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STUDIES ON A NEW OXALATE-DECOMPOSING BACTERIUM, VIBRIO OXALITICUS

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Only a few microorganisms are known to decompose oxalic acid or oxalate. Most of these are filamentous fungi, notably species of *Aspergillus* and *Penicillium*, which form oxalic acid under some conditions and decompose it under others (Wehmer, 1891; Bach and Fournier, 1935). Among bacteria the ability to decompose oxalate is very rare. Ayers *et al.* (1919) and Den Dooren de Jong (1928) tested over 125 strains isolated from a variety of sources and did not find a single one possessing this ability. Bassalik (1913) tested 90 species of bacteria and fungi and found only three strains of bacteria that could attack oxalate. Two of these decomposed oxalate very slowly in a synthetic medium, but the third, called *Bacillus extorquens*, decomposed oxalate rapidly and completely under the same conditions.

Bassalik made a rather thorough study of *Bacillus extorquens* and its action on oxalate. Since many of his observations can be directly compared with ours, it is worth while to summarize them briefly. *B. extorquens* was isolated originally from the excreta of an earthworm that had ingested plant material containing crystals of calcium oxalate; later it was shown to be present in forest and garden soils. The bacterium was enriched by inoculating the excreta or soil into a mineral medium containing ammonium oxalate as the only organic compound. Great difficulty was experienced in the isolation of pure cultures. Repeated attempts to use agar media failed, but the organism was finally isolated by the use of silica gel plates containing ammonium oxalate.

B. extorquens was a slightly bent, nonsporulating rod, averaging 1.5 by 3.0 microns in size and having a single polar flagellum. It formed a rose-red to blood-red pigment and, in a liquid oxalate medium, it grew characteristically as a film on the bottom and walls of the flask, leaving the liquid clear. Growth was poor on ordinary agar and gelatin media but was rapid and abundant on synthetic media containing one of the following compounds as a sole energy source: oxalate, glyoxalate, malonate, succinate, fumarate, maleate, oxamate, phenylacetate, formate, oxamide, methyl and ethyl alcohols, glycerol, sorbitol, mannitol, and glucose. Less vigorous growth was obtained with a variety of other compounds including glycolate and formaldehyde in low concentration. Both soluble and relatively insoluble oxalates, like the calcium and barium salts, were readily decomposed. The optimal concentration of soluble oxalates was between 0.1 and 0.3 per cent, although higher concentrations could be tolerated

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provided the alkali resulting from the decomposition was neutralized. The decomposition of oxalate was shown to be an oxidation to carbon dioxide and water, the only other product being cell material. The rate of oxygen consumption on oxalate corresponds to $Q_{O_2} = 32$ to 53 at 29 C. Finally it was shown that toluene-treated bacteria and even cell-free culture filtrates were capable of decomposing oxalate, a fact indicating the presence of a soluble enzyme.

Since the work of Bassalik very little has been done with oxalate-decomposing bacteria. Scholder and Linström (1930) observed the disappearance of oxalate from a dilute solution, apparently as a result of bacterial action, but they were unable to isolate the causative organism. Barber and Gallimore (1940) showed that when an oxalate solution is inoculated with fecal material, oxalic acid rapidly disappears under both aerobic and anaerobic conditions. The process was evidently due to nonsporulating bacteria since it was prevented by pasteurizing the inoculum. No attempt was made to isolate or identify the bacteria.

The present paper deals with the isolation and characteristics of an aerobic soil bacterium, *Vibrio oxaliticus*, nov. spec., which is similar to *Bacillus extorquens* in its ability to decompose oxalate, but which differs from this organism in several important respects.

EXPERIMENTAL METHODS AND RESULTS

Enrichment and isolation of V. oxaliticus. For the enrichment of this organism a medium (No. 1) of the following composition in g per 100 ml was used: potassium oxalate hydrate, 0.1; $(NH_4)_2SO_4$, 0.05; K_2HPO_4, 0.05; $MgSO_4 \cdot 7H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.002; $CaSO_4 \cdot 2H_2O$, 0.001; pH 7; made up with distilled water. A few drops of phenol red indicator were also added. The medium was inoculated with a small quantity of garden soil and incubated aerobically in a shallow layer at 28 C. Within 24 hours the medium became turbid and the pH rose to about 8.4. A permanganate titration showed that more than 85 per cent of the oxalate had been decomposed.

After one transfer in the same medium, the culture was streaked on an oxalate agar medium containing 0.1 per cent yeast extract. In 48 hours many small colonies of at least six different types appeared. Representative colonies were transferred by means of capillary pipettes into tubes of sterile liquid oxalate medium with yeast extract. In only two out of eight tubes was the oxalate decomposed, and microscopic examination showed that in both positive cultures at least two organisms were present, one a very small vibrio and the other a somewhat larger straight rod.

In order to facilitate the separation of these two organisms, which proved to be somewhat difficult, the plating medium was altered slightly by the addition of a small amount of sterile calcium chloride solution (2 ml M/10 per 100 ml of medium 1). This resulted in the formation of a fine crystalline precipitate of calcium oxalate that made the agar slightly opaque. In such a medium the colonies of oxalate-decomposing bacteria can be easily recognized after a few days' incubation by the formation of a clear zone or halo about each as a result of the disappearance of the calcium oxalate crystals (figure 1). The dissolution

of the crystals in the immediate vicinity of the colonies can be observed microscopically even before the microscopic halos appear.

By means of this plating technique, the vibrio was separated from the straight rod, and it was found that only the former was able to decompose oxalate. The available evidence indicates that the vibrio was the only oxalate-decomposing bacterium present in the enrichment cultures. The other five types of bacteria must have been living on organic materials synthesized by the vibrio.

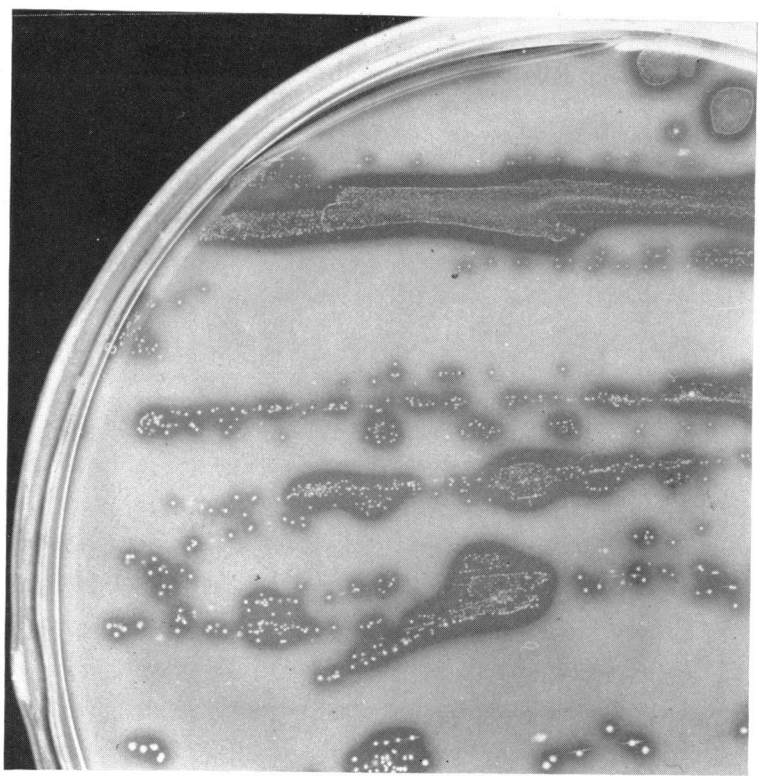

FIG. 1. CALCIUM OXALATE AGAR PLATE STREAKED FROM AN ENRICHMENT CULTURE AND INCUBATED 15 DAYS AT 28 C

Growth was slow because all the oxalate was in the form of the calcium salt.

Four other strains of the vibrio were isolated from three soil samples obtained in Berkeley and one from the garden of the Massachusetts General Hospital in Boston. A number of other soil samples were examined for the presence of the vibrio by using them as inocula for enrichment cultures that were then examined microscopically. Positive results were obtained with all samples tested. Attempts to demonstrate the presence of oxalate-decomposing bacteria in air were unsuccessful.

Most of the experiments reported in this paper were done with strain 2.

Morphology, staining, and cultural characteristics. All five strains are similar in appearance and behavior. They are typical small vibrios, the average dimen-

sions being 0.4 × 1.3 microns (figure 2). The cells in young cultures are actively motile by means of a single polar flagellum, 6 to 8 times the length of the cell body. No capsules or spores were observed. The cells were gram-negative at all times.

The organism grows on nutrient agar to form small, pin-point colonies in about 48 hours that were moist, raised, and had entire edges. The colonies grow slowly on further incubation and reach a maximal diameter of 1.5 mm in about 6 days. Colonies were never pigmented. Moderate growth was obtained in nutrient broth; after 24 hours it was mostly confined to a thin surface film, and after another 24 to 48 hours a slight general turbidity developed. The organism does not reduce nitrate, and does not form indole or hydrogen sulfide.

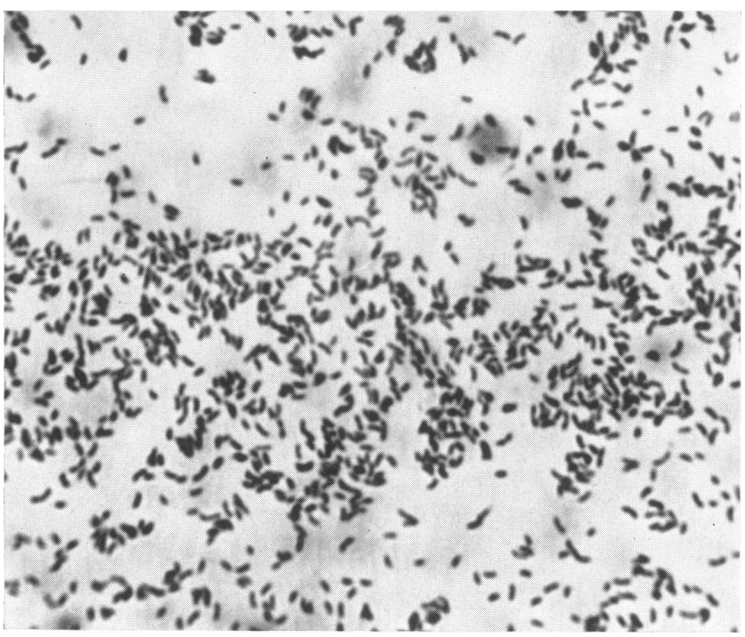

FIG. 2. V. OXALITICUS, STRAIN 1, FROM 2-DAY-OLD CULTURE ON AN OXALATE MEDIUM
Gentian violet stain, 1,000 ×

On calcium oxalate agar containing 0.1 per cent yeast extract growth is rapid, but the colonies remain small because of the limited quantity of available nutrients. The colonies are similar in size and appearance to those on nutrient agar. It should be noted that oxalate-containing media always tend to become alkaline as the anion is decomposed. When phenol red is present, the color changes from yellow to red. In a liquid oxalate medium with or without yeast extract, a slight surface film forms first, and later a general turbidity develops. Growth is more rapid in shaken than in stationary cultures, probably because of better aeration. In a culture incubated on a shaker, 0.1 per cent potassium oxalate is decomposed almost completely within 24 hours at 28 C.

Substrates utilized for growth. A number of organic compounds were tested as

growth substrates by substituting them for oxalate in medium 1 with and without the addition of 0.05 per cent Difco yeast extract. Utilization of the substrate was judged by an increased turbidity over the control with no added substrate. It was found that in the presence of yeast extract the only compounds that support growth within 3 to 4 days are oxalate, formate, and pyruvate. In this period of incubation the organism is unable to utilize acetate, butyrate, citrate, lactate, malate, malonate, succinate, tartrate, or glucose. However, acetate at least does support abundant growth when the incubation period is further extended. At the end of 3 to 4 days there is only a slight effect of acetate, but growth continues slowly and reaches a maximum on the twelfth day. This indicates that an adaptation is required for acetate utilization. The lag period can be considerably reduced by transferring the bacteria several times in acetate media. When the various compounds were tested as growth substrates in the absence of yeast extract, the results were qualitatively the same except with formate, which does not support growth when it is the sole carbon source.

Taxonomy. The morphology of the oxalate-decomposing organism places it in the genus *Vibrio* as defined in *Bergey's Manual* (1939). It could not be identified with any species described therein. There is a generic similarity between our organism and Bassalik's *B. extorquens*, but they differ in many details. For example, *B. extorquens* is a relatively large vibrio that forms a red pigment and metabolizes a great variety of organic compounds in a mineral medium. Our organism, on the contrary, is very small, does not form any pigment, and only grows well with oxalate and pyruvate in a mineral medium. In view of these obvious differences, we must regard our organism as a new species for which we propose the name, *Vibrio oxaliticus*.

Quantitative experiments on growth with various substrates. It has already been pointed out that *V. oxaliticus* can grow in a mineral medium with oxalate, acetate, or pyruvate as the sole energy source. Yeast extract also supports growth. The amount of growth depends upon the substrate and its concentration. The data presented in table 1 show that, at a given concentration, pyruvate supports the heaviest growth, acetate is next, yeast extract third, and oxalate gives the poorest growth. Pyruvate is more than ten times as effective as oxalate. This is no doubt a reflection of the different energy contents of the two compounds. It should be noted that the rate of growth is not necessarily parallel with the cell yield. Acetate is second only to pyruvate in efficiency of utilization for cell synthesis, but it is metabolized much more slowly than are the other compounds studied.

V. oxaliticus prefers relatively dilute media. With oxalate, acetate, and pyruvate the heaviest growth is obtained at or below 0.3 per cent. With yeast extract, growth continues to increase with concentration up to about 2 per cent and then falls off at higher concentrations. The low concentration optimum for oxalate might be explained by a need for calcium, which is made progressively more unavailable as the oxalate is increased. There is no obvious explanation however, for the similarly low optima with acetate and pyruvate.

Several experiments were done in order to find out what constituent of the

yeast extract is used as a growth substrate. These experiments have not led to a definite conclusion, but they indicate that the active constituent is not an amino acid or other nitrogenous compound. This may be deduced from the fact that no ammonia is formed from yeast extract, and also from the observation that growth is not improved by the addition of casein hydrolyzate, which consists largely of amino acids. Acetate, formate, and pyruvate are not present in yeast

TABLE 1

*Influence of substrate concentration on growth**

SUBSTRATE CONC.	MAXIMAL TURBIDITY†			
	Difco yeast extract	Potassium oxalate	Sodium acetate	Sodium pyruvate
%				
0.1	21	9	83.5	118
0.2	33	21	102	187
0.3	51	25	78	235
0.4	65	24	59	235
0.6	84			168

* Medium 1 with the oxalate replaced by the indicated amounts of the various substrates.

† (2-log G) × 100. Determined with an Evelyn photocolorimeter.

TABLE 2

*Influence of formate and yeast autolysate on growth**

YEAST EXTRACT	MAXIMAL TURBIDITY†		
	− Formate	+ Formate‡	Δ
g/100 ml			
0	0	0	0
.05	11	35	24
0.1	21	56	35
0.2	33	88	55
0.4	65	138	73
0.8	121	143	22
1.6	184	184	0

* Medium 1 was used without oxalate and with the indicated additions.

† (2-log G) × 100.

‡ 100 mg sodium formate per 100 ml.

extract in sufficient quantities to account for more than a minute fraction of its activity.

The utilization of formate in the presence but not in the absence of yeast extract has already been noted. This effect was studied in more detail by determining the maximal growth obtained with various concentrations of yeast extract with and without formate. The data are given in table 2. It can be seen that growth on yeast extract is greatly increased by the addition of formate. Furthermore the effect of a given amount of formate increases with the concentration

of yeast extract up to 0.4 per cent and then falls off to zero at much higher concentrations. In a second experiment the yeast extract concentration was kept constant at two levels, whereas the formate was varied over a wide range. The data presented in table 3 show that, with 0.1 per cent yeast extract, growth increases with formate concentration up to 0.2 per cent and then declines. With 0.4 per cent yeast extract, maximal growth is obtained when 0.1 per cent formate is present; growth decreases progressively as the concentration is raised further.

These results are consistent with the view that formate is an incomplete substrate that serves as an energy source but does not provide all the compounds essential for cell synthesis. When yeast extract is provided as a source of essential metabolites, the energy derived from the oxidation of formate can be used with an efficiency that increases with the supply of other compounds. However, the addition of an excess of yeast extract undoubtedly provides other energy

TABLE 3

*Influence of formate concentration on growth with different amounts of yeast extract**

SODIUM FORMATE	MAXIMAL TURBIDITY†	
	0.1% yeast extract	0.4% yeast extract
g/100 ml		
0	21	67
0.1	49	108
0.2	81	78
0.3	37	69
0.5	25	49
0.8	7	25

* Medium 1 without oxalate and with the indicated amounts of formate and Difco yeast extract.

† (2-log G) × 100.

sources, which make formate superfluous. The diminished growth at higher formate concentrations may be due to formate toxicity.

As a supplement to formate, yeast extract could not be replaced by any of the known water-soluble growth factors, either alone or in combination.

Respiration of cell suspensions. The ability of cells grown on a medium containing 0.2 per cent potassium oxalate and 0.1 per cent yeast extract to oxidize a number of organic compounds was tested manometrically using alkali to absorb carbon dioxide. Only three compounds caused an oxygen uptake greater than that of the control without substrate, namely, oxalate, formate, and pyruvate. The following compounds were not oxidized at a measurable rate: oxamate, oxamide, oxalurate, allantoin, allantoate, acetate, succinate, and hydrogen.

The respiratory rates on oxalate, formate, and pyruvate were determined using cells grown on each of these three substrates. The data given in table 4 show that the enzymes involved in the utilization of oxalate and formate are formed to about the same extent on all three growth substrates. The pyruvate enzyme,

also, is always formed, but its activity varies considerably with the substrate. It is curious that the highest activity toward pyruvate was exhibited by cells grown on formate.

The absolute oxygen uptakes and respiratory quotients observed in the same experiment are given in table 5. The values obtained with the substrates have not been corrected for the endogenous respiration. The data indicate that oxalate and formate are almost completely oxidized to carbon dioxide. With pyruvate the respiratory quotient is close to the theoretical value of 1.2, but the

TABLE 4

Respiratory rates for cells grown on different substrates

GROWTH SUBSTRATE	Q_{O_2} (N)			
	Respiration substrate (0.1 ml M/10 per 2 ml)			
	None	Oxalate	Formate	Pyruvate
Yeast extract + oxalate*....	81.4	225	235	135
Yeast extract + formate*....	148	270	310	630
Yeast extract + pyruvate*..	67	290	270	430

* Medium 1 with 0.1 per cent Difco yeast extract and 0.2 per cent of potassium oxalate, sodium formate, or sodium pyruvate.

TABLE 5

Oxygen uptake and respiratory quotients

GROWTH SUBSTRATE	RESPIRATORY SUBSTRATE (10 μM)							
	None		Oxalate		Formate		Pyruvate	
	O_2 uptake μM	R.Q.	O_2 uptake μM	R.Q.	O_2 uptake μM	R.Q.	O_2 uptake μM	R.Q.
Yeast extract + oxalate.....	1.60	1.02	4.43	3.39	4.61	1.81	(3.32*)	1.29
Yeast extract + formate.....	2.87	1.10	5.34	—	6.12	1.75	12.4	1.38
Yeast extract + pyruvate.....	1.64	1.04	7.17	3.02	6.61	1.60	10.5	1.37
Theoretical for complete oxidation.....			5.0	4.0	5.0	2.0	25.0	1.2

* The substrate decomposition was incomplete.

oxygen uptake and carbon dioxide production are only about half those required for complete oxidation. The other half of the pyruvate carbon is probably assimilated. The results are entirely consistent with the behavior of growing cultures. We have seen that the quantity of cells obtained with pyruvate is much greater than with oxalate or formate.

DISCUSSION

Our observations, considered in conjunction with those of Bassalik, indicate that oxalate-decomposing bacteria are commonly present in soil. This is not unexpected in view of the fact that substantial quantities of oxalate are being

continually added to soil in plant residues. The presence of these bacteria can be demonstrated very easily by the use of an enrichment medium containing oxalate as the only energy source. The isolation of the bacteria in pure culture is somewhat more difficult but is greatly facilitated by the use of a solid medium containing crystals of calcium oxalate.

Vibrio oxaliticus, which we have isolated from California and Boston soils, is probably closely related to Bassalik's *Bacillus extorquens*, isolated in Switzerland. In spite of its name, the latter organism is a typical *Vibrio* and it should be called *Vibrio extorquens* to fit into modern systems of bacterial taxonomy. The details of the morphology and physiology of the two organisms are, however, sufficiently different so that there can be no doubt that they represent distinct species.

Oxalate and formate are very simple substrates. The removal of two electrons from either compound will result in its conversion to carbon dioxide. This leads to the idea that the metabolism of *V. oxaliticus* may be similar to that of the autotrophic bacteria in the sense that energy is obtained by the oxidation of a simple compound, whereas the cell materials are built up from carbon dioxide. There is no a priori reason why energy for the reduction of carbon dioxide could not be obtained from the oxidation of oxalate just as well as from the oxidation of hydrogen or nitrite, for example. There is no definite evidence at present, however, in favor of this view. We do not know whether in fact *V. oxaliticus* is able to utilize carbon dioxide, although this could be determined by experiments with carbon isotopes. There is one bit of evidence against the foregoing view, namely, the fact that the organism cannot grow with formate alone. If carbon dioxide can be reduced with oxalate as an energy source, one would expect the same to occur with formate. Since formate cannot replace oxalate completely, it is quite possible that oxalate serves as a starting point for synthetic reactions as well as an energy source. In any event it is clear that the metabolism of simple substrates like oxalate presents many intriguing and fundamental questions for which there are as yet no answers.

SUMMARY

The isolation and characteristics of a new oxalate-decomposing bacterium, *Vibrio oxaliticus*, are described. The organism is able to grow on oxalate, pyruvate, and acetate in an otherwise mineral medium. Its growth is also increased by the oxidation of formate in a medium containing yeast extract. The oxalate and formate enzymes are constitutive, whereas the activity of the pyruvate enzyme system varies with the growth substrate. Oxalate and formate are almost completely oxidized to carbon dioxide; pyruvate is about half-oxidized and half-used for cell synthesis.

REFERENCES

AYERS, S. H., RUPP, P., AND JOHNSON, W. T. 1919 A study of the alkali forming bacteria found in milk. U. S. Dept. Agr., Bull. 782, 1–38.

BACH, D., AND FOURNIER, J. 1935 Sur l'assimilation de l'acide oxalique par l'*Aspergillus niger*. Compt. rend., **20**, 982–984.

BARBER, H. H., AND GALLIMORE, E. J. 1940 The metabolism of oxalic acid in the animal body. Biochem. J., **34**, 144–148.

BASSALIK, K. 1913 Über die Verarbeitung der Oxalsäure durch *Bacillus extorquens* n. sp. Jahrb. wiss. Botan., **53**, 255–302.

DOOREN DE JONG, L. E. DEN 1926 Bijdrage tot de Kennis van het Mineralizatie-process. Dissertation, Delft. Tabel. XVII.

SCHOLDER, R., AND LINSTRÖM, C. F. 1930 Über die Oxydation von Oxalaten durch Bakterien. Ber. deut. chem. Ges., **63**, 2730–2737.

WEHMER, C. 1891 Entstehung und physiologische Bedeutung der Oxalsäure im Stoffwechsel einiger Pilze. Botan. Z., **49**, 233–246.