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Microbial Decomposition of 2,4-Dichlorophenoxyacetic Acid

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The inactivation of 2,4-dichlorophenoxyacetic acid (2,4-D) and other hormone-type herbicides in soil has been shown to result largely from microbial action. Brown and Mitchell (1948) and Hernandez and Warren (1950) showed that 2,4-D was not inactivated in autoclaved soils. DeRose and Newman (1948) found that 2,4-D, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 2-methyl-4-chlorophenoxyacetic acid (MCPA) were inactivated in 3 to 6 weeks in nonautoclaved soils, and that these compounds persisted more than 9 to 15 weeks in autoclaved soils. Many studies on the effect of soil moisture and soil temperature also have indicated that microorganisms are the primary cause for the inactivation of 2,4-D. Under some conditions, leaching or absorption by soil constituents, particularly by organic matter, are important factors in the inactivation of 2,4-D and other growth regulators.

Audus (1949), Newman, *et al.* (1952), and Rogoff and Reid (1952), found 2,4-D to be decomposed more rapidly in soils in which it had decomposed previously. Audus (1950, 1951, 1952) isolated an organism capable of decomposing 2,4-D and MCPA which was identified as being a member of the *Bacterium globiforme* group. Jensen and Petersen (1952a, 1952b) isolated two organisms which were capable of decomposing 2,4-D. One of these was identified as *Flavobacterium aquatile*, which was capable of rapidly decomposing 2,4-D, but did not attack MCPA. The other organism was not identified. It resembled *Corynebacterium* but was gram negative, and was capable of slowly decomposing 2,4-D and MCPA. Rogoff and Reid (1954) also isolated an organism tentatively identified as a *Corynebacterium* species. The present paper describes a bacterium capable of decomposing 2,4-D in soil and in liquid substrates. This organism is culturally distinct from the bacteria described by the authors cited.

METHODS AND RESULTS

Isolation. Isolation of the bacteria employed in these experiments was accomplished by an enrichment technique. A 1:1000 dilution of a sample of Duffield silt loam, pH 6.2, in which 2 applications of 2,4-D had decomposed was plated out. The medium (no. 1) used for plating had the following composition per L: 2,4-D, 1 g; $NH_4H_2PO_4$, 1 g; KCl, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; and agar, 15 g. The pH of the medium was 7.1. The sodium salt of 2,4-D was used in all experiments, but amounts were based on the acid equivalent.

After an incubation period of 17 days at 25 C, small, white bacterial colonies were evident. By streaking on plates of the same medium, 7 bacterial cultures were separated from fungal contaminants. Of these, 4 cultures appeared to be identical except for minor strain variations. These 4 cultures (A, C, D, and F) were able to decompose 2,4-D and have been maintained on artificial substrates for more than 50 months without

loss in vigor. Culture B, which was similar to A, C, D, and F both culturally and morphologically, was not able to decompose 2,4-D. The remaining cultures, E and G, initially grew more rapidly than the other 5 cultures on a medium which contained glucose as well as 1000 ppm 2,4-D. These 2 cultures ceased growing on subsequent transfers.

Morphology and cultural characteristics. The 4 strains of bacteria capable of decomposing 2,4-D are gram negative nonsporeforming rods, averaging 2.1 to 4.6 μ in length by 0.5 to 1.0 μ in width. The cells are frequently banded when stained with carbol fuchsin. Branched forms and gram positive granules have been noted occasionally, particularly in liquid media containing only 2,4-D and inorganic salts. The cells are motile, having from 1 to 6 polar flagella.

This organism fails to grow, or produces extremely scanty growth on the ordinary laboratory media. Best growth is obtained on an agar medium (no. 2) of the following composition per L: 2,4-D, 0.2 to 1 g; yeast extract, 0.5 g; KNO_3, 1 g; K_2HPO_4, 1 g; NaCl, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; and agar, 15 g with the pH adjusted to 7.1. On slants of this medium, moderate growth is obtained in 3 days. The growth is dirty white, becoming cream-colored in older cultures, with no color change of the medium. The growth is flat, glistening, somewhat mucoid, echinulate with little tendency to grow out from the line of inoculation, and produces no odor. This organism, using agar or its impurities as its energy source, produces a very scant growth on medium 1 without 2,4-D. It is inhibited or grows poorly in most instances on phenol, various chloro- and hydroxyphenols, and phenoxyacetic acid (table 1). In general the growth is scant or moderate on the 2- and 4-chlorophenoxyacetic acids. The organism is able to grow on

TABLE 1. *Growth of 2,4-D decomposers on various energy sources* following incubation of 9 or 10 days*

Energy Source	PPM of Energy Source			
	50	100	200	1000
Phenol	+	+	+	−
2,4-Dichlorophenol	−	−	−	−
3-Chlorophenol	+	−	−	−
4-Chlorophenol	+++	−	−	−
2-Chlorophenol	+	+	−	−
Phenoxyacetic acid	+	−	−	−
2-Chlorophenoxyacetic acid	+	++	+	++
4-Chlorophenoxyacetic acid	+++	+++	+++	++
Resorcinol				−
Catechol				−
2,4-D	+++	+++	+++	+++
Basal substrate control	+	+	+	+

− = extremely scant to no growth, + = very scant growth, ++ = scant growth, +++ = moderate growth.

* Values are means of 3 replicates of 3 or 4 cultures on medium 1, substituting the indicated compounds for 2,4-D.

MCPA, although the growth is not as good as on 2,4-D. The growth on 2,4,5-T is no greater than on agar alone.

Concentrations in the growth factor range of yeast extract, beef extract, sodium pyruvate, paraamino benzoic acid, and soil extract stimulate growth somewhat. Higher concentrations of yeast extract and beef extract are inhibitory. Growth is restricted by a 2,4-D concentration of 5000 ppm and becomes very scanty at concentrations of 10,000 and 25,000 ppm. Addition of a vitamin mixture, 18 amino acids, or various inorganic ions to medium 1 as a liquid, did not enhance growth.

The optimum temperature for growth is 28 to 30 C. Liquid cultures containing 2,4-D exhibit a slight turbidity with a scanty, viscid sediment but no surface growth. There is very scant or no growth on nutrient agar or in nutrient broth. Gelatin was not liquified in 1 month. Growth on potato is very scanty, echinulate, glistening, and buff-colored. No acid or gas is produced from carbohydrates, and starch is not hydrolyzed. Nitrite is produced from nitrate but no gas is formed. An alkaline reaction is produced in milk. Indole and H_2S are not produced.

Taxonomy. On a morphological basis this organism belongs in the genus *Mycoplana* as described in *Bergey's Manual* (Breed *et al.*, 1948). It differs from the described species, which have been examined, in several important features, including ability to decompose 2,4-D, lack of growth on standard cultural media, and ability to reduce nitrates.

This organism is morphologically and culturally distinct from the 2,4-D decomposing bacteria isolated by Audus (1951) and Jensen and Petersen (1952b). The bacterium isolated by Audus was a nonmotile, gram negative, short rod, which grew in liquid media only in the presence of agar or yeast extract, and grew on sugars but with loss of ability to decompose 2,4-D. One of the organisms isolated by Jensen and Petersen was *Flavobacterium aquatile*, which grew readily on the standard laboratory media; the other was a small, nonmotile, nonsporeforming irregular rod suggestive of *Corynebacterium*, predominantly gram negative but sometimes with gram positive granules. Thus the organisms of Audus and Jensen and Petersen differ from those of the present paper in major morphological and cultural characteristics.

Decomposition of 2,4-D in soil. The ability of the organisms isolated in this experiment to decompose 2,4-D in soil and on various other solid substrates has been determined in a number of experiments. Determination of the extent of 2,4-D decomposition was assayed by measuring the residual compound by the cucumber root elongation test of Ready and Grant (1947). At each sampling date an aliquot of the perfusate, or of soil in nonperfusion experiments, was diluted to give a theoretical concentration of 0.05 or 0.5 ppm

of 2,4-D, assuming that no decomposition had taken place.

In a preliminary experiment, samples of Duffield silt loam in flasks were treated with 1 mg 2,4-D per 100 g soil, autoclaved, and then inoculated with cultures A, C, and D. After 1 month's incubation at 28 C and 25 per cent moisture in the soil, the residual 2,4-D was determined. Decomposition was complete with all 3 cultures, and no decomposition had occurred in uninoculated controls.

Additional studies were conducted employing the perfusion apparatus of Lees (1947), except that the apparatus was operated with air pressure instead of suction. The experiments were run at room temperature, generally between 22 and 27 C, using 100 ml of perfusate and 20 g of the 1- to 5-mm aggregates from an 0- to 3-inch sample of Duffield silt loam, which had been in sod for several years. The supply of soil was stored in the moist state at 5 C.

In a preliminary perfusion experiment, nonsterile soil was inoculated with pure cultures and perfused with a solution containing 5 ppm 2,4-D. Decomposition of 2,4-D was complete by the third day of perfusion with cultures A, C, D, and F, but no decomposition was evident with culture B.

Additional experiments were conducted in which 100 ppm 2,4-D solutions were perfused. This amount of 2,4-D is equivalent to 2000 lb per acre with respect to the soil. Two lb per acre or less is commonly applied for weed control. The results from 2 such experiments are presented in table 2. Organisms A, C, D, and F speeded up the 2,4-D decomposition process in nonsterile soil. Inoculation of sterile soil with cultures A, C, and F resulted in the complete decomposition of 2,4-D. Inoculation of nonsterile soil with culture B had no effect on the length of persistence of 2,4-D. This organism probably does not decompose 2,4-D, although it is capable of growing on the synthetic 2,4-D medium.

TABLE 2. *Decomposition of 2,4-D in inoculated soil in perfusion units**

Culture	Time Required for Decomposition	
	Nonsterile soil	Sterile soil
	days	*days*
A	3	3
B	8	—
C	5	2
D	2	—
F	4	2
Normal population	8	9
Soil suspension	5	—

* In nonsterile soil, the values are means of single determinations from 2 separate experiments, except for D and soil suspension, which are values for a single determination. The soil suspension was of a soil in which 2,4-D had decomposed. The values for sterile soil are means of triplicates. The soil was sterilized with ethylene oxide.

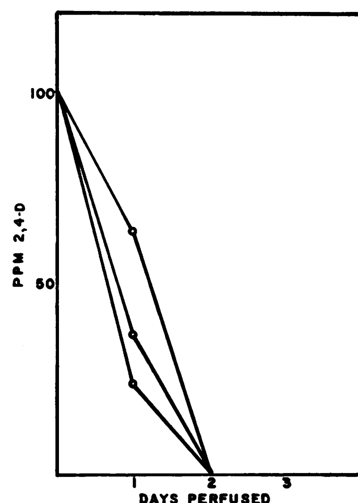

FIG. 1. Decomposition of 2,4-D in soil inoculated with a heavy suspension of culture F in perfusion units.

The initially sterile soils were retreated with a 1000-ppm solution of 2,4-D. The 1000-ppm solutions were decomposed completely in 10 to 14 days, including a lag period of 4 to 5 days during which there was no detectable change in the concentration.

The rate of decomposition of 2,4-D in autoclaved soils inoculated with a heavy suspension of culture F is shown in figure 1. The perfusate contained 100 ppm of 2,4-D. From 35 to 75 per cent of the compound was decomposed during the first day of incubation, with the remainder being decomposed during the second day. This is in contrast with the results of other perfusion experiments with the normal soil population in which there was a 3- to 8-day lag period prior to onset of decomposition.

Further perfusion experiments were conducted employing Ottawa sand, fine gravel, glass beads, and glass wool in place of soil, and using liquid medium 1 with 100 ppm 2,4-D as the perfusate. The pure cultures were capable of decomposing 2,4-D under these conditions, but generally the variation among replicates and the time required for complete decomposition were greater than with soil.

Decomposition of 2,4-D in liquid substrates. A series of investigations was made to ascertain the ability of this organism to decompose 2,4-D in liquid substrates. In these studies, the concentration of residual 2,4-D was determined by the spectrophotometric method of Bandurski (1947), after first removing suspended cells by centrifugation at 10,000 rpm. This method is more accurate at high concentration of 2,4-D than the cucumber bioassay procedure, but could not be employed in the perfusion experiments because of the presence of interfering substances in soil.

A comparison was made of the ability of culture F to decompose 2,4-D in medium 2, with and without yeast-extract. Culture tubes containing 10 ml substrate

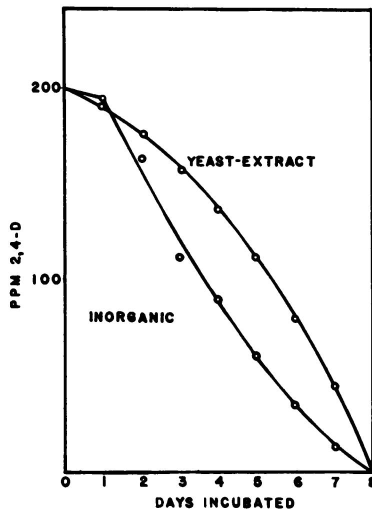

FIG. 2. Effect of yeast extract on decomposition of 2,4-D by culture F in liquid media measured spectrophotometrically.

were inoculated with a loopful of growth from slants of medium 2. At daily intervals, 2 replicate tubes were pooled for assay. During the first day of incubation, the decomposition of the 200-ppm solution of 2,4-D was slow as would be expected (figure 2). The maximum rate of decomposition was attained on the third day for completely synthetic substrate and on the eighth day for the substrate containing yeast extract, but in both substrates decomposition was complete after 8 days' incubation. Maximum turbidity after 8 days' incubation was 60 per cent greater in the yeast-extract medium.

In parallel experiments it has been shown that decomposition of 2,4-D will proceed in the presence of small amounts of beef extract and peptone. In one experiment 50 per cent of a 1000-ppm solution was decomposed in 16 days. Spectrophotometric determinations made at intervals during the next 10 days showed that no further decomposition had occurred nor was there a shift in pH. The remaining cultures were incubated for 60 days, at the end of which time another spectrophotometric determination showed that decomposition was complete. The organism will grow in other liquid substrates, but studies on the decomposition of 2,4-D in these media have not been made.

Decomposition of 2,4-D by other microorganisms. The number of species of microorganisms capable of decomposing 2,4-D appears to be quite limited when one considers that Audus (1952) found only 1 species; Jensen and Petersen (1952) found 2 species in one soil and 1 species in another and Rogoff and Reid (1954) found 1. In the initial experiments in this laboratory, only 1 species was found in Duffield silt loam. It was noted, however, that in plating out dilutions from perfusion units and enriched soils a number of other colonial types were seen. These colonies represented organisms which either were able to attack 2,4-D or were tolerant to the amount present. Further isolations were made using the techniques previously described, and 10 additional cultures were obtained and tested for their ability to decompose 2,4-D. After isolation on medium 1, the organisms were grown and maintained on medium 2, but these new cultures also grew well on nutrient agar with or without 2,4-D.

The ability of the cultures to decompose 2,4-D was determined in 5-g quantities of autoclaved Duffield silt loam contained in 18- by 150-mm culture tubes. Six-day growth from 2 to 4 slants (depending upon density of growth) was suspended in a solution of 2,4-D and 2 ml of this suspension was placed in each of 10 replicate tubes of soil. This suspension contained 50 μg of 2,4-D, which is equivalent to 20 lb per acre of 2,4-D, and brought the soil to 70 per cent of the water-holding capacity. The tubes were incubated at 28 C. The residual 2,4-D was assayed at intervals, using the cucumber root elongation procedure. This experiment was conducted within 6 weeks of the isolation of the cultures. Other cultures which had been held on artificial substrates for approximately 1 year were tested at the same time.

Of the 10 new isolates, 3 were fungi (*Penicillium* sp, *Trichoderma viridis*, and *Alternaria tenuis*) which gave no indication of ability to decompose the 2,4-D in 8 weeks. The remaining 7 cultures were bacteria, and of these 4 were capable of decomposing 2,4-D in 1 week's time. Three of these cultures were gram negative, motile rods and appeared to be strains of the organism previously described in this paper. Detailed cultural studies were not made, however. The fourth culture was grossly different from those previously described here and in the literature. This organism was a gram positive, nonmotile, short rod. It produced a dull, white, flat growth on medium 2. This culture and 1 of the 3 others capable of decomposing 2,4-D were subsequently lost when they failed to grow on any medium after several transfers.

Nine bacterial cultures isolated from 9 different agricultural soil types in perfusion units were unable to decompose 2,4-D in this experiment. Shortly after isolation one of these cultures was able to decompose 2,4-D in liquid substrate and another was able to metabolize 2,4-D in a manometric experiment. The ability of the remaining 7 cultures to decompose 2,4-D had not been ascertained previously. It seems likely that some of these cultures originally had the ability to attack 2,4-D but subsequently lost it on repeated transfer. All 9 cultures produced moderate growth on medium 2 and moderate to abundant growth on nutrient agar without 2,4-D.

In the same experiment, a strain of *Cellulomonas*, 2 strains of *Bacterium globiforme* (obtained from F. E. Clark) and *Mycoplana bullata* and *Mycoplana dimorpha*

(obtained from ATCC) were unable to decompose 2,4-D.

Metabolism of 2,4-D by resting cells. Audus (1952) suggested that the first step in the breakdown of 2,4-D and related compounds, such as MCPA and 2,4,5-T, is the removal of the acetic acid side chain by hydrolysis with the subsequent oxidation of the side chain leaving the corresponding phenols. More recently, Evans and Smith (1954) have detected a phenolic acid in a mineral salt medium containing 2,4-D which they suggest, on the basis of chromatography, to be 6-hydroxy-2,4-dichlorophenoxyacetic acid. Their evidence also indicates that the chloride radicals are not eliminated from the molecule until it has lost its aromatic character. Rogoff and Reid (1954) have also shown that the chloride is released quantitatively. Respiration studies of the metabolism of 2,4-D and some related compounds by a culture of the organisms described have been made.

For the preliminary studies, culture F was incubated at 30 C for 3 days on slants of medium 2. The cells were harvested, washed several times, suspended in 0.02 M Na-K-phosphate buffer, pH 7.1, and then stored overnight at 5 C. All experiments were run in air in the customary Warburg vessels at 30 C. The various substrates were tested at a concentration of 2 μmoles. In later experiments, the cells were grown in liquid medium 2 with heavy aeration and were harvested by filtration, using the technique of Cohen (1953). In all cases the yield of cells was low. After 3 to 4 days' incubation in aerated medium 2 only about 100 mg of cells (wet weight) were obtained per L of substrate. Attempts to grow the culture on medium 2 in Roux bottles were only moderately successful, due to the tendency of the culture to grow down into the agar, making the harvesting of the cells extremely unsatisfactory. Changing the agar concentration did not improve growth characteristics.

The oxidation of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-dichlorophenol (2,4-DCP) begins without a lag period and is characterized by an initial rapid oxygen uptake (figure 3). During the oxidation 6.5 and 5.5 M of oxygen were consumed per M of 2,4-D and 2,4-DCP respectively. Respiratory quotients were determined as 1.23 for 2,4-D and 1.09 for 2,4-DCP. These values agree with the theoretical calculated values for complete oxidation of these substrates. These data indicate that the ring is rapidly and completely metabolized. Further evidence to support this conclusion has been obtained in experiments utilizing 2,4-D labeled in 3 positions with C^{14} and in other experiments in which the residual chloride was determined potentiometrically. The results of these experiments will be published at a later date.

This culture metabolized 2-methyl-4-chlorophenoxyacetic acid (MCPA) but did not appear to carry out a complete decomposition. As shown in figure 3, the rate

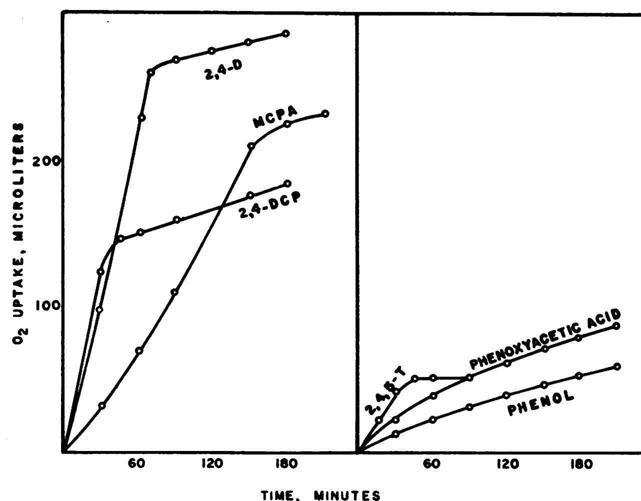

FIG. 3. Oxidation of 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-dichlorophenol (2,4-DCP), 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), phenoxyacetic acid, and phenol. Amounts of substrates employed: 2 μ moles. All curves corrected for endogenous respiration.

of oxidation of MCPA was much lower than for 2,4-D. Approximately 5 M of oxygen were consumed per M of substrate, and the respiratory quotient was 1.15 as compared to a theoretical 1.20 for complete decomposition. Additional substrates tested and found to be metabolized much the same as 2,4-D were: 2-chlorophenoxyacetic acid, 4-chlorophenoxyacetic acid, and 2-hydroxy-4-chlorophenoxyacetic acid. This culture was found to have only low activity toward 2,4,5-trichlorophenoxyacetic acid (figure 3). The oxidation was characterized by a rapid initial oxygen uptake but was only of the order of 1 M per M of substrate. Phenol and phenoxyacetic acid were metabolized slowly and the oxidation was not characterized by an initial rapid oxygen uptake. It seems unlikely that these compounds are involved as intermediates in the decomposition of 2,4-D.

Discussion

The results presented are in general agreement with those reported by Audus (1949–1951), Jensen and Petersen (1952a, b), and Rogoff and Reid (1952, 1954) and show that 2,4-D is decomposed by soil microorganisms. The organisms responsible for the decomposition of 2,4-D in Duffield silt loam are the result of either adaptation or mutation. If the ability of the organisms to decompose 2,4-D resulted from an adaptation phenomenon, the lag period would be the same for each addition of 2,4-D, since the adapted cells would revert to normal activities following the removal of the 2,4-D. In the case of a mutation, a residual population would remain after the decomposition of 2,4-D which would provide the nucleus of an active population capable of more rapid breakdown of subsequent appli-

cations. Results from field and laboratory data previously reported (Newman *et al.*, 1952), showed that 2,4-D disappeared from soil more rapidly with the second application.

Although the organisms capable of decomposing 2,4-D, as described in this paper and by several other investigators, represent a restricted group of microorganisms, results at this laboratory indicate that a more varied flora may have this ability. The isolation technique employed would preclude obtaining organisms sensitive to high concentrations of the herbicide, yet capable of decomposing it at lower concentrations. Several 2,4-D-decomposing organisms have been isolated which failed to grow after a few transfers. This indicates that the media used for isolation and maintenance of these cultures are lacking in some factor(s) carried over from the initial isolation. This material then falls below the required level upon subsequent transfer and results in loss of viability. The cultures which are maintained must therefore be less fastidious in their growth requirements as the result of a selection process. The apparent inability of the described cultures to grow well without 2,4-D points strongly toward a mutational origin.

It should be pointed out that the amounts of herbicide applied in these experiments are fantastically high as compared to the amounts normally used in weed control work. In the perfusion studies, the amount applied was equivalent to a treatment rate of 2000 lb per acre as compared to the 2 lb or less normally used for weed control. The conditions prevailing in perfusion studies approach the ideal with regard to moisture, temperature, and aeration. The results of such experiments cannot always be interpreted in the light of what happens under field conditions, where these ideal conditions are not the rule. As an example, in our perfusion experiments 2000 lb. per acre of 2,4-D was completely decomposed in 7 to 13 days in uninoculated soil; in the field 2 lb per acre is decomposed in 3 to 6 weeks (DeRose and Newman, 1948).

Acknowledgment

The authors are indebted to Dr. F. E. Clark, U.S.D.A., Agricultural Research Service, Beltsville, Maryland, for his comments concerning the organism described and for furnishing several cultures of *Bacterium globiforme* and *Cellulomonas* sp.

Summary

The isolation and culture of a microorganism tentatively identified as a species of *Mycoplana* are described. The bacterium was a gram negative, pleomorphic, motile rod which rapidly decomposed 2,4-D in soil and in solid and liquid synthetic media. Resting cell suspensions were shown to rapidly metabolize 2,4-D completely, and other similar compounds to a lesser degree. Requirements for optimum growth indicated that the organism was a mutant.

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