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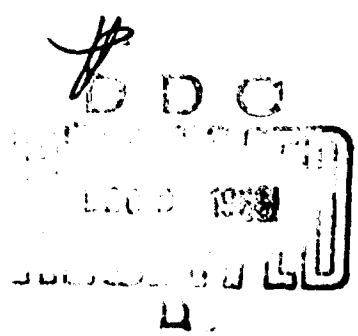

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

Stapp, C. & G. Spicher 1954. Untersuchugen uber die Wirkung von 2,4-D im Boden [Research on the activity of 2,4-D in the soil], Zentralblatt für Bakteriologie, Parasitenkunde, &c. II. Abt. Bd. 108. Heft 4/7, pages 113-126.

Resume

The stability of the selective hormonal herbicide, 2,4-D, in the soil is certainly one of its most important properties; for only so long as the 2,4-D acid maintains itself in the soil can the desired weed control be effected. The use of 2,4-D is advisable, since it affords a weed-free environment for planting, but it can be used repeatedly only with the assumption that, after the desired period of control, the herbicide is inactivated or removed from the soil. Since the 2,4-D must be below a certain concentration before planting, this problem deserves a lot of attention. A slight over-abundance in the substrate can ruin the crop (Audus, 1949; Bouillene-Walrand, 1952; Jensen & Petersen, 1952).

It is well known that most 2,4-D disappears from treated soil within a few weeks of treatment. This disappearance is attributed to microorganisms which metabolize 2,4-D. Using the "perfusion" technique, Audus (1949) has plotted the 2,4-D concentration against time; after treatment, he distinguishes three phases: (a) a slight reduction of the detectible 2,4-D, the result of adsorption onto soil colloids; (b) a phase during which the 2,4-D level remains about the same; (c) a rapid disappearance of 2,4-D. The curve is typica.

of those reflecting biological processes in the soil [Page 114] and Audus attributes the "detoxication" to the work of microorganisms. With repeated treatment of the soil, the second phase is hastened. Autoclaving or addition of a bactericide inhibits the activity.

Im impure culture of soils innoculated with the active organism 2,4-D was broken down in the same way (Newman & Thomas, 1950); Audus (1950:1951) finally isolated an organism which was able to survive on synthetic media with 2,4-D as its only carbon-source. According to his work, this bacterium is obviously a member of the Bacterium globiforme group of Lochead & Taylor. Later Jensen & Petersen (1952) identified two more 2,4-D bacteria, a "Flavobacterium aquatile," and a so-called "Bacterium 2," which they stated bore some relationship to the form described by Audus.

Identification of a 2,4-D Bacterium

Proceeding from the earlier work (Stapp & Freter, 1952; Stapp & Wetter, 1953) ... also succeeded in isolating the active organism which in pure culture on solid or liquid media was able to inactivate the sodium salt of 2,4-D (when not otherwise specified, "2,4-D" will here signify the sodium salt thereof). Composition of the medium: $Na_2HPO_4 \cdot 2H_2O$ 0.2%, NH_4NO_3 0.1%, KCl 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, 2,4-D 0.1%, Agar 2.0 to 0.1%.

The demonstration of the possibility of fluid-media culture was a significant advance. This was possible by spreading the medium with sufficient surface-area for a good oxygen supply (as the work of Akamine, 1951 and of Stapp & Wetter 1953 indicate is required), and above all an addition of 0.1% agar, which Audus (1949, 1951) had

had already indicated. It is also a good idea to permit the autoclaved culture flasks to stand at room temperature for a day before innoculation so that the media can absorb oxygen.

The yield of the impure culture of 2,4-D bacteria can be enhanced by repeated moistening of the sample with a 0.1% solution of 2,4-D, with a cress test following each moistening. This procedure can be repeated until finally when a "small amount" of the "enriched Soil2 is introduced into the flask containing the 2,4-D-containing medium, there is an immediate reduction in the 2,4-D concentration in the flask.

Puro culture at first was difficult, until it became evident that the 2,4-D bacterium is very sensitive to temperature. The unusually low upper limit of temperature tolerance was demonstrated thusly: For each temperature series, 2 cc of sterile medium was inocculated with a young culture, and this was allowed to remain 10 minutes in a constant-temperature water bath. The following different temperatures were used: 25, 30, 35, 40, 45, 50, and 55° C. Solid 2,4-D medium was then inncoulated from the temperature-treated aliquot, and the survival of the bacterium noted. The test was replicated four times. In all cases the bacteria survived temperatures of 40°C or less. A ten-minute exposure at 40°C greatly reduced the bacteria, while at 45°C the tolerance limit was already exceeded.

Even in the most diverse soils, the addition of 2,4-D led to a similar increase in the 2,4-D bacteria. This was accompanied, in all impure cultures, by an increase in Bacillus megaterium.

The tables of the "Manual of Determinatige Bacteriology" by Bergey, and the so-called "Skerman-key" (1949) or its German translation

by Meyn (1952) were used in the determination of genus to which the isolated 2,4-D bacterium belongs. Both indicate the genus Flavobacterium. Specific determination was considerably more difficult. The key of the "Manual of Determinative Bacteriology" takes us to three different species: F. aquatile, F. breve, and F. solare.

All three are non-motile, do not dilute gelatine, or do so only very slowly, leave litmus unchanged, and do not reduce nitrate to nitrite. Only in their temperature optima are they clearly differentiable.

From a further investigation of properties (Table 2), it was inferred that our 2,4-D bacterium is closest to Flavobacterium aquatile, insofar as the inference could be drawn on the basis of the published descriptions of properties. We contrast the two species below:

The colony of F. aquatile has a yellow-brown center and a colorless margin; our bacterium has a pure yellow colony.

F. aquatile dilutes gelatine only very slowly, our bacterium not at all.

F. aquatile occurs as singles, pairs, and chains; our bacterium occurs as singles or pairs, not as chains.

F. aquatile tolerates but does not require oxygen; our bacterium requires oxygen.

Above all, they differ in size (or length-width ratio): when F. aquatile is 0.5 micra wide it is about 2.5 long; when our bacterium is that wide, it is only about 0.8 micra long.

The 2,4-D bacterium identified by Jensen & Petersen (1952) as F. aquatile fits the description of the "Manual of Determinative Bacteriology" in size (larger than ours). Curiously, the bacterium of

Jensen & Petersen does not grow on a semisolid agar-medium nor on earth-extract, but develops well on a glucose medium. Our bacterium, on the other hand, does very well on nutrient solution plus 0.1% agar, and is markedly held back when 1% glucose is added.

In an attempt to determine how our 2,4-D bacterium compares with strains which have been determined by other authorities as Flavobacterium aquatile, in a series of tests four of these strains were compared with our bacterium (Table 3). All five strains are very similar, but display a few distinctions, these having to do with chain-formation, colony-color, ability to thin gelatine, and growth in nutrient bouillon. Strain 1 is closely related to strain 2, and strain 3 to strain 35. Our 2,4-D bacterium is intermediate, with a special form of colony in agar and in gelatine.

In an examination of properties not specified in the "Manual", more extensive differences became evident, when neither acid nor gas formed in the bouillon-carbohydrate media (Table 4). Here (in Table 4) our bacterium is again intermediate, but closer to strain 3 and 35. The descrease or retardation of its development in the presence of glucose and lactose is especially remarkable. Only our 2,4-D bacterium persists on the 2,4-D medium; even after a week's incubation, none of the others develops an adaptation to 2,4-D.

Audus (1949) hypothecated that the long first phase in the break-down of 2,4-D in previously untreated soil was the time during which the normally inactive bacteria adapt themselves to 2,4-D metabolism. Jensen & Petersen (1952) considered it unlikely that an organism with an enzyme system capable of this break-down would be common in a previously

untreated soil. They considered the 2,4-D bacterium to be a Flavobacterium aquatile adapted for the break-down of cyclic compounds.

Since, however, none of the investigated strains of Flavobacterium aquatile from untreated soils has ever succeeded in utilizing 2,4-D, we consider it appropriate to look upon our 2,4-D bacterium as new, and propose for it the name Flavobacterium peregrinum, new species.

The Break-down of 2,4-D by Flavobacterium peregrinum.

It seemed to us very important to compare the activity of pure and impure culture of Flavobacterium pergrinum, to see whether the impure culture may eventually contain still other organisms capable of 2,4-D break-down. The possibility arises, that if the 2,4-D bacterium can be induced to break down 2,4-D in fluid medium, the portion of the break-down attributable to the 2,4-D bacterium (Strain D) can easily be ascertained.

The nutrient described on page 114 was doubled in concentration, but with the same amount of earth-extract, and 20 cc placed in each of a number of 100-cc flasks, and autoclaved. One group was innoculated with Strain D, the other with a small amount of a soil sample enriched with the 2,4-D bacterium.

In pure culture the principal phase of 2,4-D break-down usually starts the first day, and after 2.5 days the bacteria have inactivated half of the 2,4-D supplied. In impure culture, on the other hand, the principal phase begins on the third day, and the half-way point is reached between the fourth and fifth days. Therefore Flavobacterium peregrinum is solely responsible for the nutrient-break-down, and in this respect Bacterium megaterium, which is usually found with it, is insignificant.

It is widely known that the disappearance of 2,4-D in various soils proceeds at different rates. However, the results of research on the reason for these differences, up until now, have been equivocal. [There follows a short review of literature: Kries, 1947; Akamine, 1951; Stapp & Wetter, 1953; Mitchell & Marth, 1946; Hanks, 1947; Brown & Mitchell, 1948; Krone & Hamner, 1947.]

With the aid of fluid culture it can be shown that soils of various origins contain a factor which, depending on the origin of the soil, differentially affects the rate of growth of the 2,4-D bacterium.

The finely sifted soil samples were placed in twice their weight of distilled water, were heated 30 minutes in the autoclave, centrifuged, and filtered through a membrane filter (No. 10 of the membrane filter company in Gottingen). A part of the resulting extract was added to a similar amount of the doubly-concentrated nutrient solution, and 20 cc placed in a number of 100-cc. flasks, sterilized, innoculated with Flavobacterium peregrinum, and incubated at 25°C.

The addition of soil extract speeds up the breakdown (see fig. 3a. and 3b.); if the time is propitious, the time required to break down half the 2,4-D is reduced from 72 to 40 hours. The most strongly active soils were, in our experience, humic to very humic fine sandy loams, with no carbonates, probably alluvial, and used as meadows. The The results of several other workers have been taken to indicate that the rate of 2,4-D inactivation is dependent on the sum of several factors whose total effect determines the effective activity. In our experience, one of these factors, as already pointed out by Audus (1952) is a water-soluble growth-stimulant which occurs in varying amounts. Our factor

Table 2. Comparison of the Flavobacterium aquatile, F. breve, F. solare, and F. peregrinum.

	F. aquatile	F. breve	F. solare	F. peregrinum
Bacillus	0.5x2.5 micra non-motile gram negative single, pairs, chains	0.8-1.0x2.5 micra non-motile gram-negative with terminal spots	0.3-0.4x1.0 micra non-motile gram negative single, pairs, chains	0.5x0.8-2.4 micra non-motile gram negative singles, pairs no terminal spots.
Gelatine-plate	center yellow-brown, margin colorless, with radiate fasciculate filaments	pale yellow	yellow emitting rays.	yellow margin with raidate furrows
Gelatin-stab	yellow surface-slow liquefaction	yellow surface pearly growth in the stab-canal no liquefaction	yellow treelike growth in the stab-canal no liquefaction	yellow surface no special growth in the stab-canal no liquefaction
Nutrient agar	yellow, smooth, lustrous	yellow	pale yellow branches like tree	yellow with a glassy margin; smooth, lustrous
Nutrient bouillon	turbid; white precipitate	turbid; whitish precipitate	unchanged	turbid; white precipitate
Litmus	unchanged	unchanged	unchanged	unchanged
Potato	yellow streaks	no growth	yellowish-brown streaks	yellowish streaks
Indole	not formed		not formed	not formed
Nitrate-reduction	no nitrite		no nitrite	no nitrite
Oxygen-requirement	facultative	facultative	facultative	obligate
Temperature optimum	25° C	35° C	30° C	25° C

Table 3. Comparison of the characteristics of four strains of Flavobacterium aquatile and Flavobacterium peregrinum.

	Strain 1	Strain 2	Strain 3	Strain 35	Fl. peregrinum
		of Fl. aquatile			
Bacillus	0.5x0.8-1.8 micra non-motile gramnegative single, pairs, and chains up to 8 micra long	0.5x0.8-1.8 micra non-motile gramnegative singles, pairs, and chains up to 12 micra long	0.5x0.8-2.4 micra non-motile gramnegative singles, pairs, and chains	0.5x0.8-2.4 micra non-motile gramnegative singles, pairs, and chainge	0.5x0.8-2.4 micra non-motile gramnegative singles, pairs not chains
Gelatine plate	brownish, smooth round, lustrous	brownish, smooth round, lustrous	yellowish-smooth round, lustrous	yellowish, smooth round, lustrous	yellow; the margin with radiate furrows round, lustrous
Gelatine-stab	brownish surface-growth no liquefaction	brownish surface-growth no liquefaction	yellowish surface-growth slow liquefaction	yellowish surface-growth slow liquefaction	yellowish surface-growth no liquefaction
Nutrient agar	brownish smooth lustrous, not slimy	brownish, smooth lustrous, not slimy	light yellow, smooth lustrous, not slimy	light yellow, smooth lustrous, not slimy	yellow with clear margin, smooth, lustrous, very slimy
Nutrient bouillon	unchanged	slightly turbid slight yellow precipitate	turbid white precipitate	turbid white precipitate	turbid white precipitate
Litmus	unchanged	unchanged	unchanged	unchanged	unchanged
Potato	poor growth	poor growth	yellow streaks	yellow streaks	yellow streaks
Indole	not formed	not formed	not formed	not formed	not formed
Nitrate-reduction	not nitrite	no nitrite	no nitrite	no nitrite	no nitrite
Oxygen-requirement	positive requirement	positive requirement	positive requirement	positive requirement	positive requirement

Table 4. Comparison of the characteristics of four strains of Flavobacterium aquatile and Flavobacterium peregrinum.

	Flavobacterium aquatile				Flavob. peregrinum
	Strain 1	Strain 2	Strain 3	Strain 35	
Nitrate agar	brown-yellow poor growth (surface only)	brown yellow poor growth (surface only)	might yellow good growth (surface only)	light yellow good growth (surface only)	pure yellow slow growth (surface only)
Dextrose agar	brown yellow poor growth (surface only)	brown yellow poor growth (surface only)	light yellow good growth (surface only)	light yellow good growth (surface only)	pure yellow slow growth (surface only)
Bouillon	unchangchanged	slightly turbidate slight yellow precipitate	turbid precipitate white precipitate	turbiditate white precipitate	turbiditate slight white precipitate
Bouillon plus soluble starch	unchanged	unchanged	turbid 1 hydrolysis much white precipitate	turbid hydolysis8 much white precipitate	turbid m no hydrolysis much white precipitate
Bouillon plus glucose	turbid slight yellow precipitate	turbid slight yellow precipitate	turbid much white precipitate	turbid much white precipitate	slightly turbid no precipitate
Bouillon plus sacchacose	unchanged	unchanged	turbid much white precipitate	turbid much white precipitate	turbid slight white precipitate
Bouillon plus lactose	turbid slight yellow precipitate	turbid slight yellow precipitate	turbid much white precipitate	turbid much white preciptate	unchanged
Bouillon plus glycerine	unchanged	unchanged	turbid much white precipitate	turbid much white precipitate	turbid slight white precipitate
Bouillon plus manitol	unchanged	unchanged	turbid much white precipitate	turbid much white precipitate	turbid slight white precipitate
Bouillon plus potassium nitrate	slightly turbid	slightly turbid	turbid much white precipitate	turbid much white precipitate	turbid
Peptone water	turbid	turbid	turbid	turbid	turbid
Mineralized glucose nutrient	no growth	slight growth	slight growth	slight growth	slow growth
2,4-D nutrient	no growth	no growth	no growth	no growth	good growth

No formation of gas or acid

No formation of gas or acid

No formation of gas or acid

No formation of gas or acid

No formation of gas or acid