Original Article

The Mode of Metabolism of the Herbicide Molinate by Four Strains of Microorganisms Isolated from Soil*

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The relationships between growth conditions and degradability of 4 isolated strains of the herbicide molinate (S-ethyl perhydroazepin-1-carbothioate)-degrading microorganisms, *Mycobacterium* sp. (B-1), *Flavobacterium* sp. (B-2), *Streptomyces* sp. (A-1), and *Fusarium* sp. (F-1), and the mechanisms of molinate degradation by them were investigated. None of these microorganisms could utilize molinate as a sole source of carbon and energy for growth and they needed other carbon sources. The three strains of microbes, B-2, A-1 and F-1, also needed other nutrient sources for the degradation of molinate while B-1 did not. The mode of molinate metabolism by these strains was found to be co-metabolism. Excess concentrations of the nutrient sources repressed the degradation by B-1 and B-2, but promoted by A-1. Adaptive enzymes seemed to be responsible for the first step of the molinate metabolism by A-1, whereas both constitutive and adaptive enzymes by B-1 and B-2. These four strains also responded differently to pH and temperature conditions.

INTRODUCTION

The herbicide molinate (S-ethyl perhydroazepin-1-carbothioate) is widely used for the control of barnyard grass in paddy rice fields. Soderquist *et al.*¹⁾ reported that molinate applied in the paddy field seemed to dissipate primarily through volatilization, and secondarily through microbial degradation. Thomas *et al.*²⁾ and we³⁾ reported on its degradation in soils. We revealed that the herbicide was degraded rapidly by microorganisms under aerobic conditions in soils. Golovleva *et al.*⁴⁾ and we⁵⁾ reported that the degradation of molinate was caused mainly by co-metabolizing soil microorganisms. Golovleva *et al.*⁴⁾ and Skryabin *et al.*⁶⁾ isolated several strains of

molinate-degrading bacteria from pond water and mud, and studied in detail their co-substrate⁴⁾ and degradation pathways.^{4,6)} We⁵⁾ elucidated that molinate was degraded quite rapidly in Anjo soil under aerobic condition, where approximately one-third of the microbial population was engaged in the degradation. Four strains of degradation microbes, *Mycobacterium* sp. (B-1), *Flavobacterium* sp. (B-2), *Streptomyces* sp. (A-1) and *Fusarium* sp. (F-1) were isolated.

In the present study, in order to further understand the molinate degradation in field soil, we investigated the relationships between culture conditions and kinetic patterns in the pure culture of the microorganisms.

MATERIALS AND METHODS

1. Chemicals

Purified molinate was prepared as described in our previous paper.³⁾

* Microbial Degradation of the Herbicide Molinate (Part 2).

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2. Culture Conditions

The isolated strains of *Mycobacterium* sp. (B-1), *Flavobacterium* sp. (B-2), and *Streptomyces* sp. (A-1) were incubated in a molinate-inorganic salt medium (K_2HPO_4 0.5 g, $NaNO_3$ 0.5 g, $MgSO_4$ 0.2 g, $FeSO_4$ trace, molinate 40 mg in 1 l of water, pH 7.0), nutrient broth (meat extract 10 g, peptone 10 g, $NaCl$ 0.5 g in 1 l of water, pH 7.2), or its 1/10 diluted nutrient broth. Unless specified, the 1/10 diluted nutrient broth was used. The strain of *Fusarium* sp. (F-1) was incubated in the Czapek-Dox medium. The media were supplemented with molinate at 40 ppm if not otherwise indicated. Several loopfuls of the cells of B-1, B-2, or A-1 were scraped from the stock slant and suspended in a small volume of phosphate buffer (67 mM, pH 7.0). An aliquot of this suspension was inoculated into 10 ml of liquid medium in a test tube (i.d. 18 mm) and incubated on a reciprocal shaker. In the washed cell experiment, the cells were preincubated in 100 ml of the medium with or without molinate in a Sakaguchi flask on a rotary shaker and harvested at a particular growth phase by centrifugation ($2000\times g$, 15 min). The sediment was washed 3 times with the phosphate buffer, finally suspended in the same buffer at a particular optical density and inoculated into the medium for the determination of molinate degradation. The weight of the cells inoculated were 7.2, 5.1 and 5.9 mg (dry cell basis) in a test tube containing 10 ml of the culture solution of B-1, B-2 and A-1, respectively.

In the case of F-1, small pieces of its hyphae were inoculated into 100 ml of the Czapek-Dox medium supplemented with molinate (50 ppm) in a Sakaguchi flask and incubated on a rotary shaker at 30°C for 7 days. After the incubation, the medium containing flocks of hyphae was homogenized with a Waring blender for 10 sec. The cells were collected by centrifugation ($2000\times g$, 15 min) and suspended in the phosphate buffer. Subsequently, an aliquot of the suspension was inoculated into the medium for determining the molinate degradation. Nine milligrams of hyphae (dry weight basis) was inoculated to 10 ml of culture solution, unless otherwise described.

3. Analysis of Molinate

Residual molinate in the culture was determined with gas chromatography (NP-FID) as reported previously.³⁾

4. Determination of the Microbial Growth

The growth of B-1, B-2 and A-1 was determined by measuring the optical density of the culture at 660 nm (OD_{660}). When the culture of A-1 turned dark presumably by melanoid formation, the culture samples were centrifuged ($14,000\times g$, 15 min), the cells were suspended into the fresh medium, and OD_{660} was measured for determining the population. In some cases, the cells were collected by centrifugation, dried at 80°C for 24 hr and their dry weight was measured.

RESULTS

1. Effects of Nutrient Sources on the Growth and the Degrading Activity of B-1, B-2, and A-1

In the inorganic salt medium supplemented with molinate as a sole source of carbon, none of the 3 strains grew, and neither B-2 nor A-1 degraded molinate but B-1 did (Fig. 1). The growth in this medium was not observed until the 30th day of the incubation. The result was the same in the culture of the microorganisms in another inorganic salt medium containing NH_4^+ replacing NO_3^- as a nitrogen source. Microbes of every strain grew and degraded molinate with ease in the 1/10 diluted nutrient broth. In the non-diluted nutrient broth, the microbes grew vigorously. The degradation rate, however, was low in B-1 and B-2 but high in A-1 when compared to the culture in the 1/10 diluted nutrient broth (Fig. 1).

2. Effects of Temperature on the Growth and Degradability of B-1, B-2 and A-1

Both microbial growth and molinate degradation of B-1 and B-2 proceeded smoothly at 20 and 30°C but were suppressed at 37°C (Fig. 2). In contrast, at 37°C A-1's growth was somewhat retarded but its molinate degradation went well, while at 20°C the degradation was delayed.

Fig. 1 Effect of nutrient on molinate degradation and microbial growth.

Initial concentration of molinate: 40 ppm, the incubation at 30°C. (1): *Mycobacterium* sp. B-1, OD after 1/4 dilution. (2): *Flavobacterium* sp. B-2, (3): *Streptomyces* sp. A-1, OD after 1/10 dilution. Molinate remaining (%) (—), growth of microbes (-----), in an inorganic salt medium (■, □), in 1/10 diluted nutrient broth (●, ○), and in non-diluted nutrient broth (▲, △).

Fig. 2 Effect of temperature on molinate degradation and microbial growth.

Initial concentration of molinate: 40 ppm, the incubation with 1/10 diluted nutrient broth. (1): *Mycobacterium* sp. B-1, (2): *Flavobacterium* sp. B-2, (3): *Streptomyces* sp. A-1. Molinate remaining (%) (—), growth of microbes (-----), at 20°C (■, □), at 30°C (●, ○), and at 37°C (▲, △).

3. Effects of pH on the Growth and Degradability of B-1, B-2 and A-1

The strains were incubated in the 1/10 diluted nutrient broth adjusted to different values of pH with HCl and NaOH. During the incubation of B-1, the pH value decreased by 1–1.5 units, while the pH value increased by 0–1 and 0–3 by the incubation by B-2 and A-1, respectively. The relationships among the initial pH, the microbial growth and the degrading activity are shown in Fig. 3. Al-

though the microorganisms of these 3 strains grew and degraded molinate over a wide range of pH, the optimum pH for the microbial growth was 7.9, 5.1, and 7.5 for B-1, B-2, and A-1, respectively.

4. The Washed Cell Experiment with B-1, B-2, and A-1

The molinate degradation by washed cells of B-1, B-2 and A-1 preincubated with or without molinate was investigated in the phosphate

Fig. 3 Effect of pH on molinate degradation and microbial growth.

Residual molinate and growth: after a 6-day, 2-day, 2-day, and 7-day incubation at 30°C for (1), (2), (3), and (4), respectively. (1): *Mycobacterium* sp. B-1, OD after 1/3 dilution, (2): *Flavobacterium* sp. B-2, (3): *Streptomyces* sp. A-1, (4): *Fusarium* sp. F-1, weight of inoculated hyphae = 1.0. Molinate remaining (%) (●), and growth of microbes (○).

Fig. 4 Molinate degradation by washed cells.

The amount of cells inoculated in a test tube: 7.2 mg, 5.1 mg, and 5.9 mg for (1), (2), and (3), respectively. Initial concentration of molinate: 40 ppm, the incubation at 30°C. (1): *Mycobacterium* sp. B-1, (2): *Flavobacterium* sp. B-2, (3): *Streptomyces* sp. A-1. Preincubation with molinate, in the 1/10 diluted nutrient broth (●) and in phosphate buffer (▲). Preincubation without molinate, in the 1/10 diluted nutrient broth (○), and in phosphate buffer (△).

buffer at pH 7.0 and in the 1/10 diluted nutrient broth (Fig. 4). Preincubated with molinate, the resting cells of B-1 in the phosphate buffer degraded molinate as rapidly as the vital cells in the 1/10 diluted nutrient broth. In the cases of B-2 and A-1, the cells degraded molinate slowly in the phosphate buffer compared with in the 1/10 diluted nutrient broth, suggesting that they seemed to need additional nutrient or energy sources for the rapid degra-

dation (black circles and black triangles in Fig. 4). When preincubated without molinate, the cells of A-1 did not degrade molinate in the phosphate buffer, while those of B-1 and B-2 did, though not rapidly (white triangles in Fig. 4). The cells of every strain preincubated without molinate degraded molinate rapidly after a lag time in the 1/10 diluted nutrient broth.

5. Experiments with *Fusarium* sp. (F-1)

F-1 showed no evidence of growth in the inorganic salt agar medium containing molinate, and the hyphae grown in the Czapek-Dox medium with molinate hardly degraded the herbicide chemical in the phosphate buffer (Fig. 5). However, it grew and degraded the chemical in the Czapek-Dox medium with pH values of 3 to 10 (Fig. 3). The fungi of F-1 grew and degraded molinate vigorously at 30°C, continued to degrade without growth at

20°C, and grew but degraded only slowly at 37°C (Fig. 6).

DISCUSSION

A decrease of the concentration of molinate in a medium was mainly due to its degradation, which was confirmed by other experiments using ^{14}C -molinate.⁷⁾ The decrease was caused neither by the adsorption of molinate to microbial cells nor by its uptake by cells, but by its transformation in the microbes. During the 7-day incubation at 30°C, about 10% of the initial molinate was volatilized from the medium, so the residual molinate was expressed as a ratio to molinate in the blank medium.

Horvath⁸⁾ used the term "co-metabolism" to describe a metabolic process in which a microorganism oxidizes a substance without utilizing the energy derived from the oxidation to support the growth. Every strain in this study showed no evidence of growth in the inorganic salt medium with molinate as a sole carbon source (Fig. 1), so these microbes must degrade molinate through co-metabolism. The mode of co-metabolism of chemicals has been studied from various viewpoints.⁸⁻¹¹⁾ Skryabin *et al.*¹²⁾ pointed out the possible necessity of other energy sources for substrate transport, enzyme synthesis, or enzyme functioning in a microorganism which needs co-substrate for the degradation of another substrate.

Since *Mycobacterium* sp. (B-1) degraded molinate rapidly in the phosphate buffer (Fig. 4), the chemical may be degraded by extracellular enzymes or by cellular enzymes after rapid penetration through the cell membrane of the bacteria with no energy sources needed for the enzyme functioning. On the other hand, to other 3 strains, *Flavobacterium* sp. (B-2), *Streptomyces* sp. (A-1) and *Fusarium* sp. (F-1), other energy sources may be essential presumably for substrate transport or for proper functioning of the degrading enzymes as well as for synthesis of the enzymes (Figs. 4 and 5).

Among these strains, B-1 degraded molinate under the broadest range of conditions. If competitive, this strain may contribute greatly to the degradation of molinate in heterogeneous soils. The other strains, however, may be able to degrade molinate rather rapidly in the rhizosphere, where amino acids, sugars,

Fig. 5 Effect of nutrient on molinate degradation and fungal growth of *Fusarium* sp. F-1.

Initial concentration of molinate: 40 ppm, the incubation at 30°C. Molinate remaining (%) (—), fungal growth (----), in Czapek-Dox medium (●, ○), and in phosphate buffer (▲, △).

Fig. 6 Effect of temperature on molinate degradation and fungal growth of *Fusarium* sp. F-1.

Initial concentration of molinate: 40 ppm, the incubation with Czapek-Dox medium. Molinate remaining (%) (—), fungal growth (----), at 20°C (■, □), at 30°C (●, ○), and at 37°C (▲, △).

and other nutrients exist at high concentrations to support the favorable conditions for their activity.¹³⁾ Golovleva *et al.*⁴⁾ reported that the effect of co-substrates on the microbial activities depends on the kinds of microbes. Therefore, the exact contribution of these strains to the molinate degradation in soil may be a subject yet to be elucidated.

It is well known that the microbial degradation of various xenobiotics is either suppressed or promoted by concomitant nutrient sources depending on their kinds or concentrations.¹⁴⁻¹⁷⁾ This study also showed that excess nutrients to the bacterial strains, B-1 and B-2, suppressed the degradation of molinate, while ones to the actinomyces strain, A-1, promoted the degradation. In the washed cell experiment of A-1 using the 1/10 diluted nutrient broth (black circles and white circles in Fig. 4), where the greater amount of cells was inoculated in each test tube than in any test tubes during the incubation with non-diluted nutrient broth (triangles in Fig. 1), molinate was degraded at a smaller rate and to a lower extent than in the non-diluted nutrient broth (Fig. 1). Thus the molinate degradation of A-1 with excess nutrient was not only enhanced by an increase in the population, but also by an increase in the activity in each individual cell.

It may be presumed that in the former types of microbes such as B-1 and B-2, the enzymes responsible for the catabolism to get energy may transform molinate, since the degradation of molinate is suppressed with excess amounts

of preferable nutrients. In the latter types of microbes such as A-1, the degrading enzymes may transform xenobiotics or other compounds without involving any energy producing systems. The activities of these latter microbes are promoted by excess nutrients through enhancement of the general microbial activities.

It has been established that a pesticide is degraded by constitutive enzymes in a decomposing microbe in some cases and by adaptive enzymes induced by a substrate in other cases.^{9,18)} In the case of A-1, its cells grown without molinate showed no evidence of degradation in the phosphate buffer where no further proteins could be synthesized, while the cells degraded molinate in the 1/10 diluted nutrient broth after a lag time, during which degrading enzymes were probably induced (Fig. 4). It can be concluded that in this strain, adaptive enzymes are primarily responsible for the degradation of molinate. In the case of B-1 or B-2, molinate was partially transformed even when adaptive enzymes hardly existed, that is, in washed cells grown without molinate in the phosphate buffer (Fig. 4). Therefore, it is presumed that in these strains, the rapid transformation of molinate is caused mainly by adaptive enzymes, although a part of molinate might be degraded by constitutive enzymes.

The metabolism experiment using ¹⁴C-molinate⁷⁾ indicated that there were several simultaneous metabolic pathways of molinate in each of these microbes, and how each of these metabolic pathways is controlled is still left to

Table 1 Comparison of mode of molinate degradation among 4 strains of molinate-degrading microorganisms.

Microorganism	General mode of molinate metabolism	Demand of other nutrients for molinate degradation	Enzymes ^{a)} for the 1st step of molinate degradation	Effect of excess nutrients on molinate degradability	Relation with the energy yielding system ^{b)}
<i>Mycobacterium</i> sp. B-1	Co-metabolism	No demand	A, C	Suppressive	With relation
<i>Flavobacterium</i> sp. B-2	Co-metabolism	Demand	A, C	Suppressive	With relation
<i>Streptomyces</i> sp. A-1	Co-metabolism	Demand	A	Promotive	No relation
<i>Fusarium</i> sp. F-1	Co-metabolism	Demand	—	—	—

^{a)} A: Adaptive enzymes, C: Constitutive enzymes.

^{b)} Possible relation of the molinate degradation system to the energy yielding system.

be solved.

Table 1 summarizes the mode of molinate degradation (transformation) in these microbes. We have shown that 4 strains isolated from a soil degrade molinate in various modes of metabolism differently responding to certain factors in the pure culture experiment. A question as to how these microbes participate in molinate degradation in soil under actual conditions, however, still remains to be solved in the future.

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要 約

土壌から分離した 4 種の除草剤モリネート分解菌によるモリネート代謝機構*

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土壌から分離した 4 種のモリネート分解菌, *Mycobacterium* sp. (B-1), *Flavobacterium* sp. (B-2), *Streptomyces* sp. (A-1) および *Fusarium* sp. (F-1) の生育条件とモリネート分解活性の関係および分解機構について研究した。これらの菌はいずれもモリネートを炭素源として利用できず、生育に他の炭素源を必要とした。B-1 以外の 3 種の菌ではモリネートの分解にも他の栄養源を必要としたが、B-1 では必要としなかった。過剰な栄養は B-1 および B-2 でモリネートの分解を抑制したが、A-1 では促進した。モリネートの代謝の第一段階の反応は A-1 では適応酵素、B-1 および B-2 では適応酵素と構成性酵素の両方によると推定した。これら 4 種の菌は温度と pH に異なる反応を示した。

* 除草剤モリネートの微生物分解 (第 2 報)