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Comparative studies on the oxidative activities of glucose and succinate by psychrophilic and mesophilic pseudomonad

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Facultative psychrophilic bacteria, *Pseudomonas* sp. strain 351 (P-351) was examined in respect of growth rate, oxidative activities of glucose and succinate, and enzymatic activities of NADH, succinate and isocitrate dehydrogenases at various temperatures. As a control, mesophilic bacteria *Pseudomonas aeruginosa* was used.

1. P-351 could grow well at 0°C and had an optimum temperature for growth at 30° to 35°C and a maximum temperature at 37°C, while *P. aeruginosa* having an optimum temperature at 37°C could not grow at 10°C or below.

2. Temperature-activities relationships in glucose oxidation by P-351 and *P. aeruginosa* were found to be markedly different, namely, both intact cells and cell-free extracts of P-351 oxidized glucose most rapidly at 30°C and even at 5°C the oxidative activity remained at about 30% of the maximum, while in *P. aeruginosa* the rate of glucose oxidation decreased remarkably with lowering temperature.

3. Several inhibitors were used to determine which pathways were operating for glucose degradation. These results indicated that glucose was oxidized *via* TCA cycle and respiratory chain in P-351, but the early processes of glucose degradation were not clarified.

4. P-351 was recognized to be a bacterium which was easily allowed to autolyze at higher temperatures, and this nature seemed to be one of the causes which made P-351 be thermolabile.

5. Temperature-activity relationships in succinate and NADH oxidation by both the bacteria were similar as the results obtained in glucose oxidation.

6. Apparently, relative activity of succinate dehydrogenase of P-351 was always higher than that of *P. aeruginosa* at lower temperatures, but it was due to the thermolability of the enzyme of P-351 at higher temperatures. In respect of specific activity at lower temperature, any difference was not observed between P-351 and *P. aeruginosa*.

7. From these results, it was concluded that P-351 had psychrophilic oxidation systems, and this property may be at least a factor which permitted P-351 grow well at lower temperatures. As to the reason why P-351 can not grow at higher temperatures, two factors, thermolability of some enzymes and autolyzability, are assumed to participate.

Since FORSTER (1887) firstly reported the bacteria capable of growth at or near 0°C, which were termed "psychrophiles" by SCHMIDT-NIELSEN (1902), studies on the biochemical basis of psychrophily have been performed. Many problems, however, remain unsolved. Psychrophily of micro-organisms has two characters: (a) they can grow at low temperatures as low as 0°C or below, and (b) they cannot grow at moderate temperatures. Therefore, psychrophily should be investigated from these two aspects. Many works on psychrophily in the past were concerned with the latter, thermolability. Although there are many explanations on thermolability (EVISON and ROSE, 1965; LANGRIDGE and MORITA, 1966; HAGEN and KUSHNER, 1964; SEGA *et al.*, 1972; BEST and MATTINGLY, 1973 and so on), as to the natures of "cold-loving" or "cold-stability" of psychrophilic microorganisms, only a few investigations have been performed. Namely, it was reported that psychrophiles contained much more unsaturated fatty acids in the cell membranes than mesophiles (CHAN *et al.*, 1971; KATE and HAGEN, 1964), and HUG *et al.* (1974 a, 1974 b) reported that *Pseudomonas putida*, known as a psychrophile, had psychrophilic enzymes such as histidine ammonia-lyase and urocanase. Both the activities of these enzymes at 0°C or 1.5°C were about 30% of their maximum activities. HUG *et al.* concluded that the faculty of these enzymes from *P. putida* to function well at low temperatures was a factor in the ability of this microorganism to grow on histidine medium at 0°C.

Furthermore, it was reported that some psychrophiles exhibited higher efficiency of substrate transport at lower temperatures than mesophiles (BAXTER and GIBBONS, 1962). However, as stated above, there are only a few studies being concerned with psychrophily and the mechanisms by which psychrophiles can grow at lower temperatures have not yet been clarified. The purpose of this work is to obtain some informations on the energy-acquiring systems of psychrophiles.

Materials and Methods

Organisms: *Pseudomonas* sp. strain 351 (P-351), which was isolated from cold-stored milk and given by Prof. T. YASUI (Faculty of Agriculture, Hokkaido University), and *Pseudomonas aeruginosa* were used as psychrophile and mesophile, respectively. The stock culture of the former strain was stored at 2°C and the latter at room temperature on the ordinary peptone broth agar slants.

Reagents: KCN, NaF and $K_3Fe(CN)_6$ were obtained from the Wako Pure Chemical Ind., Ltd. Sodium succinate and malonic acid were purchased from Nakarai Chemicals, Ltd. Sodium isocitrate was obtained from Sigma

Chemical Co. $NADP^+$ and NADH were the products of Boehringer Mannheim. All other reagents were of analytical grade.

Growth measurements: 20 m*l*-test tube containing 6.0 m*l* of liquid medium and a teflon-coated cylindrical magnet was sterilized and thermostabilized in a temperature-controlled water-bath. After 0.06 m*l* of preculture was inoculated to each test tube, the cultures were aerated by vigorous stirring on an Acrobat Stirrer. At the estimation time, 0.2 m*l* of the culture was carefully sucked up by a steril syringe and poured into 1.8 m*l* of the same medium. Immediately after mixing, the absorbance at 600 nm was estimated by a Hitachi Perkin-Elmer spectrophotomer Type 139.

Intact cell suspensions: The bacteria were cultivated in the medium at 20°C for 20 hours (P-351) or 24 hours (*P. aeruginosa*) with vigorous shaking. Bacterial cells were collected, washed by centrifugation, and suspended in suitable amount of 50 mM phosphate buffer, pH 7.0.

Preparation of cell-free extracts: The washed cell suspensions were exposed to sonic oscillation in a 20 kc Umeda Sonor for 5 min (P-351) or 7 min (*P. aeruginosa*) in an ice-bath. Supernatants obtained after centrifugation at 12,000 rpm for 20 min at 0°C were used as cell-free extracts.

Protein concentrations: Protein was determined by the method of LOWRY *et al.* (1951) with bovine serum albumin as the standard.

Determination of oxidation rates: Oxidation of glucose or succinate was measured by usual manometric techniques. Each vessel contained 1.2 m*l* of cell suspensions or cell-free extracts, 0.1 m*l* of substrate, and 0.2 m*l* of deionized water in main compartment and 0.5 m*l* of 20% KOH in center well.

Determination of bacterial autolysis: Bacterial cell suspensions were adjusted to have absorbance of 0.8 at 600 nm in a Spectronic 20 photometer and incubated at 2, 15, 24, 30 and 37°C. Absorbance changes of each suspension were estimated at intervals.

Assay of succinate dehydrogenase: Activity of succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of SINGER *et al.* (1962). Reduction rate of ferricyanide was determined by measuring the change of absorbance at 400 nm using a Hitachi spectrophotometer Type 139 with a temperature-controlled cell. The reaction mixture contained 2.5 mM sodium succinate, 10 mM KCN, 1 mM potassium ferricyanide, 67 mM phoshate buffer, pH 7.24 and cell-free extracts in a final volume of 2.0 m*l*. The reaction was initiated by addition of cell-free extracts.

Determination of NADH oxidation: Oxidation of NADH was determined by measuring the change of absorbance at 340 nm. Reaction mixture contained 67 mM phosphate buffer, pH 7.4, 1 mM NADH and cell-free extracts

in a final volume of 2.0 m*l*. The reaction was initiated by addition of NADH.

Assay of isocitrate dehydrogenase: Activity of isocitrate dehydrogenase (EC 1.1.1.42) was assayed by measuring the change of absorbance at 340 nm. Reaction mixture contained 67 mM phosphate buffer, pH 7.4, 0.1 mM $NADP^+$, 2 mM sodium isocitrate and cell-free extracts in a final volume of 2.0 m*l*. The reaction was started by addition of isocitrate.

Results

Growth rates: Growth rates of P-351 and *P. aeruginosa* were determined in the temperature range of 0° to 37° at intervals of 10° or 5°C. Although growth of P-351 was very slow at 0°C, it reached to maximum in 220 hours. P-351 had an optimum temperature for growth at 30° to 35°C and could not grow at 37°C. On the other hand, *P. aeruginosa* having an optimum temperature at 37°C or above could not grow at 10°C or below.

Glucose oxidation by intact cells and cell-free extracts: Glucose oxidation by both the bacteria was examined in the temperature range of 5° to

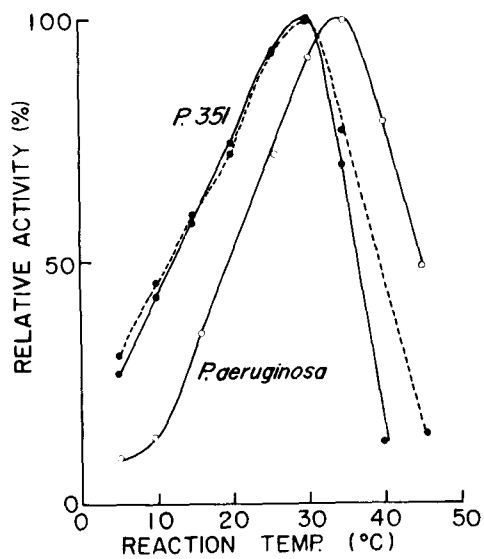

Fig. 1. Effect of temperature on the oxidation rate of glucose by intact cells (—●—) and cell-free extracts (···●···) of P-351 and *P. aeruginosa* (○). Reaction mixture in main compartment consisted of following components in a final volume of 1.5 m*l*: 30 mM phosphate buffer, pH 7.0; 16.7 mM glucose; 2.4 mg dry weight of cell suspension or cell-free extracts (20.4 mg protein for P-351, 18.7 mg for *P. aeruginosa*). In center well, 0.5 m*l* of 20% KOH was used.

TABLE 1. Effect of several inhibitors on glucose and succinate oxidation by P-351

Experimental conditions were the same as described in the legend to Fig. 1, except that the indicated concentrations of inhibitors were added. Figures in the table represent per cent of inhibition.

Substrate	Glucose, 16.7 mM			Succinate, 16.7 mM	
Inhibitor (conc.)	Malonate 16.7 mM	NaF 8 mM	KCN 1 mM	Malonate 33.3 mM	KCN 1 mM
Intact cells	14.3	18.9	87.3	—	—
Cell-free extract	15.8	18.5	58.7	76.6	48.4

45°C at intervals of 5°C. Both intact cells and cell-free extracts of P-351 oxidized glucose most rapidly at 30°C and the oxidative activities at 5°C were retained about 30% of the maximum activities (Fig. 1). Glucose oxidation by *P. aeruginosa*, however, was maximum at 35°C and rapidly decreased as the temperature was lowered. As seen in Fig. 1, decrease of glucose oxidation at higher temperatures by cell-free extracts of P-351 was less rapid than that of intact cells. This phenomenon was assumed to be caused by autolysis which was proved to be facilitated at higher temperatures.

Effects of inhibitors: To determine the degradative pathway of glucose and succinate oxidation in P-351, several inhibitors were examined. As shown in Table 1, inhibition by NaF was weak in both intact cells and cell-free extracts of P-351, therefore, glucose might be degraded *via* any other pathway than EMP pathway. Malonate also inhibited glucose oxidation only weakly, but inhibited intensely succinate oxidation. KCN inhibited remarkable both glucose and succinate oxidation.

Autolysis: As P-351 was assumed to autolyze at moderate temperatures from the data of growth curves and glucose oxidation, bacterial autolysis was investigated. It was found that P-351 rapidly autolyzed at moderate temperatures. However, *P. aeruginosa* did not so remarkably autolyze and the rate at 37°C was about one fifteenth of the rate at 37°C in P-351.

Succinate oxidation: Succinate oxidation by cell-free extracts of P-351 and *P. aeruginosa* was examined. Succinate oxidation system in P-351 seemed to be functionable more efficiently than that in *P. aeruginosa* at lower temperatures. At 5°C, Qo_2 of P-351 was about seven times of *P. aeruginosa* (Fig. 2).

Effects of temperature on enzyme activities:

1) Succinate dehydrogenase: Although the data were not represented here, relative activity of succinate dehydrogenase in P-351 seemed to have

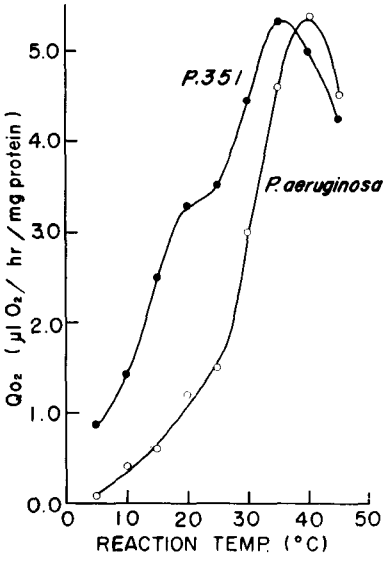

Fig. 2. Effect of temperature on Q_{O_2} values of succinate oxidation by cell-free extracts of P-351 (●) and *P. aeruginosa* (○). Q_{O_2} was defined as μl of O_2 uptake per hr per mg of protein.

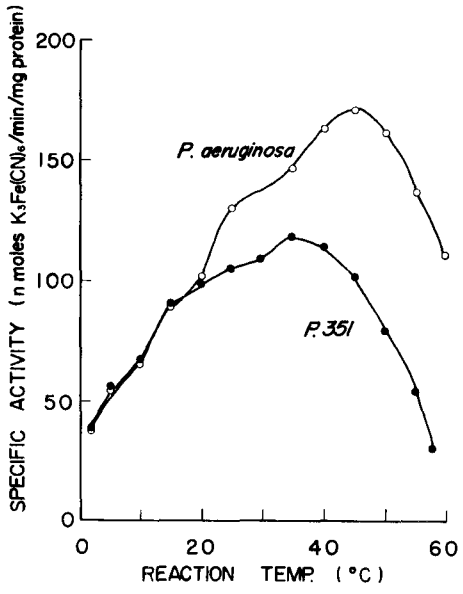

Fig. 3. Effect of temperature on the specific activities of succinate dehydrogenase in P-351 (●) and *P. aeruginosa* (○). An unit of activity was defined as the amount of enzyme which reduced 1 nmole of $NADP^+$ per min, and specific activity was as units of enzyme activity per mg of protein.

considerably high activity at lower temperatures as compared with those of *P. aeruginosa.* However, specific activities of both succinate dehydrogenases were much the same below 20°C (Fig. 3). Namely, both succinate dehydrogenases could function with same activities at lower temperatures, but at moderate temperature succinate dehydrogenase of P-351 was inactivated.

2) Isocitrate dehydrogenase: Isocitrate dehydrogenases of P-351 and *P. aeruginosa* were assayed at various temperatures. The activities of both the enzymes did not show marked differences at lower temperatures as was observed in succinate dehydrogenases (Fig. 4). Isocitrate dehydrogenase of P-351 as well as succinate dehydrogenase was inactivated at elevated temperatures.

3) NADH oxidation: NADH oxidation by cell-free extracts of P-351 and *P. aeruginosa* was also examined. NADH oxidation by P-351 was more active in comparison with *P. aeruginosa* at all the assay temperatures (Fig. 5).

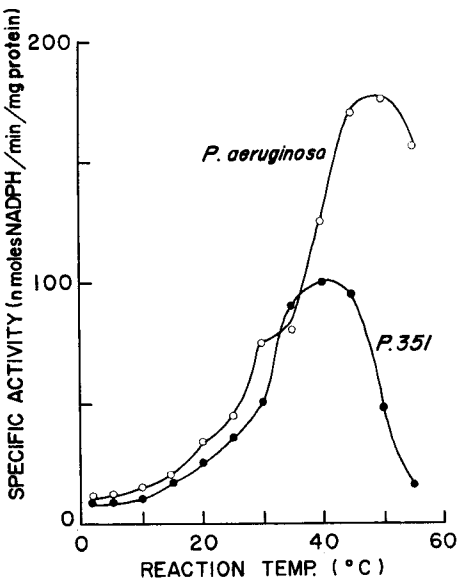

Fig. 4. Effect of temperature on the specific activities of isocitrate dehydrogenase in P-351 (●) and *P. aeruginosa* (○). Specific activities were determined as described in the legend to Fig. 3.

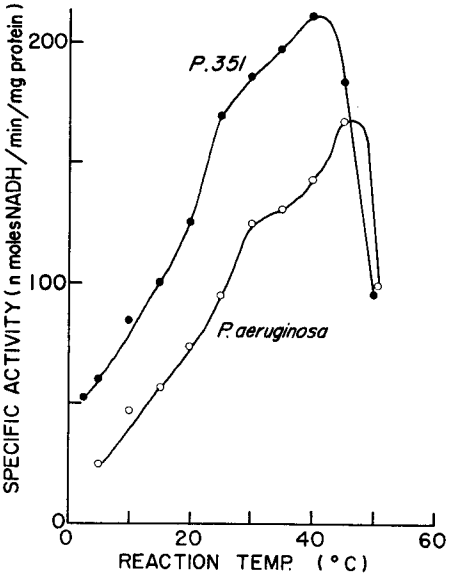

Fig. 5. Effect of temperature on the specific activities of NADH oxidation by cell-free extracts of P-351 (●) and *P. aeruginosa* (○). Specific activities were the same as described in the legend to Fig. 3.

Discussion

One of the remarkable properties of psychrophiles is their ability to grow well at 0°C or below. According to HUCKER *et al.* (1954) psychrophiles capable of growth at 0°C were subdivided into two groups, *i. e.* obligate psychrophiles and facultative psychrophiles. The former could not grow at 32°C, whereas the latter could grow. INGRAHAM and STOKES (1959) proposed that obligate psychrophiles were defined as the bacteria that grew well at 0°C and most rapidly at temperatures below 20°C, and facultative psychrophiles were only capable of growth at 0°C. Employing these definitions, P-351 belongs to facultative psychrophiles.

That psychrophiles can grow at lower temperatures suggests that they have a certain energy-acquiring system functionable at the temperatures. The results represented in Figs. 1, 2, and 5 support the suggestion. The glucose oxidation rate of P-351 at 5°C is 30% of the maximum activity observed at 30°C (Fig. 1), and this value is rather higher than that of *Bacillus psychrophilus* which is a obligate psychrophile (ref. STOKES and LARKIN,

1968). High activity of glucose oxidation by P-351 at 5°C was observed not only in relative activity but also in specific activity, Q_{O_2} (Table 2).

As for degradative pathway of glucose in *Pseudomonas*, it has not been clarified what oxidative pathways are operative, but P-351 does not seem to have EMP-glycolytic pathway, since NaF inhibited the enolase reaction only weakly as shown in Table 1. According to ENTNER and DOUDOROFF (1952), glucose is degraded in *Pseudomonas via* ENTNER-DOUDOROFF pathway but not by EMP pathway. Whichever pathway was operating in the early processes of glucose degradation in P-351, subsequent oxidation of intermediates such as pyruvate seemed to be carried out *via* TCA cycle and respiratory chain because of the results of inhibition experiments with malonate and cyanide, though malonate did not intensely (Table 1).

There was marked difference in the activity of succinate oxidation between P-351 and *P. aeruginosa* (Fig. 2). Nevertheless, the specific activities of both succinate dehydrogenases between 0°C and 20°C were much the same (Fig. 3). Furthermore, isocitrate dehydrogenases did not exhibit marked differences in their specific activities except that the enzyme of P-351 was thermolabile. Consequently, higher activities of oxidation systems in P-351 at lower temperatures were not due to difference at dehydrogenase level.

According to INGRAHAM and BAILEY (1959), the temperature difference for the oxidation of glucose between psychrophilic and mesophilic *Pseudomonas* disappeared when cell-free extracts were used. Then they concluded that the differences in temperature response for the oxidation of glucose between psychrophiles and mesophiles were probably dependent upon a result of some aspect of cellular organization rather than of enzymatic differences. But in our results, it is assumed that the marked differences in the activities of succinate oxidation by both the bacteria at lower temperatures may attribute to the differences of activities of electron transfer systems rather than cellular structure. In deed, the results of NADH oxidation by cell-free extracts of P-351 and *P. aeruginosa* support the presumption (Fig. 5).

It has been reported that several psychrophiles tend to autolyze at higher temperatures (HAGEN *et al.*, 1964; BEST and MATTINGLY, 1973), and

TABLE 2. Effect of temperature on Q_{O_2} values of glucose oxidation by intact cells of P-351 and *P. aeruginosa*

Temp. (°C)	5	10	15	20	25	30	35	40	45
P-351	26.2	39.2	53.5	68.5	102.9	115.9	49.6	10.1	—
P. aeruginosa	5.6	8.1	33.4	61.4	63.6	81.0	91.4	71.4	42.8

in our studies on P-351 the same phenomenon was also observed, that is, the remarkable autolysis was occurred at higher temperatures. Although detailed studies on the autolysis of P-351 were not performed, the temperature-induced autolysis and thermolability of some enzymes such as succinate dehydrogenase or isocitrate dehydrogenase would not permit P-351 grow at moderate temperatures.

HAGEN *et al.* (1964) reported that some psychrophiles could transport substrates at lower temperatures more efficiently than mesophiles. But in P-351 the permeability of substrates did not seem to participate in psychrophily, because no differences between the activities of glucose oxidation by intact cells and those by cell-free extracts were observed at lower temperatures.

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