

## MICROBIOLOGICAL STUDIES OF PHYTOPATHOGENIC BACTERIA

### 1. Taxonomy of Genus *Erwinia* and Oxidative Metabolism of Carbohydrate

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In the previous paper (Suzuki and Uchida, 1964), it was found that several strains of the *Erwinia amylovora* group of bacteria produced a remarkable amount of 2-ketogluconic acid during the fermentation of glucose under aerobic conditions such as the shaking culture. The ability of the genus *Erwinia* to form ketogenic acid has not been reported by previous workers.

The present paper deals with the comparative investigations on the fermentation patterns of carbohydrate between the bacteria of *Erwinia amylovora* group and *Erwinia carotovora* group. The differences in the cultural and physiological properties between two groups were also reported.

#### METHODS

##### *Microorganisms*

Three strains of *Erwinia amylovora* ATCC 7400, 7401 and 7398, and



Fig. 1. Gall on stem of *Milletia* plant

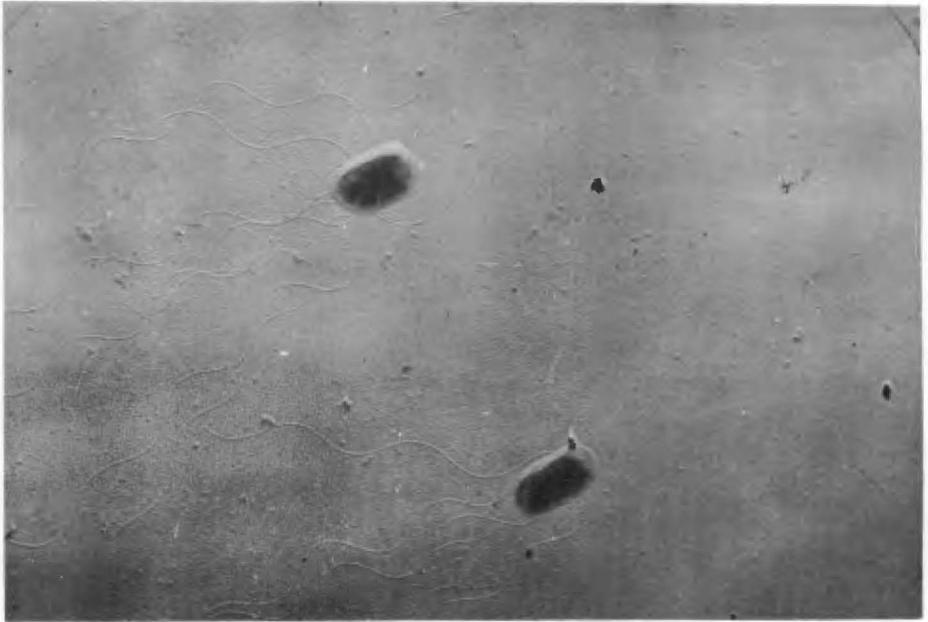


Fig. 2. Electron microscopic photograph of *E. milletiae*

*Erwinia tracheiphila* ATCC 11417 were taken from the lyophilized collections of phytopathogenic bacteria which were furnished by the American Type Culture Collection. One strain of *Erwinia milletiae* was kindly supplied by Dr. M. Goto of Shizuoka University and the other strains of *E. milletiae* were isolated from the galls on the *Milletia* plants in the garden of the Ohara Institute (Figure 1). All of these strains isolated were motile with 7-8 peripheral flagella. Flagellation was observed with a electronmicroscope of the Hitachi HS-6 type (Figure 2) and also with the application of the staining method of Löffler. Inoculation experiments with these organisms on young stems of the plant gave typical tumor growth from which the same organisms were reisolated. The other microorganisms used in this work were as follows: *Erwinia carotovora*, *Erwinia aroideae*, *Escherichia coli*, *Aerobacter aerogenes* and *Serratia marcescens*.

#### *Analytical methods*

Sugar determination was carried out in the usual manner by the Bertrand's method. 2-Ketogluconic acid, acetic acid,  $\alpha$ -ketoglutaric acid and protein were estimated by the methods of Stubbs et al. (7), Friedemann and Brook (1), Shimizu (5) and Lowry et al. (4), respectively.

## RESULTS

### 1. *Production of 2-Ketogluconic Acid by Shaking Cultures of Various Strains of*

TABLE 1  
Production of 2-ketogluconic acid from glucose by various strains of *E. amylovora* group of bacteria

Organisms	Incubation days	Consumption of glucose (%)	2-Ketogluconic acid produced (g/100 ml)
<i>E. amylovora</i>			
ATCC 7400	6	95.6	5.78
ATCC 7401	6	93.3	1.45
ATCC 7398	6	92.8	2.10
<i>E. tracheiphila</i>			
ATCC 11417	6	15.3	0.08
<i>E. milletiae</i>			
No. 1	4	97.9	8.82
No. 2	4	96.7	8.05
No. 3	4	92.5	7.95
No. 4	4	94.0	8.20

TABLE 2  
Production of 2-ketogluconic acid from gluconate in growing culture of *E. milletiae*

<i>E. milletiae</i>	No. 1	No. 2	No. 3	No. 4
Incubation days	2	2	2	1
Concentration of calcium gluconate (g/50 ml)	5.00	5.00	5.00	5.00
Reduced copper (g/50 ml)	4.71	4.57	4.38	3.54
2-Ketogluconic acid produced (g/50 ml)	2.84	2.74	2.59	2.09

Cultivated at 28°C on the medium containing 10% calcium gluconate, 0.3% polypeptone, 0.1% NaCl, 0.05% K<sub>2</sub>HPO<sub>4</sub>, 0.04% MgSO<sub>4</sub> · 7H<sub>2</sub>O, 5mg% FeSO<sub>4</sub> · 7H<sub>2</sub>O and 1% CaCO<sub>3</sub>.

#### *E. amylovora* Group of Bacteria

The fermentation experiments were carried out with a medium containing 10% glucose, 0.3% polypeptone, 0.05% yeast extract, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.2% NaCl, 0.04% MgSO<sub>4</sub> · 7H<sub>2</sub>O and 5 mg% FeSO<sub>4</sub> · 7H<sub>2</sub>O. The pH of the medium was adjusted to 7.0 with sodium hydroxide. The medium was dispensed in 100 ml quantities in 500 ml shaking flasks, and then sterilized. Prior to inoculation, sterile calcium carbonate was added to each medium. After inoculation with 2 ml of a 2-3 days' culture grown aerobically in a potato sugar medium, the flasks were incubated at 28°C for 4-6 days on a shaking apparatus. After incubation, the products of fermentation were analysed. The results are shown in Table 1. All the strains of the *E. amylovora* group of bacteria used in the experiments were recognized to produce a considerable amount of 2-ketogluconic acid in the course of oxidation of

glucose. Furthermore, it will be seen in Table 2 that a remarkable production of 2-ketogluconic acid took place after 2 days' incubation, when *E. milletiae* was cultivated with gluconate medium under shaking conditions.

## 2. Isolation and Identification of 2-Ketogluconic Acid

*E. milletiae* was cultured at 28°C on a shaker with 10% glucose medium mentioned in the previous experiments. The course of the biological oxidation was followed by measuring the optical rotation of aliquots of the culture supernatant. When the maximum negative rotation was observed, the culture was centrifuged in order to remove bacterial cells and residual calcium carbonate. The supernatant was treated with decolorizing carbon at 50°C. To the transparent liquor, two volumes of ethanol were added slowly with stirring and the mixture kept to stand overnight at 0°C. A precipitate formed was collected by decantation and recrystallized from the minimal amount of water to obtain pure calcium salt of the preparation. This calcium salt had a strong reducing action on warm Fehling's solution and gave no colored products on the reaction with naphthoresorcinol, phloroglucinol and orcinol. And also it appeared as a deep olive green spot on the paper with a orthophenylenediamine spray. Then this calcium salt was dissolved in water, acidified by the addition of oxalic acid and the resulting precipitate of calcium oxalate was removed by centrifugation. The clear supernatant was then extracted with ether in order to remove the residuary oxalic acid. The ether insoluble part was neutralized with potassium carbonate and concentrated to a small volume in vacuo at 35°C, to which ethanol was added until precipitation occurred. After keeping overnight at 0°C, fine diamond-shaped crystals were separated and were purified by redissolving in water and recrystallizing with ethanol. The melting point of this crystalline preparation was 152°C, not depressed by the admixture with the authentic potassium 2-ketogluconate.

Identification of 2-ketogluconic acid was established with the preparation of its methylester by mixing 0.7 g of potassium salt in 8 ml of absolute methanol and 0.1 ml of conc. H<sub>2</sub>SO<sub>4</sub> at 70-80°C. The crystals, which separated after 3 hours' reaction, were recrystallized from hot methanol to constant melting point (171-172°C). The mixed melting point with the authentic specimen was identical. Results of elementary analysis were as follows: Found: C, 40.46; H, 6.06 Calcd. for C<sub>7</sub>H<sub>12</sub>O<sub>7</sub>: C, 40.38; H, 5.82%.

These data confirmed the fermentation product by *E. milletiae* to be 2-ketogluconic acid. And also the isolation of 2-ketogluconic acid from the culture fluid of *E. amylovora* ATCC 7400 was carried out according to the same procedure as described above and 2-ketogluconic acid was obtained as crystals of potassium salt of melting point 151-153°C. Its methylester showed a melting point 172-173°C, just the same as that of 2-ketogluconic

acid.

### 3. Chemical Changes during Fermentation of Glucose

Five hundreds ml shaking flasks containing 100 ml of 10% glucose medium were inoculated with *E. milletiae* and incubated on a reciprocating shaker at 28°C. Progress of the fermentation was followed by analyses of both reducing power and optical rotation power. Paperchromatography (Koepsell, Stodola and Sharpe, 1952) was also employed for noting the appearance of fermentation products.

From the results shown in Figure 3, it will be seen that both the consumption of glucose and the production of 2-ketogluconic acid commenced within 24 hours after inoculation and thereafter the accumulation of 2-

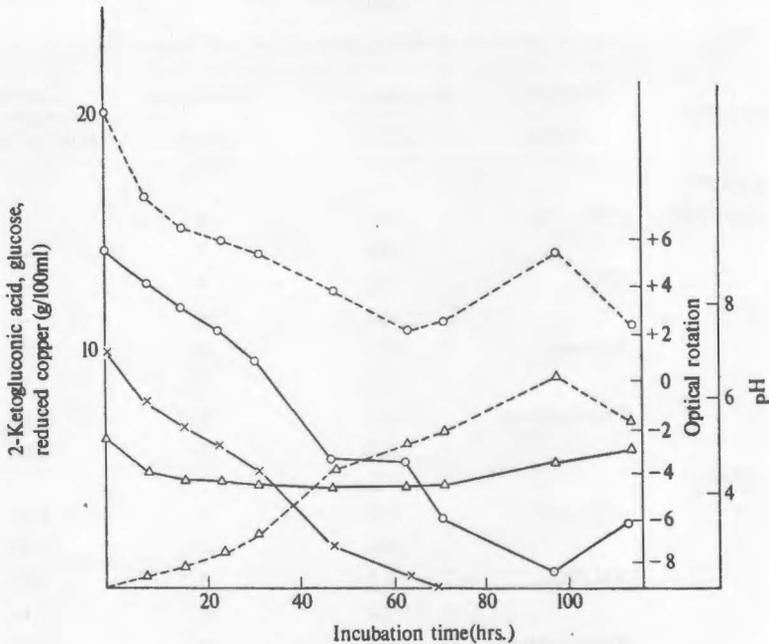


Fig. 3. Chemical changes during fermentation of *E. milletiae*  
 reduced copper ○.....○ optical rotation ○—○  
 2-ketogluconic acid △.....△ glucose ×—×  
 pH △—△

ketogluconic acid increased successively. After 4 days incubation, the amount of 2-ketogluconic acid attained a maximum value (the yield being about 85% on glucose used) and then gradually decreased. Similar phenomena were observed with *E. amylovora* ATCC 7400. 5-Ketogluconic acid was never detected on paperchromatograms of the growing cultures of *E. milletiae* and *E. amylovora*.

### 4. Effect of Nitrogen Source upon Production of 2-Ketogluconic Acid by the Bacteria

of *E. amylovora* Group

In the following experiments, the basal medium was composed of 10 g glucose, 0.1 g  $K_2HPO_4$ , 0.2 g NaCl, 0.04 g  $MgSO_4 \cdot 7H_2O$  and 5 mg  $FeSO_4 \cdot 7H_2O$  in 100 ml of distilled water, and sterile calcium carbonate in the amount of 40% of glucose used was added separately. The fermentation was carried out as described in the previous experiments. Several kinds of nitrogen sources, as listed in Table 3, were tested for their effects on the accumulation of 2-ketogluconic acid. As shown in Table 3, both the inorganic and organic nitrogen sources were very effective for acid production with *E. milletiae*. On the other hand, in *E. amylovora* the inorganic nitrogen sources resulted in negative yields. Further, the yield of 2-ketogluconic acid obtained

TABLE 3  
Effect of nitrogen sources on the production of 2-ketogluconic acid

Organisms	Nitrogen sources	Concentration (%)	Incubation days	Yield of 2-ketogluconic acid (g/100 ml)
<i>E. amylovora</i>				
ATCC 7400	$(NH_4)_2SO_4$	0.1	4	+
		0.5	4	—
	KNO <sub>3</sub>	0.1	4	—
		0.5	4	—
	Peptone	0.1	4	3.34
		0.5	4	1.77
	Yeast extract	0.1	4	5.26
		0.5	4	2.08
<i>E. milletiae</i>				
No. 4	$(NH_4)_2SO_4$	0.1	4	8.55
		0.5	4	1.90
	NH <sub>4</sub> NO <sub>3</sub>	0.1	4	7.65
		0.5	4	1.19
	Yeast extract	0.3	4	8.80
		1.0	4	2.43
	Polypeptone	0.3	4	8.10
		1.0	4	2.15

was affected markedly by the amount of nitrogen supplied on the medium. At the low levels of nitrogen, fermentation was slow but 2-ketogluconic acid was obtained in high yields, while at high levels fermentation was very much more rapid but the yields of 2-ketogluconic acid were low or non-existent.

5. Oxidation of Ethanol and Polyhydric Alcohols by Species of *E. amylovora* Group

It is well-known that the bacteria of genus *Acetobacter* and genus *Gluconobacter* produce acetic acid from ethanol and ketogenic compounds from

sorbitol, mannitol and glycerol. It has been reported by Stanier (6) that some strains of *Pseudomonas fluorescens* are also able to oxidize ethanol to acetic acid. On the other hand, the production of 2-ketogluconic acid was observed with some species belonging to the genus *Acetobacter*, *Gluconobacter* and *Pseudomonas*.

In the present paper, experiments were carried out in order to ascertain the oxidative power for ethanol and to ascertain whether ketogenic compounds would be obtained in the oxidation of polyhydric alcohols by the bacteria of *E. amylovora* group. Fermentation was instituted with the medium containing 2% absolute ethanol in 50 ml of 1% yeast extract. Acid formation was tested by the method of Friedemann and Brook (1938), after incubation at 28°C for 10 days in stationary conditions. All of the strains belonging to the *E. amylovora* group were very weak in their oxidative abilities for ethanol.

The composition of the cultural medium used in the oxidation of polyhydric alcohols was similar to the shaking culture mentioned above, except mannitol, sorbitol and glycerol were added in place of glucose. Shaking flasks containing 30 ml of the medium were inoculated with various strains of *E. amylovora* group and then incubated at 28°C for 10 days on a shaker. Reducing power of broth during incubation was determined by the Bertrand's method at two days intervals. All strains could not produce any ketogenic compounds from these polyhydric alcohols.

Moreover, the authors examined whether the substances having positive ferric chloride reaction would be obtained from glucose and fructose respectively by the bacteria of *E. amylovora* group. The bacteria were grown on 1% yeast extract medium containing the substrate mentioned above in a five percent concentration under aerobic conditions. After incubation at 28°C for 7 days, culture liquor was centrifuged. The supernatant was tested for color reaction with 1%  $\text{FeCl}_3$  aqueous solution. None of the strains tested gave a reddish violet color reaction.

#### 6. Production of $\alpha$ -Ketoglutaric Acid in Growing Culture by the Bacteria of *E. carotovora* Group

Fifty ml of the media containing, in per cent, 10 calcium gluconate or 8 glucose, 0.3-0.15 polypeptone, 0.2 NaCl, 0.05  $\text{K}_2\text{HPO}_4$ , 0.04  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.005  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  in water were placed in 200 ml shaking flasks and then sterilized. Prior to inoculation, sterile calcium carbonate was added to each medium. After inoculation with 2 ml of a 2 days' culture of the bacteria grown on bouillon, the flasks were incubated at 28°C for 7 days under shaking conditions. At the end of fermentation, the cultures were analysed. The results are shown in Table 4. A remarkable fact was that  $\alpha$ -ketoglutaric acid fermentation took place, instead of 2-ketogluconic acid fermentation, with glucose medium by *E. aroideae* which belonged to

TABLE 4  
Aerobic oxidation of glucose and gluconate  
by various species of genus *Erwinia*

Organisms	<i>E. milletiae</i> No. 1	<i>E. carotovora</i> IAM 1024	<i>E. aroideae</i> IAM 1068				
Incubation days	2	2	2	5	7	7	7
Concentration of glucose							
Initial (g/50 ml)			4.000	1.000	1.000		
Final (g/50 ml)			1.870	0.020	0.090		
Concentration of calcium gluconate							
Initial (g/50 ml)	5.000	5.000	5.000			1.250	1.250
Reduced copper (mg/50 ml)	4705	60	85				
2-Ketogluconic acid produced (mg/50 ml)	2840	0	0	0	0	0	0
$\alpha$ -Ketoglutaric acid produced (mg/50 ml)	158	595	734	659	257	608	292

the *E. carotovora* group of bacteria. The yield of  $\alpha$ -ketoglutaric acid was observed to be about 31% on glucose consumed after 5 days' incubation when 8% glucose was employed.

Furthermore, it was pointed out that all strains of *E. carotovora* group used in this study accumulated  $\alpha$ -ketoglutaric acid as a major product during the oxidative fermentation of gluconate, contrary to strains of *E. amylovora* group which produced a large quantity of 2-ketogluconic acid.

Lockwood and Stodola (3) found that certain species of *Pseudomonas* bacteria oxidized glucose to gluconate and 2-ketogluconate successively in the early stage of fermentation, and then produced  $\alpha$ -ketoglutaric acid in the yields of 0.50 to 0.55 mole per mole of glucose consumed on continued oxidation. Subsequently, paperchromatography (2) was employed for noting the appearance of 2-ketogluconic acid, pyruvic acid and  $\alpha$ -ketoglutaric acid. However, 2-ketogluconic acid was never detected at any stage of fermentation by the bacteria of *E. carotovora* group.

#### 7. Isolation and Identification of $\alpha$ -Ketoglutaric Acid from Shaking Culture

A portion of the fermentation liquor was filtered off. The filtrate, after decolorization with carbon, was concentrated to a small volume. After being kept overnight, fine needle-shaped crystals formed were collected and dissolved in a small amount of warm water, acidified with hydrochloric acid and extracted with ether. Concentration of the ether extract gave crystals of free acid. After recrystallization from water, crystals of melting point 115-116°C were obtained. No depression of melting point was observed when they were mixed with synthetic  $\alpha$ -ketoglutaric acid. For identification,

the recrystallized preparation was converted into 2,4-dinitrophenylhydrazone. An hydrochloric acid solution of 2,4-dinitrophenylhydrazine was added to the recrystallized preparation, whereby a yellow precipitate was formed. The precipitate was collected, washed with 2N-hydrochloric acid and dried under reduced pressure. The crude 2,4-dinitrophenylhydrazone was dissolved in ethylacetate and then extracted with 10% sodium carbonate solution. When the carbonate extract was acidified with hydrochloric acid solution, a pale yellow crystalline precipitate was formed. After washed with dilute hydrochloric acid and cold water, the precipitate was recrystallized from ethanol and ethylacetate. This preparation melted at 222°C, which melting point quite coincided with that of synthetic 2,4-dinitrophenylhydrazone of  $\alpha$ -ketoglutaric acid, and the mixed melting point was

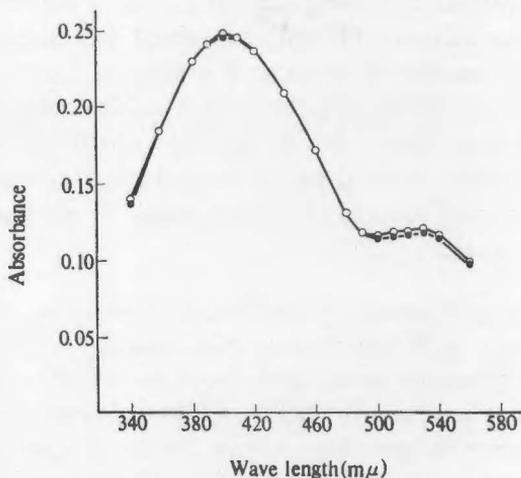


Fig. 4. Absorption spectra of 2,4-dinitrophenylhydrazones of  $\alpha$ -ketoglutaric acid and the sample

Solvent: 10%  $\text{Na}_2\text{CO}_3 + 1.5 \text{ N-NaOH}$

Sample ○—○  
Standard ●—●

not depressed. Ultraviolet absorption spectrum of this preparation agreed well with that of 2,4-dinitrophenylhydrazone of a synthetic (authentic)  $\alpha$ -ketoglutaric acid as shown in Figure 4.

#### 8. Production of $\alpha$ -Ketoglutaric Acid from Various Carbonaceous Compounds by Washed Cells of *E. aroideae*

*E. aroideae* IAM 1068 was grown at 28°C in a medium consisting of 10% glucose, 0.3% polypeptone, 0.05% yeast extract, 0.1%  $\text{K}_2\text{HPO}_4$ , 0.2%  $\text{NaCl}$ , 0.04%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 mg%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and 4%  $\text{CaCO}_3$  (sterilized separately). After inoculation with 2 ml of a 1-2 days' culture of the bacteria on bouillon and incubation on a shaker for 4-5 days, the bacterial cells were

TABLE 5  
Production of  $\alpha$ -ketoglutaric acid from varied sources  
of carbon by washed cells of *E. aroideae*

Carbon sources	Glucose	Pectin	Pectic acid	Sodium pyruvate
Substrate (mmoles)				
Initial	4.170	500*	500*	3.000
Used	2.221			3.000
$\alpha$ -Ketoglutaric acid produced (mmoles)	0.892	57*	91*	0.488
2-Ketogluconic acid produced (mmoles)	0	0	0	

\*mg per 40 ml of reaction mixture

harvested by centrifugation, washed and then suspended in distilled water. Experiments with washed cells were carried out on a shaker at 28°C for 17 hours. The reaction mixture (40 ml) contained 4-5 mmoles of phosphate buffer (pH 7.0), 100  $\mu$ moles of  $MgSO_4$ , 4  $\mu$ moles of  $FeSO_4$ , the suspension of washed cells (100 mg of dry weight) and requisite amounts of substrates, which are illustrated in Table 5. It can be recognized in Table 5 that washed cells of *E. aroideae* were capable of producing  $\alpha$ -ketoglutaric acid in a high yield from varied sources of carbon, such as glucose, pectin, pectic acid or sodium pyruvate.

9. *Aerobic Oxidation of Gluconate by the Enzyme Preparation such as Dried Cells and Cell-free extracts of E. aroideae and of E. milletiae*

The experimental results mentioned above, in which  $\alpha$ -ketoglutaric acid was produced without any accumulation of 2-ketogluconic acid in the course of oxidation of glucose in growing culture by the bacteria of *E. carotovora* group, led to the authors to enquire whether 2-ketogluconic acid would be an intermediate product in  $\alpha$ -ketoglutaric acid fermentation by the bacteria of *E. carotovora* group. Experiments were at first carried out with dried cells of *E. aroideae* IAM 1068 and of *E. milletiae* grown aerobically on glucose medium in the same manner as already mentioned. The bacterial cells obtained by centrifugation were dried under an electric fan for 5-7 hours and placed in a desiccator over concentrated sulfuric acid for one night. After suspending in water, dried cells were added to the reaction mixture containing 2 mmoles of potassium gluconate, 4 mmoles of phosphate buffer (pH 6.0) and 100  $\mu$ moles of  $MgSO_4$ , and the volume was made up with water to 20 ml. Incubation was performed on a shaker at 28°C for 24 hours. Results are presented in Table 6, from which no production of 2-ketogluconic acid from gluconate with dried cells of *E. aroideae* was observed, while in *E. milletiae* dried cells were capable of producing 2-ketogluconic acid in a high potency (yield being about 0.8 mole per mole of gluconic acid after 24 hours' incubation).

TABLE 6  
Aerobic oxidation of gluconate by dried cells and cell-free preparations of *E. aroideae* and *E. milletiae*

Organisms	Enzyme preparation	Incubation time (hours)	Concentration of potassium gluconate (mmoles)	Reduced copper (mg/20 ml)	2-Ketogluconic acid produced (mmoles)	$\alpha$ -Ketoglutaric acid produced (mmoles)
<i>E. aroideae</i>	Dried cells	4.5	1.923	0	0	0
		9.0	1.923	0	0	0.116
		24.0	1.923	0	0	0.328
IAM 1068	Cell-free extracts	15.0	1.923	0	0	0.088
		4.5	1.923	75	0.226	0
<i>E. milletiae</i>	Dried cells	9.0	1.923	165	0.479	0
		24.0	1.923	537	1.588	+
		15.0	1.923	150	0.407	

In the case of cell-free extracts, total volume of reaction mixture was 15ml.

Oxidation of gluconate was further investigated with cell-free preparations of the bacteria. Cell-free extracts were prepared by exposing the washed cell suspension to sonic vibration in a Kubota 10 Kc. oscillator. After 20 minutes' treatment, cellular debris was removed by centrifugation at  $10,000\times g$  for 30 minutes and discarded. The resulting crude extracts containing 28 mg of protein per ml were used for the following experiments.

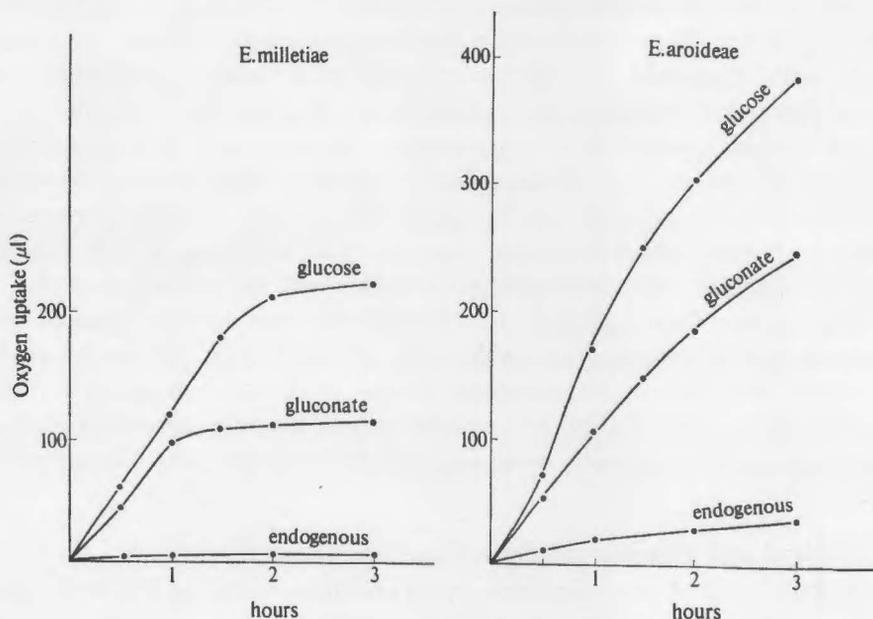


Fig. 5. Oxidation of glucose and gluconate in Warburg manometer by cell-free extracts of *E. aroideae* and *E. milletiae*. After 30 and 60 minutes' incubation, fermentation products were examined by paperchromatography.

The reaction mixture (15 ml) in a shaking flask contained 2 mmoles of potassium gluconate, 3 mmoles of phosphate buffer (pH 6.0), 100  $\mu$ moles of  $MgSO_4$ , 5 ml of crude cell-free extracts and water. Incubation was carried out at 28°C for 15 hours on a shaker. One ml aliquot of the reaction mixture was withdrawn at certain intervals and oxidative products from gluconate were examined by the application of paperchromatography. In the case of *E. aroideae*, any olive green spot which was given by 2-ketogluconic acid was never detected on the paper with a o-phenylenediamine spray. In contrast to the lack of activity in *E. aroideae*, the 2-ketogluconic acid-producing activity of *E. milletiae* extracts was very high.

These results were further ascertained in the following manometric experiments. Warburg vessels contained 10  $\mu$ moles of potassium gluconate, 50  $\mu$ moles of phosphate buffer (pH 6.0), 2  $\mu$ moles of  $MgSO_4$  and one ml of cell-free extracts, and the total volume was 2.3 ml. Oxidation was measured in presence of 0.2 ml of 15 % KOH in centre well. Incubation was performed at 30°C in air. The results are shown in Figure 5. When glucose and gluconate were oxidized in the Warburg vessel by cell-free extracts of *E. milletiae*, about one and 0.5 moles of oxygen per mole of substrate were consumed, respectively. These quantities of oxygen are the amounts necessary in each case for oxidation of the substrate to 2-ketogluconic acid. A paperchromatographic analysis of the cup contents showed 2-ketogluconic acid as the sole detectable product of glucose or gluconate oxidation, and 2-ketogluconic acid was present in a high concentration. While, the oxygen uptake with gluconate by cell-free extracts of *E. aroideae* proceeded well beyond theoretical values for the conversion of gluconate to 2-ketogluconate, and no oxygen uptake of 2-ketogluconate was observed. 2-Ketogluconate could not be obtained as an intermediate product even in the case of the oxidation of large quantities of gluconate (100  $\mu$ moles), and the pentose-like substance having positive orcinol reaction was detected in this reaction mixture. Further properties of this substance will be published later.

The above data indicate that *E. milletiae* metabolized glucose into gluconate and 2-ketogluconate oxidatively, whereas in *E. aroideae* 2-ketogluconic acid was not an intermediate in the oxidative fermentation. And it is strongly suggested that *E. carotovora* group may be distinguished from *E. amylovora* group principally by reason of its failure to produce 2-ketogluconic acid.

#### 10. Cultural and Physiological Properties of the Genus *Erwinia*

Cultural and physiological properties are summarized in Tables 7 and 8. All experiments were run in duplicate at 30°C.

Bouillon agar slants. All the bacteria, except *E. milletiae* and two strains of the genus *Serratia*, produced greyish-white to milky colored growth

TABLE 7  
Cultural and physiological characters of various species of the genus  
*Erwinia* and some related nonpathogenic bacteria

Species	Bouillon agar	Potato-sucrose agar	NO <sub>3</sub> to NO <sub>2</sub>	Production of H <sub>2</sub> S	Secretion of protopectinase	Hydrolysis of starch	Indole formation	Voges-Proskauer test	Utilization of citrate
<i>Erwinia amylovora</i> group									
<i>Erwinia amylovora</i>									
ATCC 7400	White	White	—	+	—	—	—	+	—
ATCC 7401	White	White	—	+	—	—	—	+	—
ATCC 7398	White	White	—	+	—	—	—	+	—
<i>Erwinia tracheiphila</i>									
ATCC 11417	White	White	—	±	—	—	—	—	—
<i>Erwinia milletiae</i>									
No. 1	Yellow	Yellow	+	+	—	—	—	+	+
No. 2	Yellow	Yellow	+	+	—	—	—	+	+
No. 3	Yellow	Yellow	+	+	—	—	—	+	+
No. 4	Yellow	Yellow	+	+	—	—	—	+	+
<i>Erwinia carotovora</i> group									
<i>Erwinia carotovora</i>									
IAM 1024	White	White	+	+	+	—	—	+	+
No. B-21	White	White	+	+	+	—	—	+	+
<i>Erwinia aroideae</i>									
IAM 1068	White	White	+	+	+	—	—	+	+
No. S-1	White	White	+	+	+	—	—	+	+
<i>Escherichia coli</i>									
G-2, E-8, E-9	White	White	+	+	—	—	+	—	—
<i>Aerobacter aerogenes</i>									
ATCC 7256, 8308	White	White	+	+	—	+	—	+	+
<i>Serratia marcescens</i>									
No. B-30, B-31	Red	Red	+	+	—	—	—	+	—

TABLE 8  
 Fermentation of carbohydrates by various species of the  
 genus *Erwinia* and some related nonpathogenic bacteria

Species	Glycerol	D-Xylose	D-Glucose	D-Fructose	D-Galactose	Lactose	Maltose	Sucrose	Mannit	Formation of reducing substance from gluconate under shaking culture
<i>Erwinia amylovora</i> group										
<i>Erwinia amylovora</i>										
ATCC 7400	—	±	++	++	—	—	—	++	—	+++
ATCC 7401	—	—	++	++	—	—	—	++	—	+
ATCC 7398	—	—	++	++	—	—	—	++	—	++
<i>Erwinia tracheiphila</i>										
ATCC 11417	—	—	+	+	—	—	—	+	—	+
<i>Erwinia milletiae</i>										
No. 1	+	+	++	++	+	—	+	+++	+	+++
No. 2	+	+	++	++	+	—	+	+++	+	+++
No. 3	+	+	++	++	+	—	+	+++	+	+++
No. 4	+	+	++	++	+	—	+	+++	+	+++
<i>Erwinia carotovora</i> group										
<i>Erwinia carotovora</i>										
IAM 1024	+	+	++	++	+	+	±	++	+	—
No. B-21	+	+	++	++	+	+	±	++	+	—
<i>Erwinia aroideae</i>										
IAM 1068	+	+	++	++	+	+	±	++	+	—
No. S-1	+	+	++	++	+	+	±	++	+	—
<i>Escherichia coli</i>										
No. G-2, E-8, E-9	+	++	+++	+++	++	++	+++	+	++	—
<i>Aerobacter aerogenes</i>										
ATCC 7256, 8308	+++	++	+++	+++	++	++	+++	+	++	—
<i>Serratia marcescens</i>										
No. B-30, B-31	+	+	++	++	+	—	+	+	+	+++

on bouillon agar slants. *E. milletiae* was yellow. *Serratia* bacteria were orange red. The bacteria of *E. carotovora* group produced a rather more abundant growth than *E. amylovora* group. But none of these bacteria grew as luxuriantly as *coli-aerogenes* bacteria.

Potato-sucrose agar slants. All cultures grew much more abundantly on potato-sucrose agar slants than on bouillon agar slants. *E. milletiae* produced a characteristic yellow pigment on potato-sucrose agar after 2 days' cultivation.

Reduction of nitrates. The bacteria were cultured for 2 or 5 days in bouillon containing 0.1 %  $\text{KNO}_3$  and these cultures were tested on nitrite production from nitrate using  $\alpha$ -naphthylamine and sulfanilic acid. *E. amylovora* and *E. tracheiphila* did not reduce nitrate even in several trials, but *E. milletiae* reduced. All strains of *E. carotovora* group, *coli-aerogenes* bacteria and *Serratia* reduced nitrate to nitrite.

Formation of hydrogen sulfide. The ability of bacteria to produce hydrogen sulfide was examined by growing on bouillon containing 0.05% cysteine solution. The lead acetate-soaked paper strips were used as the indicators of hydrogen sulfide. All of the bacteria produced hydrogen sulfide sufficient to blacken the paper strips suspended over the media during the incubation.

Production of protopectinase. In the present study protopectinase activity was tested by means of the softening of potato slice. Raw potato slices were placed in petri dishes containing two layers of water-soaked filter paper and inoculated by placing a loopful of bacterial suspension, obtained by washing the bacteria from a 2 days-old agar culture, in a drop of water on the top of each slice of potato. Both *E. aroideae* and *E. carotovora* caused a visible softening of potato slices within 2 days, that is to say, they were able to secrete protopectinase in amount sufficient to soften raw potato slices, whereas the other yielded no trace of softening activity.

Hydrolysis of starch. On the bouillon agar plate containing 0.2 % of soluble starch, test organisms were incubated. A weak iodine solution was dropped on each culture plate to test for hydrolysis, but it remained blue.

Indole formation. The microorganisms were inoculated in 2 % peptone water and tested by the Ehrlich Bohme method after 5 days' incubation. None of the plant pathogens studied produced indole under the condition, whereas *Escherichia coli* produced a positive reaction.

Voges-Proskauer test. The Voges-Proskauer test was determined by growing the organisms in the medium containing 0.5 % glucose, 0.5 % polypeptone and 0.5 %  $\text{K}_2\text{HPO}_4$ . Determinations were conducted with 2, 4 and 7 days' cultures using 40% KOH and  $\alpha$ -naphthol. With the exception of *E. milletiae*, the Voges-Proskauer test was not as clear-cut when

used with the plant pathogens as it was with *coli-aerogenes* bacteria. The bacteria of *E. carotovora* group seemed to be definitely positive. *E. milletiae* gave a strong positive reaction identical with the *Aerobacter* species of the *coli*-form bacteria. In *E. amylovora*, the results of the Voges-Proskauer test were doubtful, but pyruvic acid was observed to give rise to a large quantity of acetoin which was identified as nickeldimethylglyoxime, when fermentation was carried out at 28°C under stationary conditions with the reaction mixture (30 ml) containing 3 mmoles of sodium pyruvate, 5 mmoles of phosphate buffer (pH 5.3), 100  $\mu$ moles of  $MgSO_4$ , 100  $\mu$ moles of  $MnSO_4$  and washed cells (100 mg of dried weight) of *E. amylovora* grown aerobically at 28°C for 4 days on 10 % glucose medium mentioned in the previous experiment. Consequently *E. amylovora* was also found to be positive.

Growth on the Koser's citrate medium. A citrate medium composed of 0.3% sodium citrate, 0.15 %  $Na(NH_4) HPO_4$ , 0.1 %  $KH_2PO_4$  and 0.02 %  $MgSO_4$  was incubated after inoculation from young agar slant cultures. After 2 days' incubation, serial transfers to fresh citrate medium were made. Growth, as evidenced by turbidity, in the medium was examined in the second serial transfer after 2 days' incubation. *E. milletiae*, *E. aroideae*, *E. carotovora*, *Aerobacter aerogenes* and *Serratia marcescens* which were able to utilize citrate as a sole source of carbon grew vigorously within 24 hours, but *E. amylovora* and *Escherichia coli* showed no growth after the lapse of 7 days.

Fermentation of carbohydrates. Culture medium employed for the fermentation of carbohydrates was peptone water in test tube containing 0.5 % of various carbohydrates and brom cresol purple as the indicator. Production of acid was detected by the color change of culture liquid, purple to yellow. All fermentation tube cultures were incubated at 30°C for 3 days. Results are listed in Table 8. *E. amylovora* and *E. tracheiphila* fermented only a limited number of substrates tested with acid production, while all of the other bacteria attacked a majority of the substrates tested. The tendency to ferment only a few of the substrates indicates that *E. amylovora* and *E. tracheiphila* are physiologically more highly specialized than the other bacteria studied. In *E. milletiae*, acid production was recognized on every culture out of lactose tube and the strongest was sucrose tube and the weakest glycerol tube. Both *E. carotovora* and *E. aroideae* fermented almost as wide a range of substrates as *coli-aerogenes* bacteria, but their fermentation type was much less vigorous. With respect to gluconate fermentation under shaking conditions, all of the *E. amylovora* group bacteria and *Serratia marcescens* produced 2-ketogluconic acid. The cultures of the *E. carotovora* group bacteria, *Escherichia coli* and *Aerobacter aerogenes*, on the other hand, failed to produce even a significant amount of 2-ketogluconic acid from gluconate.

The results of the cultural and physiological studies show that (1) *E. amylovora* and *E. tracheiphila* differed from the bacteria of *E. carotovora* group

in that they were able to do no reduction of nitrate, no secretion of pro-pectinase and no utilization of citrate and to attack the limited range of fermentable carbohydrate and to produce 2-ketogluconic acid; (2) *E. milletiae* showed a close relationship to *Serratia marcescens* in the biochemical activities, such as the fermentation of carbohydrates and production of 2-ketogluconic acid; (3) the production of 2-ketogluconic acid should be pointed out as one of the effective means of distinguishing the *E. amylovora* group from the *E. carotovora* group.

#### SUMMARY

Comparative investigations were carried out on the oxidative degradation of glucose by two groups of the genus *Erwinia*. The *E. carotovora* group was found to accumulate a large amount of  $\alpha$ -ketoglutaric acid from glucose (the yield being about 31 % on glucose consumed). Pectin, pectic acid and pyruvic acid were also the good carbon sources for the production of  $\alpha$ -ketoglutaric acid by the bacteria.  $\alpha$ -Ketoglutaric acid was produced without any accumulation of 2-ketogluconic acid in the course of oxidation of glucose or gluconic acid by dried cells and cell-free extracts of *E. aroideae* (belong to the *E. carotovora* group). On the other hand, cell-free extracts of *E. milletiae* (belong to the *E. amylovora* group) oxidized glucose and gluconic acid with an uptake of 1.0 and 0.5 moles oxygen per mole of the substrate, respectively. 2-Ketogluconic acid was the sole product detected at the end of oxidation by chromatographic analysis.

Cultural and physiological properties of twelve strains belonging to the genus *Erwinia* were studied comparatively with closely related non-pathogenic bacteria.

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