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Complete Microbial Degradation of Both Enantiomers of the Chiral Herbicide Mecoprop [(*RS*)-2-(4-Chloro-2-Methylphenoxy)propionic Acid] in an Enantioselective Manner by *Sphingomonas herbicidovorans* sp. nov.

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Sphingomonas herbicidovorans **MH (previously designated *Flavobacterium* sp. strain MH) was able to utilize the chiral herbicide (*RS*)-2-(4-chloro-2-methylphenoxy)propionic acid (mecoprop) as the sole carbon and energy source. When strain MH was offered racemic mecoprop as the growth substrate, it could degrade both the (*R*) and the (*S*) enantiomer to completion, as shown by biomass formation, substrate consumption, and stoichiometric chloride release. However, the (*S*) enantiomer disappeared much faster from the culture medium than the (*R*) enantiomer. These results suggest the involvement of specific enzymes for the degradation of each enantiomer. This view was substantiated by the fact that resting cells of strain MH grown on (*S*)-mecoprop were able to degrade the (*S*) but not the (*R*) enantiomer of mecoprop. Accordingly, resting cells of strain MH grown on (*R*)-mecoprop preferentially metabolized the (*R*) enantiomer. Nevertheless, such cells could transform (*S*)-mecoprop at low rates. Oxygen uptake rates with resting cells confirmed the above view, as oxygen consumption was strongly dependent on the growth substrate. Cells grown on (*R*)-mecoprop showed oxygen uptake rates more than two times higher upon incubation with the (*R*) than upon incubation with the (*S*) enantiomer and vice versa.**

Chiral (*RS*)-2-(4-chloro-2-methylphenoxy)propionic acid (mecoprop) (Fig. 1) is a systemic, postemergence herbicide developed in the 1950s for use in the control of broad-leaved weeds in cereal crops and lawns, and commercially it is often used in formulations together with other herbicides (22). Already in 1953, studies about the stereochemistry of plant growth regulators revealed that the herbicidal activity was associated only with the (*R*) enantiomer of mecoprop (11). Enantiomerically pure (*R*)-2-(4-chloro-2-methylphenoxy)propionic acid was made available commercially, beginning in 1987, as mecoprop-P, yet many commercial formulations contain mecoprop—the racemic mixture—and not mecoprop-P—the pure (*R*) enantiomer (22). The importance of considering the stereochemistry of pesticides, crop protectants, detergents, and drugs—not only for the investigation of their biological effects, but also for studying their environmental fate—has recently been emphasized (1, 2, 15). Nevertheless, such considerations are missing in many studies about the environmental fate and the microbial degradation of mecoprop (4, 7, 9, 12), and this might be one of the reasons why only limited progress has been made in unravelling the exact mechanism of microbial mecoprop degradation.

While the microbial degradation pathway of 2,4-D (2,4-dichlorophenoxyacetic acid) has been investigated in detail (3, 10, 13, 14, 20), the microbial metabolism of mecoprop remains to be elucidated. Mecoprop is known to degrade in natural environments by the action of microorganisms, and disappearance rates in soil ranging from 5 to 11 days have been reported (16, 17). Although mecoprop degradation most likely occurs in the top soil, the rather high mobility of the pollutant constitutes a risk of contamination for underlying aquifers and groundwater resources (5). Mecoprop vanished when added at low concentrations to an aerobic aquifer under laboratory conditions. In this case, a lag phase was observed after initial degradation of approximately 50% of the compound, which was followed by complete degradation. This phenomenon was attributed to microbial discrimination between the two stereoisomers of mecoprop (5).

The failure of being able to isolate pure cultures of microorganisms with the ability to use mecoprop as the sole carbon and energy source has led researchers to postulate that in natural environments the complete degradation of mecoprop may only occur through the synergistic activities of a consortium of microorganisms (19). However, two pure bacterial cultures with the ability to utilize mecoprop as a growth substrate have been recently described (6, 19). Both strains—*Sphingomonas herbicidovorans* MH (previously designated *Flavobacterium* sp. strain MH [6]) and *Alcaligenes denitrificans*—were able to use mecoprop as the sole carbon and energy source. Experiments with the pure enantiomers of mecoprop as growth substrates revealed that *A. denitrificans* exclusively degraded the (*R*) enantiomer of mecoprop, while the (*S*) enantiomer remained unaffected (19). In the case of *S. herbicidovorans* MH, only mecoprop—the racemic mixture—was tested as a substrate in growth experiments and therefore no conclusions about enantiomer-specific processes could be drawn (6).

In this study, we investigated the degradation of mecoprop by *S. herbicidovorans* MH by using enantiomer-specific analytical tools and the pure enantiomers as well as the racemic mixture of 2-(4-chloro-2-methylphenoxy)propionic acid as growth substrates. We were able to unequivocally demonstrate for the first time that a pure bacterial culture is able to degrade

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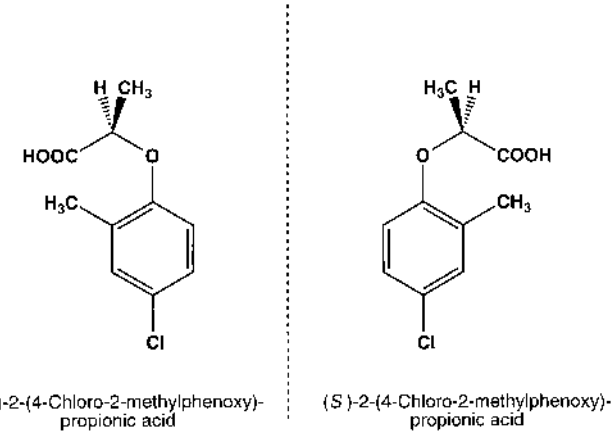

FIG. 1. Structural formulas of the (*R*) and the (*S*) enantiomer of 2-(4-chloro-2-methylphenoxy)propionic acid (mecoprop).

both enantiomers of mecoprop to completion. The data presented suggest the involvement of specific enzymes for the initial attack on each enantiomer. We intend to stress the necessity of considering stereochemistry when studying the degradation of chiral compounds.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *S. herbicidovorans* MH (previously designated *Flavobacterium* sp. strain MH) was a gift from F. Streichsbier, Vienna, Austria. It was isolated from a soil column with (*RS*)-2-(2,4-dichlorophenoxy) propionic acid as the carbon and energy source (6). Strain MH was identified by the Deutsche Stammsammlung von Mikroorganismen und Zellkulturen (DSM), Braunschweig, Germany, as a new species belonging to the *Sphingomonas* group within the α subgroup of the proteobacteria.

(i) Mineral medium. The chloride-free mineral salts medium used for growth of strain MH consisted of 20 mM phosphate buffer (KH_2PO_4-Na_2HPO_4, pH 6.5) containing $(NH_4)_2SO_4$ (0.12 g/liter), $MgSO_4 \cdot 7H_2O$ (73 mg/liter), and $Ca(NO_3)_2 \cdot 4H_2O$ (1 mg/liter). The following trace elements were supplied at 0.2 ml/liter from an acidified (H_3BO_3, 0.1 g/liter; H_2SO_4, 5 ml/liter) stock solution containing $FeSO_4 \cdot 7H_2O$ (2.5 g/liter), $MnSO_4 \cdot H_2O$ (0.75 g/liter), $ZnSO_4 \cdot 7H_2O$ (1.3 g/liter), $CuSO_4 \cdot 5H_2O$ (0.25 g/liter), $Co(NO_3)_2 \cdot 6H_2O$ (0.3 g/liter), $Na_2MoO_4 \cdot 2H_2O$ (0.15 g/liter), and $NiSO_4 \cdot 7H_2O$ (0.1 g/liter). After autoclaving, 1 ml of a vitamin solution containing pyridoxin-HCl (0.1 g/liter), thiamine-HCl (0.05 g/liter), riboflavin (0.05 g/liter), nicotinic acid (0.05 g/liter), Ca-pantothenate (0.05 g/liter), 4-aminobenzoic acid (0.05 g/liter), nicotinamide (0.05 g/liter), vitamin B_{12} (0.05 g/liter), biotin (0.02 g/liter), and folic acid (0.02 g/liter) was added by sterile filtration (0.2 μm) per liter of medium. The medium was supplemented with 20 mg of peptone (Biolife, Milan, Italy) per liter. The carbon sources were added to the mineral medium before autoclaving, as no losses could be observed upon heating to 121°C for 30 min.

(ii) Complex medium. The chloride-free complex medium consisted of 6.0 g of peptone per liter, 1.2 g of yeast extract per liter, and 0.4 g of glucose per liter in the mineral medium described above.

Chemicals. When not explicitly stated, chemicals were purchased from Fluka Chemicals, Buchs, Switzerland. Analytical standards of (*R*)-mecoprop were obtained from Riedel-de Haën, Seelze, Germany. Technical grade (*RS*)-mecoprop (92%) was donated by BASF Chemicals, Limburgerhof, Germany. It was recrystallized twice, first from 80% formic acid and then from petroleum (bp, 60 to 80°C), to form small, white crystals. The (*S*) and the (*R*) enantiomer of mecoprop were resolved from the racemic mixture by letting the recrystallized technical grade (*RS*)-mecoprop react with (*R*)- and (*S*)-phenylethylamine, respectively, to form the phenylethylamine diastereoisomeric salts by a procedure described by Matell (11). The enantiomer excess (ee) of the (*S*)- and the (*R*)-mecoprop was determined by enantiomer-specific high-performance liquid chromatography (HPLC) as 99 and 92%, respectively. The authenticity of the (*S*)- and the (*R*)-mecoprop was confirmed by ^1H- and ^{13}C-nuclear magnetic resonance spectroscopy.

Growth experiments. Precultures used for the inoculation of the media of the growth experiments were grown in chloride-free complex medium in 300-ml Erlenmeyer flasks at a temperature of 30°C. In order to remove the residual complex carbon substrate prior to the growth experiments, the precultures were harvested by centrifugation (15 min, 7,500 × *g*, 4°C) during the midexponential growth phase, washed, and resuspended in mineral medium in such a way that an optical density of 1.3 absorbance units at 546 nm (0.3 g [dry weight] per liter) was achieved. This cell suspension was used for inoculation (2% [vol/vol]). Growth experiments were performed in 1-liter Erlenmeyer flasks equipped with magnetic stirring bars. The flasks were equipped with a silicon tube that allowed easy sterile sampling. The experiments were carried out in a water bath at a temperature of 30°C with stirring held constant at 370 rpm. The initial mecoprop concentration was 0.46 mM (100 mg/liter).

Determination of growth yields. Dry weight was measured by filtering defined volumes of the culture broth through tared 0.20-μm-pore-size polycarbonate filters dried at 100°C (Nucleopore, Tübingen, Germany). Yields were calculated after determination of residual substrate and dissolved organic carbon (DOC).

Substrate consumption of resting cells. Cells were grown on (*R*)- or (*S*)-mecoprop, centrifuged, and washed. Each cell suspension (67 mg [dry weight] per liter for (*R*)-mecoprop-grown cells and 86 mg [dry weight] per liter for (*S*)-mecoprop-grown cells) was divided into three parts and the assay substrates (*R*)-, (*S*)-, and (*RS*)-mecoprop were added at a concentration of 0.2 mM in the final assay volume of 20 ml. The cell suspensions were transferred to 100-ml Erlenmeyer flasks and incubated at 30°C on a rotary shaker operated at 125 rpm.

Oxygen uptake of resting cells. Oxygen uptake was measured polarographically with a Clark type oxygen electrode (Rank Brothers, Cambridge, United Kingdom). The electrode was mounted to a reaction vessel (3 ml) whose temperature was held constant at 30°C. The assay was carried out with a substrate concentration of 1 mM and biomass concentrations in the range of 40 to 130 mg (dry weight) per liter. Specific oxygen uptake rates were corrected for endogenous rates.

Analytical procedures. (i) DOC. Samples were centrifuged (12,000 × *g* for 15 min), acidified with HCl (pH 2), freed from dissolved CO_2 by purging with N_2 for 10 min, and finally analyzed in a Tocor 2 carbon analyzer (Maihak, Hamburg, Germany).

(ii) HPLC. HPLC analyses were performed on a Waters 625 LC system with a WISP 700 autosampler and a 901 photodiodearray detector (Waters Millipore Corp., Milford, Mass.). (*R*)- and (*S*)-mecoprop were separated on a Nucleodex-α-PM column (200 by 4.0 mm) with permethylated α-cyclodextrin as the stationary phase (Macherey-Nagel, Düren, Germany) and detected at a wavelength of 230 nm. The system was operated isocratically with an eluent consisting of 70% methanol and 30% NaH_2PO_4 (50 mM, pH 3.0) at a flow rate of 0.7 ml/min. Retention times were typically 8.5 min for (*R*)- and 11.5 min for (*S*)-mecoprop. The detection limit for mecoprop was 4 μM.

(iii) Ion chromatography. Chloride concentration was measured with an ion chromatograph equipped with a conductivity detector (Dionex, Olten, Switzerland) after separation on an anion-exchange column (Dionex IonPac AS11; alkanol quaternary ammonium as functional group) with a gradient from 5 to 12 mM NaOH as the mobile phase at a flow rate of 1 ml/min. Subsequent elution of other anions was obtained with 25 mM NaOH. The detection limit for chloride was 20 μM.

(iv) ^1H- and ^{13}C-NMR. ^1H- and ^{13}C-NMR spectra were obtained with a 400-MHz Bruker AMX-400 NMR spectrometer (Spectrospin, Fällanden, Switzerland). Samples were dissolved in $CDCl_3$.

(v) UV-VIS spectroscopy. UV-visible light (VIS) spectra were obtained with an UVIKON 860 spectrophotometer (Kontron, Zürich, Switzerland).

RESULTS AND DISCUSSION

Taxonomical characteristics of strain MH. Strain MH is a yellow-pigmented, gram-negative, oxidase- and catalase-positive rod with a length of 1.5 to 4.0 μm and a width of 0.6 to 0.8 μm. It is aminopeptidase positive and ADH and urease negative, lyses only esculin but not gelatin or DNA, cannot reduce nitrate, is not able to grow anaerobically, and does not form acids from sugars. Analysis of the cellular fatty acids showed that the strain belongs to the *Sphingomonas* group within the α subgroup of the proteobacteria. Partial sequencing of the gene for the 16S rRNA allowed an unequivocal classification of strain MH as a member of this group. The highest degree of similarity (99%) was obtained with the gene for the 16S rRNA of *Rhizomonas suberifaciens* and *S. yanoikuyae*. DNA-DNA hybridization experiments between strain MH and the type strains of these two species (37.4% identity to *R. suberifaciens* DSM 7465 and 33.8% identity to *S. yanoikuyae* DSM 7462) showed that strain MH belongs to a new species within the group. Although it has been suggested that *S. yanoikuyae* should be placed into the genus *Rhizomonas* (18, 21), we decided to name strain MH *S. herbicidovorans*, because—according to opinion 14 of the *International Code of Nomenclature of Bacteria* (8)—the genus name *Rhizomonas* is a nomen rejiciendum and therefore usage of this name is in violation of rule 28a of the code. *S. herbicidovorans* MH has been deposited as

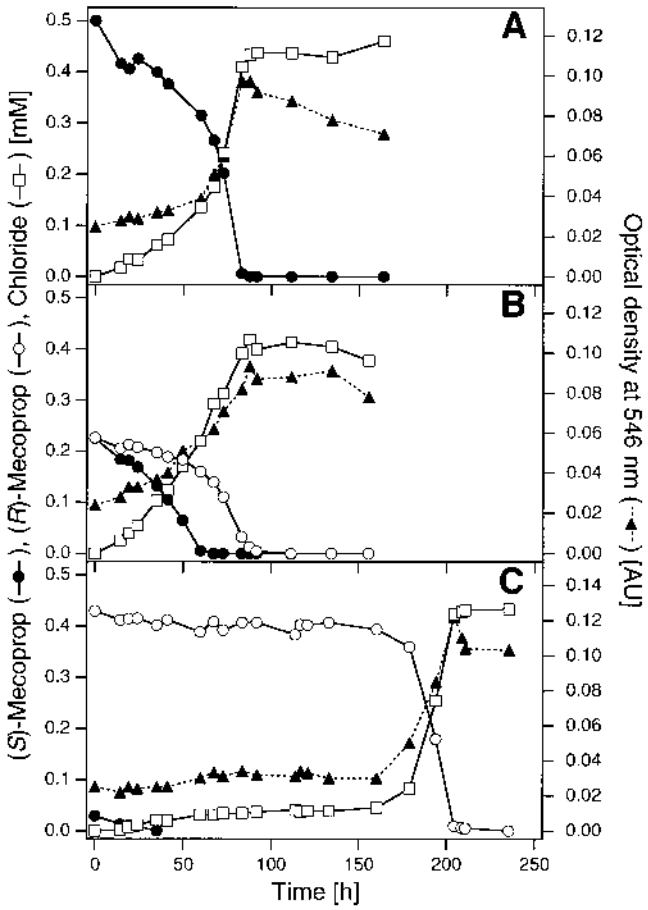

FIG. 2. Growth experiments with *S. herbicidovorans* MH incubated with (*R*)-2-(4-chloro-2-methylphenoxy)propionic acid, (*S*)-2-(4-chloro-2-methylphenoxy) propionic acid, and racemic (*RS*)-2-(4-chloro-2-methylphenoxy)propionic acid (mecoprop). (A) Growth on the pure (*S*) enantiomer. (B) Growth on the racemic mixture. (C) Growth on the pure (*R*) enantiomer. The inocula for these experiments were noninduced cells grown on complex media.

the type strain with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig (DSM 11019).

It took at least 3 days at 30°C before circular, brightly yellow colonies with a smooth surface could be observed on nutrient agar plates. The shape of the colonies changed to a flower-like appearance when plates were incubated over longer time periods (up to 7 days). Cells secreted a brownish pigment into the medium when grown on solid or liquid nutrient broth. Cells taken from the exponential growth phase were rod shaped and often sticked together forming a chain, whereas cells taken from the stationary phase were almost of a coccoid shape. In mineral salts medium with racemic mecoprop as the sole source of carbon and energy, strain MH needed supplements of vitamins and peptone (20 mg/liter) for growth. Minimal agar plates had to be incubated up to 7 days until colonies appeared. These colonies were smaller than the colonies grown on nutrient agar and not as intensely colored, but upon examination with a stereoscopic microscope, both colony types looked identical.

Growth characteristics. Growth experiments with the single pure enantiomers as well as with the racemic mixture of mecoprop as growth substrates revealed that strain MH could completely degrade both enantiomers of mecoprop (Fig. 2A to C), albeit in an enantioselective manner. The inocula for all the growth experiments were noninduced cells grown on complex media. Chloride release was stoichiometric in all growth experiments, a strong hint for the destruction of the aromatic ring. Growth on (*S*)-mecoprop was completed after about 3.5 days irrespective of whether the (*R*) enantiomer was present. When racemic mecoprop was the growth substrate, degradation of the enantiomers was sequential, with (*S*)-mecoprop being degraded first. The (*R*) enantiomer was only slowly degraded during the first 50 h of such experiments, but the degradation rate for (*R*)-mecoprop markedly increased after (*S*)-mecoprop was completely consumed (Fig. 2B). Cells started to grow after a lag phase of about 7 days, when pure (*R*)-mecoprop was the sole carbon and energy source (Fig. 2C). Once growth commenced, cells grew as fast as when (*S*)-mecoprop was the growth substrate. In all growth experiments, strain MH did not excrete any metabolites that could be detected by HPLC into the medium. The fact that *S. herbicidovorans* MH was able to utilize both enantiomers of the chiral herbicide (*RS*)-2-(4-chloro-2-methylphenoxy)propionic acid (mecoprop) as the sole carbon and energy source is in contrast to findings with *A. denitrificans*, which—besides strain MH—is the only strain maintained in pure culture that has been described to metabolize mecoprop. *A. denitrificans* could only degrade the (*R*) enantiomer of mecoprop, while the (*S*) enantiomer remained untouched (19). The enantiomer-specific degradation pattern (Fig. 2) suggests that there is more than one enzyme system involved in the degradation of (*S*)- and the (*R*)-mecoprop in *S. herbicidovorans* MH.

The growth rates of strain MH growing on (*S*)-, (*R*)-, and (*RS*)-mecoprop (0.5 mM) were 0.039, 0.032, and 0.016 h^{-1} with yields of 0.35, 0.30, and 0.30 g (dry weight) per g of substrate, respectively. Hence, both enantiomers are equally well suited as a carbon and energy source for this bacterium. The values of the growth yields are equal to the one obtained with *Alcaligenes eutrophus* JMP134 growing on 2-methylphenoxyacetic acid and about twice the ones for growth of *A. eutrophus* on 2,4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid, respectively (14). DOC levels measured at the beginning of a growth experiment and after depletion of the substrates corresponded to a complete conversion of mecoprop into biomass and presumably carbon dioxide. The decreased growth rate of 0.016 h^{-1} with racemic mecoprop as the growth substrate indicates that in this case the growth rate was affected by the concentration, which for each enantiomer was only half the value of what it was in the experiments with the pure enantiomers. Additional growth experiments showed that increasing the concentration of the racemic mixture caused an increase of the growth rate (data not shown). Apart from (*R*)-, (*S*)- and (*RS*)-mecoprop, strain MH was able to grow on (*R*)- and (*RS*)-dichlorprop [(*R*)- and (*RS*)-(2,4-dichlorophenoxy)propionic acid], 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid, and pyruvate.

Evidence for enantioselectivity in oxygen uptake and substrate consumption experiments with washed cell suspensions. The oxygen uptake by washed cells of strain MH grown on (*R*)-mecoprop was much higher for the (*R*) enantiomer than for the (*S*) enantiomer (Table 1). Accordingly, cells grown with (*S*)-mecoprop as the sole carbon and energy source were adapted to utilize the (*S*) enantiomer at much higher rates. In both cases, the rate of oxygen consumption due to the addition of the racemic mixture of mecoprop was in the same range as the one due to the addition of the pure enantiomer that was used as the growth substrate. Cells were also grown with the racemic mixture as the carbon and energy source. In this case, oxygen consumption due to the addition of the racemic mixture was higher than the one due to the addition of the single

TABLE 1. Oxygen uptake rates with (*R*)- and (*S*)-mecoprop and the racemic mixture by resting cells of *S. herbicidovorans* MH grown on these compounds[a]

Assay substrate	Absolute oxygen uptake rates (μmol of O_2/g [dry wt] per min) by cells grown on[b]:		
	(*R*)-Mecoprop	(*S*)-Mecoprop	(*RS*)-Mecoprop
(*R*)-Mecoprop	59.1 ± 3.3	23.5 ± 3.0	39.7 ± 2.0
(*S*)-Mecoprop	26.9 ± 1.9	89.9 ± 0.8	47.4 ± 3.3
(*RS*)-Mecoprop	65.3 ± 1.4	76.2 ± 12.4	56.9 ± 2.6

[a] All rates for cells grown on complex medium were below detection limit (<5 μmol of O_2 per g [dry weight] per min). Other substrates tested for oxygen uptake are mentioned in the text.
[b] Values are means of triplicates ± standard deviations.

pure enantiomers. A reasonable explanation for these findings is the cumulative effect of two enzyme systems acting each on one of the enantiomers. Cells grown on complex carbon substrates were not adapted to the utilization of (*R*)- or (*S*)-mecoprop (Table 1). The same was true for cells grown on pyruvate. When (*R*)- and (*RS*)-dichlorprop were used as the assay substrates, the oxygen uptake pattern was nearly identical to the one obtained with (*R*)- and (*RS*)-mecoprop. Strain MH was also grown with (*R*)- and (*RS*)-dichlorprop as the sole carbon and energy source. The oxygen uptake pattern with such cells and all the test substrates was nearly identical to the one with (*R*)- and (*RS*)-mecoprop as the growth substrates. Experiments with the pure (*S*)-dichlorprop could not be carried out because this enantiomer was not at our disposal. The specific oxygen uptake rates by resting cell suspensions support the existence of enantioselective degradation.

The results presented in Table 2 clearly show that cells of *S. herbicidovorans* MH grown on (*S*)-mecoprop were not able to transform the (*R*) enantiomer. Washed-cell suspensions of such cells effected disappearance of (*S*)-mecoprop and concomitant formation of chloride upon incubation with the pure (*S*) enantiomer as well as with the racemic mixture. The results of experiments with washed cells of strain MH grown on (*R*)-mecoprop (Table 2) were almost congruent to the results with cells grown on (*S*)-mecoprop—the cells preferentially transformed the enantiomer that was used as the growth substrate. Again, these data suggest the involvement of two independent enzymes for, at least, the initial transformation of each enantiomer. However, a slight uptake of (*S*)-mecoprop with concomitant chloride release by cells grown on the (*R*) enantiomer was evident (Table 2). This finding indicates that the enzymes responsible for the degradation of (*S*)-mecoprop may be constitutively expressed at low levels or that the (*R*)-mecoprop-degrading enzymes are able to slowly turn over (*S*)-mecoprop. A pathway based on racemization of (*R*)- to (*S*)-mecoprop and further degradation of the latter one would have been a third explanation, but in such a case much higher consumption rates for (*S*)-mecoprop by (*R*)-mecoprop-grown cells would have been expected. Yet, the possibility remains that the absence of a transport molecule for (*S*)-mecoprop in such cells prevented degradation. It seems unlikely, though, that the enzymes responsible for breakdown of (*S*)-mecoprop were induced without the concomitant induction of a potential transport molecule. Although not completely excluded, a degradation pathway based on racemization is not favored by the data from the substrate consumption experiments.

TABLE 2. Rates of consumption of (*R*)- and (S)-mecoprop and rates of formation of chloride by resting cells of *S. herbicidovorans* MH grown on (S)- and (*R*)-mecoprop[a]

Compound assayed	Rate of consumption or formation (μmol/g [dry wt]/min) with cells grown on[a]:	
	(*S*)-Mecoprop	(*R*)-Mecoprop
Incubations with (*S*)-mecoprop		
(*S*)-Mecoprop	−8.1 ± 0.3	−2.2 ± 0.9
Chloride	7.6 ± 0.8	1.9 ± 0.1
Incubations with (*R*)-mecoprop		
(*R*)-Mecoprop	0	−14.7 ± 1.6
Chloride	0	13.6 ± 0.3

[a] Values are parameter estimates ± standard deviations from linear regression analysis.

Conclusions. The data presented clearly demonstrate the complete microbial degradation of both enantiomers of the herbicide mecoprop in an enantioselective manner in *S. herbicidovorans* MH. This study proves the importance of employing enantiomer-specific analytical tools and the necessity of treating enantiomers as distinct molecules when studying the degradation of chiral compounds. The apparent neglect of stereochemistry has hampered progress in the elucidation of the mecoprop degradation pathway. However, we believe that progress can be made and further work on the biochemistry of mecoprop degradation is being carried out in our laboratory.

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