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Effect of temperature on the induction of isocitrate lyase

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Effect of temperature on the inductive formation of isocitrate lyase in a psychrotrophic *Flavobacterium* sp. strain G-3, and mesophilic *Proteus vulgaris* and *Pseudomonas aeruginosa* was investigated.

1. No remarkable differences between the enzymes from *F. G-3* and *P. aeruginosa* were observed in respect of thermostability, activation energy, and optimum temperature for the induction.
2. Optimum induction temperatures of isocitrate lyases in *F. G-3*, *P. vulgaris*, and *P. aeruginosa* were 10°C, 25°C and 15°C, respectively, which were 10°~20°C lower than the respective optimum growth temperature.
3. It was confirmed that decreased induction near the optimum growth temperature was not due to thermolability of induced isocitrate lyase, destruction of isocitrate lyase by some digestive enzymes, or inhibition by dialyzable small molecules accumulated at higher temperatures.

Both psychrophilic and psychrotrophic bacteria can grow well even at the low temperatures at which mesophilic bacteria can not grow. Much effort has been offered to elucidate this character, "psychrophily", in such aspects as protein synthesis (QUIST and STOKES, 1969; SZER, 1970; QUIST and STOKES, 1972; KATO *et al.*, 1972; SARUYAMA *et al.*, 1978), the structure and function of cell membranes (CULLEN *et al.*, 1971; FINNE and MATCHES, 1976), energy-acquiring systems (OKUYAMA and SASAKI, 1977), and so on. Although a few psychrophilic enzymes such as histidase (HUG and HUNTER, 1974 a), urocanase (HUG and HUNTER, 1974 b), and isocitrate dehydrogenase (OCHIAI *et al.*, 1979), and a proteinous factor, "P-factor", requiring in protein synthesis at low temperatures (SZER, 1970) have been reported, problem of psychrophily remains unsolved.

As an important approach to elucidation of psychrophily, effect of temperature on protein synthesis has been studied by estimation of amino acid uptake with whole cells or cell-free systems (SZER, 1970; SARUYAMA and SASAKI, 1976) and of inducibility of some enzymes such as a protease (KATO *et al.*, 1972), formic hydrogenlyase (QUIST and STOKES, 1969), hydrogenase (QUIST and STOKES, 1972), and benzoate-oxidizing enzymes (QUIST and STOKES, 1972). All these reports exhibited that protein-synthesizing system

in the psychrophiles was actively operating at or near 0°C, but not in the mesophiles. In our laboratory, however, SARUYAMA *et al.* (1976) investigated the temperature-activity relationships of protein synthesis in intact resting cells of psychrotrophs and mesophiles and observed that protein-synthesizing system of the mesophiles, which were cultivated at 20°C, could operate to some extent even at 0°C. Moreover, NISHI *et al.* (unpublished data) found that polyphenylalanine synthesis in cell-free extracts of *Pseudomonas aeruginosa* was active and stable at 0°C in contrast with *Escherichia coli* (SZER, 1970). To evaluate the relationship between temperature and protein-synthesizing activity, we examined inducibility of isocitrate lyase (EC 4.1.3.1) in psychrotrophic *Flavobacterium* sp. strain G-3 and mesophilic *P. aeruginosa* and *Proteus vulgaris* at several temperatures. In this study, we found that the optimum induction temperature was about 10°~20°C lower than the optimum growth temperature not only in the psychrotroph but also in the mesophiles.

These results are described in this paper.

Materials and Methods

Bacteria: *Flavobacterium* sp. strain G-3 (SARUYAMA *et al.*, 1978) was used as a psychrotrophic bacterium. *Pseudomonas aeruginosa* and *Proteus vulgaris* were used as mesophilic bacteria.

Medium: Peptone-broth medium, which consisted of 1% each of peptone and meat extract, was used for preculture and stock culture. The same medium supplemented 50 mM sodium acetate was used as the induction medium.

Preparation of cell-free extract: Cells were harvested, washed with 20 mM potassium phosphate buffer (pH 7.9) by centrifugation and suspended into the same buffer containing 10 mM MgCl₂. The suspension was exposed to sonic oscillation, and centrifuged at 11,000 × *g* for 20 min. The supernatant was frozen until use for determination of isocitrate lyase activities, since malate synthetase activity was known to be lost by freezing and thawing (DIXON *et al.*, 1960), but not isocitrate lyase.

Glyoxylate assay: Calibration curve was made as follows: The reaction mixture (2.0 ml) consisted of 40 mM phosphate buffer (pH 6.4), 0.2 mg NADH, 0.18 units glyoxylate reductase, and standard glyoxylate solution (2 to 10 μmoles). The reaction was started by addition of the enzyme at 30°C and decrease of optical density was determined at 340 nm.

Assay of isocitrate lyase: The reaction mixture consisted of 7 mM glyoxylate, 7 mM succinate, 20 mM phosphate buffer (pH 7.9), 1.3 mM MgCl₂,

and an appropriate amount of cell-free extract. After 5 min of incubation at 30°C, the reaction was stopped by heating. The supernatant solutions obtained by centrifugation were subjected to an assay of glyoxylate. One unit of enzyme activity was defined as 1 nmole of glyoxylate consumed per min. Specific activity was defined in terms of units per mg protein.

Protein determination: Protein concentration was determined by the method of LOWRY *et al.* (1951) with bovin serum albumin as a standard.

Detection of Keto acids: After deproteinization, 2,4-dinitrophenylhydrazones derivatives of keto acids were extracted by the method of SAZ and HILLARY (1956), and detected by paper chromatography (SASAKI *et al.*, 1961; MEISTER and ABENDSHEIN, 1956) or by the method of KATSUKI *et al.* (1971).

Chemicals: NADH and glyoxylate reductase were purchased from Boehringer Mannheim. Sodium glyoxylate was a product of Sigma Chemical Co.

Results

Inducibility of isocitrate lyase: To estimate induced isocitrate lyase, we measured residual glyoxylate by the method of KATSUKI *et al.* (1971). Fig. 1 shows that isocitrate lyase could be induced by acetate in all three strains of bacteria. Isocitrate lyase reaction proceeded linearly at least for initial 5 min. However, this assay method was ambiguous if any other carbonyl compounds besides glyoxylate could be produced by the reaction. Then,

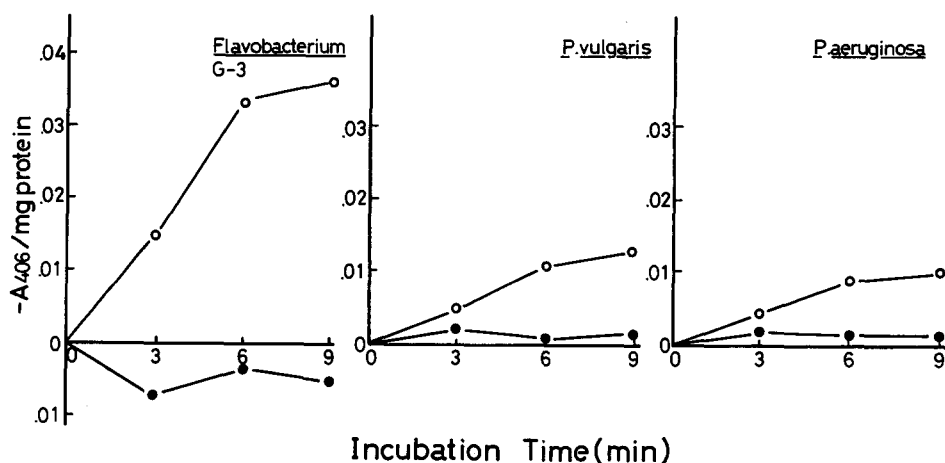


Fig. 1. Induction of isocitrate lyase on the peptone-broth medium (●) or the induction medium (○). Isocitrate lyase was assayed as described in Materials and Methods. Sonic extracts were prepared from *F. G-3* grown at 10°C and from *P. aeruginosa* and *P. vulgaris* grown at 20°C.

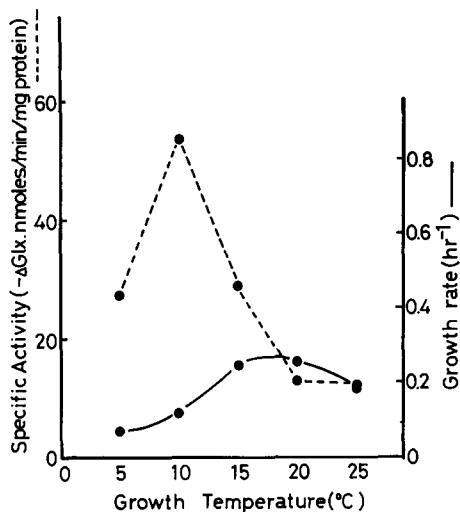


Fig. 2. Effect of temperature on induction of isocitrate lyase of *F. G-3*.

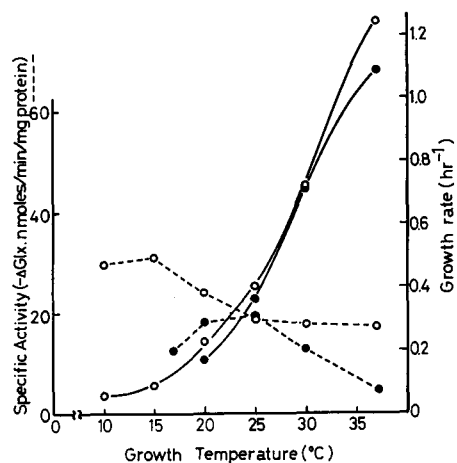


Fig. 3. Effect of temperature on induction of isocitrate lyase of *P. aeruginosa* (○) and *P. vulgaris* (●).

the reaction products were analyzed by paper chromatography with respect to carbonyl compounds. Consequently, α -ketoglutarate and pyruvate besides glyoxylate were identified as the products by paper chromatography and by their absorption spectra in alkaline pH. By reason of these above results, hereafter, glyoxylate was determined enzymatically.

Effect of temperature on the induction of isocitrate lyase: Bacteria were cultivated in the induction medium at different temperatures between 5° and 37°C, harvested at suitable intervals, washed, resuspended in phosphate buffer (pH 7.9), and exposed to sonic oscillation. Sonic extract obtained after centrifugation was subjected to an assay of isocitrate lyase. Figs. 2 and 3 show growth rate and amount of isocitrate lyase induced at different temperatures. In *Flavobacterium G-3*, the optimum induction temperature of isocitrate lyase was 10°C, while the optimum growth temperature was 20°C. In *Proteus vulgaris* and *Pseudomonas aeruginosa*, the optimum induction temperatures of the lyase were 20°~25°C and 15°C, respectively, and the optimum growth temperature of both the bacteria was 37°C.

Thus, each optimum induction temperature of isocitrate lyase was found to be fairly lower than each corresponding optimum growth temperature in these psychrotrophic and mesophilic bacteria. Amount of isocitrate lyase induced at different temperatures shown in Figs. 2 and 3 may be only apparent one, that is, it may not exhibit net synthesis of isocitrate lyase, because following possibilities have not been excluded: a) newly synthesized

isocitrate lyase may be thermolabile enzyme; b) low molecular inhibitors such as pyruvate, phosphoenolpyruvate and oxalacetate, which are known to inhibit isocitrate lyase activity (TANAKA *et al.*, 1977), may be accumulated in large quantities at higher temperatures; and c) proteases may operate actively at higher temperatures. Attempt was made to clarify whether these questions were correct or not.

Effect of temperature on isocitrate lyase activity: Isocitrate lyase activities in sonic extracts of above three bacteria grown at their optimum induction temperatures, were assayed at different temperatures. The enzymes from *F. G-3* and *P. aeruginosa* showed the maximum activity at 30°~40°C and about 20% of the maximum activity remained at 10°C (Fig. 4). Activation energies of the enzymes in both the bacteria were almost the same

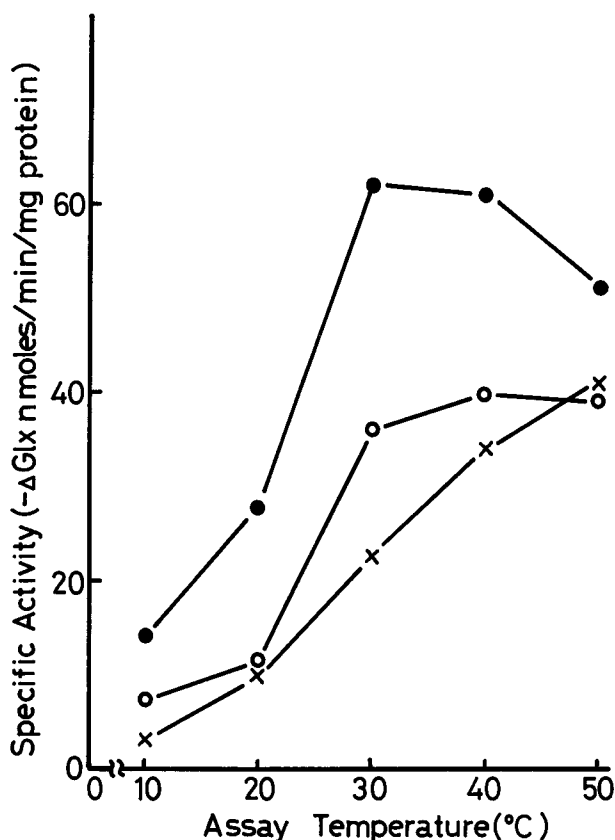


Fig. 4. Effect of temperature on isocitrate lyase activity. Sonic extracts were prepared from *F. G-3* (●), *P. aeruginosa* (○) and *P. vulgaris* (×) grown at each optimum temperature.

(12.94 and 12.55 Kcal·mole⁻¹). On the other hand, isocitrate lyase of *P. vulgaris* showed the maximum activity at 50°C and only 8% of the maximum activity remained at 10°C. Activation energy of *P. vulgaris* enzyme was 17.85 Kcal·mole⁻¹. Anyhow, all these enzymes were not thermolabile so much. Then the residual activities of isocitrate lyase were measured after the sonic extracts of *F. G-3* and *P. aeruginosa* were preincubated at different temperatures for 10 min, and after the sonic extracts of *F. G-3* and *P. vulgaris* were preincubated at 60°C as a function of time. These results are represented in Fig. 5 A and B. Isocitrate lyases of *F. G-3* and *P. aeruginosa* seemed to be slightly thermolabile, but it was not so labile as to support the above possibility (a).

Isocitrate lyase activity after dialysis: To elucidate whether decreased induction of isocitrate lyase in the bacteria grown at higher temperatures (see Figs. 2 and 3) may be due to inhibition of the enzyme by accumulated low molecular inhibitor(s), the sonic extracts were dialyzed for 12 hours against 20 mM phosphate buffer (pH 7.9) and then isocitrate lyase activity was measured. Isocitrate lyase activities in all these bacteria were reduced by dialysis, so it might be concluded that any dialyzable inhibitor was not accumulated in these extracts (Table 1).

Possibility of a presence of protease-like activity: If any digestive enzymes such as protease are more produced in the cells grown at higher

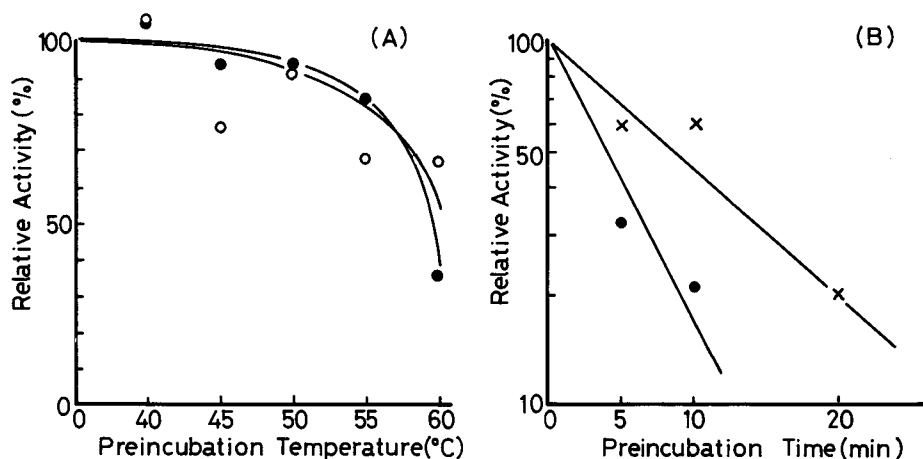


Fig. 5. Thermostability of isocitrate lyase. Sonic extracts of *F. G-3* (●) and *P. aeruginosa* (○) were preincubated in phosphate buffer (pH 7.9) at each temperature indicated in the figure for 10 min and then residual activity of isocitrate lyase was measured (A), and sonic extracts of *F. G-3* (●) and *P. vulgaris* (×) were preincubated at 60°C as a function of time and residual activity of the lyase was measured (B).

TABLE 1. Effect of dialysis on the activity of isocitrate lyase

Strain	Relative activity (%)	
	Control	Dialysis
<i>F. G-3</i>	100	75
<i>P. aeruginosa</i>	100	66
<i>P. vulgaris</i>	100	87

Sonic extracts from the bacteria grown at higher temperature were dialyzed against 20 mM phosphate buffer, pH 7.9, at 4°C for 12 hours, and residual activity of isocitrate lyases was measured.

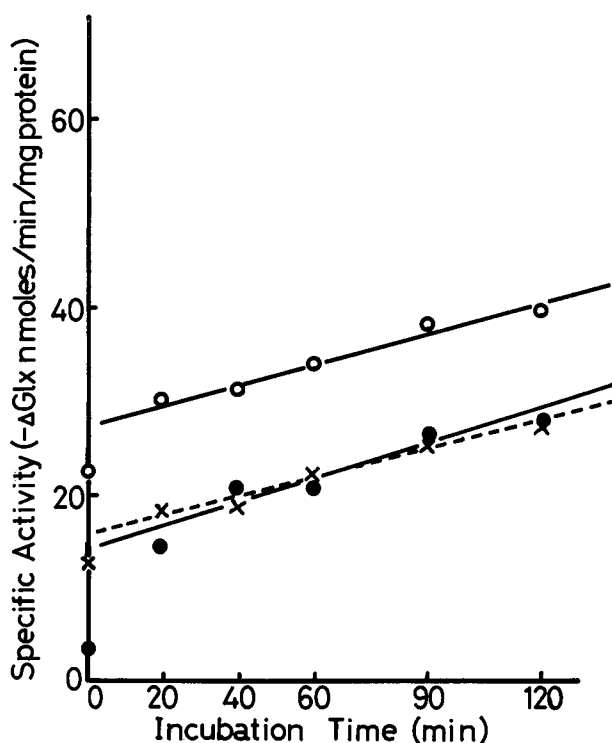


Fig. 6. Effect of sonic extract from *F. G-3* grown at 20°C. (○), sonic extract from the cells grown at the optimum induction temperature, 10°C; (●), sonic extract from the cells grown at 20°C; and (⋯×⋯), mixture of these above sonic extracts. Details are in the text.

temperature (high temperature cells) than in those at low temperature (low temperature cells), the lyase activity in the extract from the low-temperature cells should be more or less decreased by addition of the extract from the high-temperature cells.

That was not the case, and the activity in the mixture did not change in all three bacteria (data not shown). Furthermore, any effect of the extract from the high-temperature cells on partially purified isocitrate lyase was not observed. Using the freshly prepared extracts from the high-temperature cells of *F. G-3* under pH 7.0 instead of pH 7.9, the lyase activity in the mixture gradually increased with the time of incubation (Fig. 6). From these results, decreased lyase activity at higher temperatures may not be due to action of such digestive enzymes.

Discussion

In the present studies, we examined inducibility of isocitrate lyase at different temperatures in a psychrotrophic *Flavobacterium G-3* and mesophilic *Pseudomonas aeruginosa* and *Proteus vulgaris*. Optimum induction temperature of isocitrate lyase in *F. G-3* was about 10°~15°C lower than that of *P. vulgaris* and the induction of isocitrate lyase in *F. G-3* was higher than in *P. vulgaris* at lower temperature (Figs. 2 and 3). Similar results have been reported with other inducing enzymes in psychrophilic and mesophilic bacteria (QUIST and STOKES, 1969; QUIST and STOKES, 1972; KATO *et al.*, 1972). These results give support to the hypothesis that temperature characteristics of protein synthesis is an important determinant in growth of psychrophilic bacteria at lower temperatures. However, in this study, isocitrate lyase induction of *P. aeruginosa*, one of the mesophiles, was found to resemble those of psychrotrophic *F. G-3* (Figs. 1 and 2). This may be due to high adaptability of *P. aeruginosa*, which is renowned for the capacity to utilize an enormous of compounds as energy sources and probably for the adaptability to wide ranges of physical environmental factors. In our laboratory, it was also observed that the optimum temperatures for amino acid uptake and incorporation into hot TCA-insoluble fraction were 5°C lower than those for growth in psychrotrophic strains N-I-9 and *Pseudomonas 351* (SARUYAMA and SASAKI, 1976). These results could be explained by the thermolability of their protein-synthesizing systems, and in fact some thermolabile steps in protein-synthesizing systems have been reported (MALCOLM, 1968; MALCOLM, 1969; NASH *et al.*, 1976; and so on). However, this explanation is unsatisfactory to elucidate the difference between the optimum temperatures for growth and those for induction of isocitrate lyase in *F. G-3*, *P. aeruginosa* and *P. vulgaris*, because it has been proved that the protein-synthesizing system in *F. G-3* was not fairly thermolabile as well as *P. aeruginosa* (SARUYAMA and SASAKI, 1976). Then, we supposed that the amount of isocitrate lyase estimated at higher temperature may

not exhibit net synthesis of isocitrate lyase. This supposition, however, were almost completely denied in this study, hence inducibility-temperature relationships (Figs. 2 and 3) represent net amount of isocitrate lyase synthesized at each temperature. Although it has not been examined whether isocitrate lyase is *de novo* synthesized from free amino acids, *de novo* synthesis of inducing enzymes is generally accepted. Here, new puzzling problem has arisen, that is, from the results of this study and the experiment by SARUYAMA and SASAKI (1976) two different temperature-dependent protein-synthesizing systems must be present in the same organisms (*F. G-3* and *P. aeruginosa*), because the optimum temperature for amino acid incorporation to hot TCA-insoluble fraction in both *F. G-3* and *P. aeruginosa* was 30°C but those for induction of isocitrate lyase in *F. G-3* and *P. aeruginosa* were 10°C and 15°C, respectively.

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