medium by HPLC analysis. *A. flavus* and *A. terrícola* have been also described as metolachlor-degrading fungi, reducing the half-life of this herbicide from 189 to 3.6 and 6.4 days, respectively (*42*). Coupled with data showing that some fungicides significantly reduce metolachlor dissipation in soils (*31*), results from our studies are consistent with the notion that soil yeast and other fungi may be responsible for significant transformation of metolachlor in soils. Moreover, because degradation of metolachlor by *C. xestobii* was fairly rapid and resulted in the mineralization of this herbicide, our data suggest that this yeast may eventually prove to be useful for metolachlor bioremediation efforts. More studies, however, are needed to determine whether this yeast is also able to metabolize and mineralize other aniline herbicide compounds and to identify metabolites produced during the degradation process.

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