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The Isolation and Characteristics of an Oxalate-decomposing Organism

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SUMMARY: The isolation from soil of three strains of oxalate-decomposing organisms different from other known oxalate-decomposing organisms is described; only one strain was studied in detail. This organism grew in concentrations of potassium oxalate up to 2% (w/v) with an optimum of c. 1% (w/v). Growth was preceded by a lag period of c. 18 hr. which was not decreased by addition of yeast autolysate, bicarbonate or formate. Of several other substrates tested only glycolate, DL-lactate, pyruvate, DL-malate and succinate supported growth. Washed cell suspensions oxidized oxalate and the gas exchanges observed corresponded with an assimilation of some of the oxalate used. The oxidizing powers of washed cells varied somewhat with the carbon source used for growth.

Very few bacteria have been reported as able to utilize oxalate as the sole source of carbon and energy. The only bacteria which have been isolated in pure culture and shown to decompose oxalate are: *Bacillus extorquens* (Bassalik, 1913); *Vibrio oxaliticus* (Bhat & Barker, 1948); *Pseudomonas extorquens* (Janota, 1950); a species of *Proactinomyces* (Müller, 1950); *Pseudomonas oxalaticus* (Khambata & Bhat, 1953). It has been known for a long time that certain plants (e.g. rhubarb) contain oxalates. Oxalate was one of the first products to be discovered in early studies on the aerobic breakdown of carbohydrate by species of *Aspergillus* and *Penicillium* (Wehmer, 1891), and under certain conditions these fungi also decompose oxalate (Wehmer, 1891; Bach & Fournier, 1935). Although oxalic acid is of wide occurrence in nature relatively little is known of its metabolism; in order to study this process attempts were made to isolate from soil, by the enrichment technique, organisms which used oxalate as sole source of carbon and energy for growth. The present paper deals with the isolation and properties of an aerobic organism of this type. A preliminary account of this work has already been given (Jayasuriya, 1954).

RESULTS

Isolation

For the enrichment cultures a chemically-defined medium (medium A) was used; it had the following composition (mg./100 ml.): KH_2PO_4 , 136; Na_2HPO_4 , 213; $(\text{NH}_4)_2\text{SO}_4$, 50; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 0.25; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.25; potassium oxalate, $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$, 1000. Volumes (10 ml.) of medium A were inoculated with c. 5 g. samples of garden soil, one from Pacific Grove, California, and the other from a herbaceous border in Sheffield. Separate enrichment cultures were set up at 25° and 37°

under aerobic and anaerobic conditions. The aerobic enrichment cultures were carried out in 50 ml. Erlenmeyer flasks, each containing 10 ml. medium A. The anaerobic enrichment cultures were set up in 60 ml. bottles, having ground glass stoppers, completely filled with the medium.

Microscopic examination of the cultures after 48 hr. revealed the presence, in all cultures, of a large number of morphological types of organisms with no single one predominating. A loopful of each culture was transferred into fresh medium A. A second series of subcultures were also made in medium A + 0.01 % (v/v) yeast autolysate (Barker & Beck, 1942). Within 48 hr. the 25° aerobic cultures became turbid and titration with 0.1N-KMnO₄ showed that oxalate was being decomposed. There was neither marked growth of organisms nor any appreciable decrease in the permanganate titre in subcultures from the 37° aerobic cultures or in any of the anaerobic cultures.

Organisms from the 25° aerobic cultures were isolated by plating out on a calcium oxalate agar medium similar to that used by Bhat & Barker (1948). This medium contained the components of medium A + 3 % (w/v) washed agar (prepared from Davis New Zealand agar by soaking the agar in distilled water for 24 hr. followed by decanting the water and then repeating the process 3 times) + 0.1 % (v/v) yeast autolysate. Before pouring the plates 10 ml. sterile 0.1M-CaCl₂/100 ml. medium were added to convert part of the oxalate present to calcium oxalate. After incubation for 48 hr. many small colonies appeared. The slow growth of the oxalate-decomposing organisms made their isolation difficult since the colonies tended to be overgrown by contaminants. Slow growth was possibly due to the relative inaccessibility of the carbon source when present as the insoluble calcium oxalate. However, the use of this medium greatly facilitated the recognition of oxalate-decomposing organisms, the colonies of which were surrounded by a clear zone. The organisms picked from these colonies were purified by several successive platings on this solid medium and maintained by fortnightly transfers on aerobic slants.

To confirm that these isolates utilized oxalate, medium A was inoculated from a single colony and the disappearance of oxalate shown by removing samples under aseptic conditions at suitable intervals and determining the oxalic acid content by titration with permanganate. These isolates also grow on silica gel plates prepared according to Meiklejohn (1950) and containing the constituents of medium A.

Three strains of oxalate-decomposing organisms were thus isolated; two from Pacific Grove soil and designated OD1 and OD2; one from Sheffield soil and designated OD3. Organism OD2 was isolated from the second series of subcultures in the presence of yeast autolysate. All three strains were isolated from the 25° aerobic enrichment cultures; no oxalate-decomposing organisms were isolated from either the 37° aerobic enrichment cultures or from any of the anaerobic cultures. The three strains isolated are morphologically similar; the work described below was carried out entirely with the organism OD1.

Organism OD1; morphology and biological properties

Organism OD1 is a short rod of mean dimensions $0.5 \times 1.5 \mu$. It does not stain well by the usual procedures; the only method which gave good preparations was negative staining with nigrosin, but it appears to be Gram-negative. It is actively motile, especially in young cultures. Flagella staining by the method of Bailey (1929), as modified by Fisher & Conn (1942), revealed a single polar flagellum. Neither spores nor capsules were observed. Long filamentous forms were seen occasionally, especially in old cultures.

Pin-point colonies appeared between 24 and 48 hr. on nutrient agar, on 10% (v/v) yeast autolysate agar and on calcium oxalate agar; no colonies were visible up to 24 hr. on any of these media. After 72 hr. the colonies were about 1 mm. diam.; on further incubation the colonies on nutrient agar and on yeast autolysate agar grew to *c.* 2 mm. diam. while the colonies on oxalate agar grew to *c.* 1.5 mm. diam. within about 7 days. All colonies were smooth, raised, opaque and had entire edges. These observations held at 25° and 30°. The organism failed to grow on the above media at 37° and 45°.

In nutrient broth and 10% (v/v) yeast autolysate the organism formed a thin pellicle in 24 hr. at 25° and 30°. This was followed by a heavy turbidity throughout the medium, and at the end of growth there was a heavy deposit on the bottom of the culture vessel. In liquid oxalate medium A, however, only a slight turbidity developed. The organism does not produce any water-soluble pigments, but is itself coloured yellow when grown in nutrient broth or in 10% (v/v) yeast autolysate. The organism does not produce indole; methyl red and Voges-Proskauer tests were negative. Litmus milk was turned slightly alkaline. Catalase is present. Neither acid nor gas was produced in glucose, mannose, sucrose, maltose, lactose, starch or dextrin.

In the presence of 1% (w/v) potassium oxalate as source of carbon and energy, organism OD1 grew with diammonium hydrogen phosphate, ammonium nitrate, sodium nitrate, glycine, L-asparagine or peptone as nitrogen source. Any of these substances at 0.5 g./l. can replace ammonium sulphate in medium A.

A wide variety of carbon compounds was tested as growth substrates for organism OD1 by incorporating them into medium A at 0.1 and 1.0% (w/v) in place of potassium oxalate. Glycollate, DL-lactate, pyruvate, DL-malate and succinate (as sodium salts) were utilized. Organism OD1 also grew on 10% (v/v) yeast autolysate. There was a considerable lag period when media containing these substrates were inoculated with oxalate-grown cells (Table 1); this was, however, shortened by successive subculture in the appropriate medium. However, once growth had started, it was usually complete within 24 hr., as is the case with oxalate as substrate.

Methanol, formaldehyde, formate, ethanol, acetaldehyde, acetate, ethylene-glycol, glycine, glycerol, malonate, propionate, *n*-butyrate, citrate, glucose, mannose, sucrose, maltose, lactose, starch and dextrin were not utilized by organism OD1 in place of oxalate.

Classification of organism OD 1

Organism OD 1 has not yet been compared directly with other oxalate-decomposing organisms, but from the published descriptions of the latter it would appear to be a different organism; the relevant properties are set out in Table 2. Organism OD 1 differs: from *Bacillus extorquens* which is a larger

Table 1. *Lags before growth of organism OD 1 started on various substrates*

The substrate was incorporated into the medium A in place of potassium oxalate. The media were inoculated with oxalate-grown cells.

Substrate		Lag (days)	Total growth (mg. dry wt./l.)
Oxalate	(1 %, w/v)	1	66
Glycollate	(1 %, w/v)	2	41
DL-Lactate	(1 %, w/v)	7	300
Pyruvate	(1 %, w/v)	7	—
DL-Malate	(1 %, w/v)	7	350
Succinate	(1 %, w/v)	14	60
Yeast autolysate	(10 %, v/v)	4	300

Table 2. *Comparison of the properties of organism OD 1 and other known oxalate-decomposing organisms*

All four organisms are motile.

	<i>Bacillus extorquens</i>	<i>Vibrio oxaliticus</i>	<i>Pseudomonas oxalaticus</i>	Organism OD 1
Dimensions (μ) ...	1.5 $\times$ 3.0	0.4 $\times$ 1.3	0.3 to 0.4 $\times$ 0.9 to 1.5	0.5 $\times$ 1.5
Flagella ...	Single polar	Single polar	1-3 polar	Single polar
Water soluble pigment...	Blood red	—	—	—
Utilization of C sources				
Oxalate	+	+	+	+
Formate	+	+	+	—
Acetate	.	+	+	—
DL-Lactate	.	—	+	+
Citrate	.	—	+	—
DL-Malate	.	—	.	+
Succinate	.	—	.	+

. = Not reported.

organism and produces a blood-red water-soluble pigment; from *Vibrio oxaliticus* which is morphologically different and is able to use acetate and formate but not DL-lactate, DL-malate or succinate; from *Pseudomonas oxalaticus* which can use acetate, formate and citrate. However, two strains (Ox₄ and Ox₆) of *Pseudomonas oxalaticus* (Khambata & Bhat, 1953) used neither citrate nor formate but unlike OD 1 where able to use acetate. Organism OD 1 also differs from *P. oxalaticus* in being unable to grow at 37°.

The morphology and biological properties of organism OD 1 place it in the genus *Pseudomonas*, but it does not resemble any of the species of *Pseudomonas* described in *Bergey's Manual* (1948).

Features of growth of organism OD1 on oxalate

The optimum conditions for growth in liquid medium with oxalate as sole source of carbon and energy were investigated. The galvanometer reading given by the E.E.L. photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) using filter No. 404, was taken as a measure of growth. As these experiments were for the purpose of comparison, the instrument was not calibrated against dry weight and the values, which express total growth, are given in terms of E.E.L. galvanometer readings.

Fig. 1. The effect of the concentration of oxalate on the growth of organism OD1. Oxalate was incorporated into medium A at the concentrations indicated in the figure. After 72 hr. incubation at 25° the total growth was measured by the E.E.L. photoelectric colorimeter using filter no. 404.

Organism OD1 tolerated oxalate in concentrations up to 2% (w/v) with an optimal value of c. 1% (w/v) in the mineral medium A (Fig. 1). In concentrations of over 2% (w/v) there was little or no growth. Added growth factors were not required and yeast autolysate when incorporated into medium A at concentrations between 0.0001 and 5% (v/v) did not stimulate growth within 48 hr. Increasing the concentration of inorganic salts up to 15 times the strength used in medium A did not improve growth; there was hardly any growth in the absence of inorganic salts and very high concentrations of salts inhibited growth. Growth occurred when the initial pH value of the medium was in the range 6.5–9.0; optimum 7.0–8.0. The following measurements were

made on samples removed from cultures of organism OD1 during growth in liquid oxalate medium: (a) the optical density of the culture by the Hilger Spekker absorptiometer using the neutral grey Ilford filter no. H508; (b) the nitrogen content of the centrifuged cells by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943), the ammonia being distilled in the apparatus of Markham (1942) and trapped in 1 ml. of the boric acid reagent of Conway & O'Malley (1942); (c) the amount of oxalate which disappeared from the medium as measured by titration with permanganate; (d) the pH value of the culture with the glass electrode.

Fig. 2. Growth of organism OD1 on liquid oxalate medium A. Changes measured in the following ways and expressed as a percentage of the maximum values were made during growth: (a) optical density in the Hilger-Spekker absorptiometer using filter no. H508 (●—●); (b) nitrogen content of the cells (○—○); (c) the disappearance of oxalate (▲—▲). The pH value of the culture was also measured at intervals (■—■).

There was a prolonged lag phase of about 18 hr. in each case. This could not be shortened either by using heavier inocula or by reinforcing the medium with sodium bicarbonate $\pm$ sodium formate, at a concentration of 0.1 % (w/v). These substances were tested on the assumption that during the oxidation of oxalate, carbon dioxide and formate might be among the first oxidation products and that a lack of these might be the cause of the lag. The formation of cell nitrogen and oxalate utilization paralleled each other, whereas the maximum optical density was reached before all the oxalate was utilized. During growth the pH value of the medium increased from 7.0 to 9.5 (Fig. 2).

Oxidation of oxalate by washed suspensions of organism OD 1

Organism OD 1 was grown at 25° in liquid oxalate medium A and distributed in 300 ml. portions in penicillin flasks (180 mm. diam., 100 mm. deep, James A. Jobling and Co. Ltd., Sunderland, England). Each penicillin flask was inoculated with 2 ml. of a 30 hr. culture of organism OD 1 grown in the same medium. After 40 hr., and whilst the organism was in the logarithmic phase of growth, the cells were harvested by centrifuging at 2500 *g* for 15 min. The cells were washed twice with distilled water and suspended in distilled water

Fig. 3. Oxidation of oxalate by washed suspensions of organism OD 1. Manometer cups contained: main compartment, 1.0 ml. cell suspension (10 mg. dry wt./ml.), 0.5 ml. 0.05 M-triethanolamine buffer (pH 8.0) and inhibitor where indicated; side bulb, 0.1 ml. 0.1 M-oxalate (10 μ mole); centre well, 0.2 ml. 10 % KOH. Total volume of contents, 2.2 ml. Results are corrected for endogenous oxygen uptake. Oxalate alone (●—●); oxalate + 0.001 M-azide (▲—▲); oxalate + 0.0005 M-2,4-dinitrophenol (○—○).

to give 10 mg. dry weight cells/ml. Dry weight was determined by drying a sample of the cell suspension for 4 hr. at 105°. Subsequently, dry weights were estimated by referring to a standard graph which related dry weight (4 hr.; 105°) to optical density as measured in the Hilger Spekker absorptiometer with the neutral grey Ilford filter no. H508.

The oxidation of oxalate by washed suspensions of organism OD 1 was tested manometrically in 0.0125 M-triethanolamine buffer (pH 8.0) at 25°. Gas exchanges were measured by the first method of Dickens & Šimer (1930) as modified by Elsdon & Lewis (1953). The oxidation of oxalate was preceded by a lag period of about 10 min. (Fig. 3). The oxygen uptake was always 60 %

of that required for the complete oxidation of oxalate to CO_2 . In the presence of 0.001 M-sodium azide or 0.0005 M-2:4-dinitrophenol, known to be inhibitors of oxidative assimilation (Clifton, 1937), the oxygen uptake was almost the theoretical for complete oxidation and the respiratory quotients approximated to the theoretical value of 4.0 (Table 3).

Table 3. *The effect of azide and 2:4-dinitrophenol on oxidation of oxalate by washed suspensions of organism OD 1*

Manometer cups contained: main compartment, 1.0 ml. cell suspension (10 mg. dry wt./ml.), 0.5 ml. 0.05 M-triethanolamine buffer (pH 8.0) and inhibitor where indicated; side bulb, substrate; centre well, 0.2 ml. 10 % KOH. Total volume of contents, 2.2 ml.

Substrate	Cells alone		Cells + 0.001 M-azide		Cells + 0.0005 M 2:4-dinitrophenol	
	O_2	CO_2	O_2	CO_2	O_2	CO_2
	uptake ($\mu\text{l.}$)	output ($\mu\text{l.}$)	uptake ($\mu\text{l.}$)	output ($\mu\text{l.}$)	uptake ($\mu\text{l.}$)	output ($\mu\text{l.}$)
(a) None	42	58	42	61	35	45
(b) 10 μmole oxalate	102	369	146	439	135	443
(b) - (a)	60	311	104	378	100	398
Theoretical values*	112	448	112	448	112	448
R.Q. observed	5.18		3.63		3.98	
R.Q. theoretical	4.00		4.00		4.00	

* Theoretical values for complete oxidation of oxalate to CO_2 .

Table 4. *The oxidation of various substrates by oxalate-grown and lactate-grown cells of organism OD 1*

Manometer cups contained: main compartment, 1.0 ml. cell suspension (10 mg. dry wt./ml. in the case of oxalate-grown cells and 15 mg. dry wt./ml. in the case of lactate-grown cells) + 0.5 ml. 0.05 M-triethanolamine buffer (pH 8.0); side bulb, substrate; centre well, 0.2 ml. 10 % KOH. Total volume of contents, 2.2 ml.

Respiratory substrate	Q_{O_2}	
	Oxalate- grown cells	Lactate- grown cells
Oxalate	43	18
Glycollate	6	19
Acetate	5	10
Formate	6	6
Pyruvate	14	17
DL-Lactate	11	22
Succinate	10	4
Fumarate	7	3

Oxidation of other substrates by washed suspensions of organism OD 1

The ability to oxidize other substrates was tested; it varied with the carbon source used for growth (Table 4). (The lactate medium was prepared by replacing potassium oxalate in medium A by 1 % (w/v) sodium lactate.) The main differences to be observed are that when the organism was grown on lactate the Q_{O_2} ($\mu\text{l. O}_2$ consumed/mg. dry weight/hr.) on lactate or glycollate was increased two to threefold, whereas the Q_{O_2} on oxalate was decreased to about half that observed with oxalate-grown cells.

DISCUSSION

Oxalate-decomposing organisms have been isolated from a variety of habitats. Thus *Bacillus extorquens* (Bassalik, 1913), *Vibrio oxaliticus* (Bhat & Barker, 1948) and the organism described in this paper were isolated from soil, whereas the oxalate-decomposing *Proactinomyces* sp. (Müller, 1950) was isolated from sheep rumen and *Pseudomonas oxalaticus* (Khambata & Bhat, 1953) from the alimentary tract of an Indian earthworm. Organism OD1 appears to resemble most closely *P. oxalaticus*, but there are certain differences in the biochemical properties of the two organisms; and further OD1 does not stain readily, whereas Khambata & Bhat (1953) do not appear to have experienced any such difficulty with *P. oxalaticus*. On morphological grounds, e.g. its rod-like shape and polar flagellation, OD1 would appear to be a member of the genus *Pseudomonas* but, until such time as a direct comparison has been made with authentic strains of other oxalate-decomposing organisms, it has been decided to refer to this organism as *Pseudomonas* OD strain 1.

Apart from carbon dioxide, oxalate is the most oxidized carbon compound known to be used by bacteria as a sole source of carbon and energy, and it is obvious that any organism which grows upon oxalate must be capable of reducing it to the level of cell material. In this respect, the metabolism of such oxalate-decomposing organisms resembles that of the aerobic autotrophic bacteria which, as a result of the oxidation of inorganic substrates, obtain energy for the reduction of carbon dioxide to cell substance. Consequently a study of the processes involved in the metabolism of oxalate-decomposing bacteria may help towards an understanding of the metabolism of the autotrophic bacteria.

Since oxalate is such a highly oxidized compound the mechanism of its oxidation would seem likely to involve only a few reactions and a possible pathway may be:

Formate is placed in parenthesis since organism OD1 does not grow upon this substance, and it may be that an active form of formate is involved.

Table 3 shows that when oxalate is oxidized by washed suspensions of organism OD1 the amounts of carbon dioxide produced and of oxygen consumed are below those required for complete oxidation. On the other hand, the respiratory quotient is greater than the theoretical, 5.18 as opposed to 4.0. In the presence of either azide or 2:4-dinitrophenol the gaseous exchanges approached the theoretical values for complete oxidation to CO_2 and the respiratory quotients approximated to 4.0. These calculations have been made on the assumption that the amounts of oxygen consumed and carbon dioxide produced by cell suspensions in absence of substrate could be deducted from the appropriate figures obtained in presence of substrate. This would appear to be justified, at least in so far as the figures for oxygen are concerned, since in the presence of both azide and 2:4-dinitrophenol the total oxygen consumed in the presence of substrate was greater than that required for complete oxidation

of the substrate. The fact that the respiratory quotient is greater than theoretical is in agreement with the view that some of the substrate is being reduced to cell material.

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