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Citation	Journal of the Faculty of Science, Hokkaido University. Series 5, Botany, 14(2), 115-124
Issue Date	1988
Doc URL	<a href="https://hdl.handle.net/2115/26436">https://hdl.handle.net/2115/26436</a>
Type	departmental bulletin paper
File Information	14(2)_P115-124.pdf



## Growth and acetate utilization by a psychrophilic bacterium, *Vibrio* sp. strain ABE-1

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*Vibrio* sp. strain ABE-1 exhibited unusual growth when acetate was given as the sole carbon source. Length of lag phase and growth rate were greatly influenced by concentration of acetate in a medium. The more acetate was present in the medium, the longer lag phase was observed, and the specific growth rate increased progressively but not linearly. Supplement of acetate to the culture in the middle logarithmic phase brought about rapidly growth inhibition. When carbon source was shifted from succinate to acetate, the bacterium grew biphasically, slow in the first phase and fast in the second phase. Length of the first phase depended on the concentration of acetate, and the cells began to consume acetate at about the end of the first phase. Furthermore, induction rates of isocitrate lyase and isozyme-I of isocitrate dehydrogenase induced by acetate were affected inversely by acetate concentration in the medium.

We previously reported that *Vibrio* sp. strain ABE-1 (*Vibrio* ABE-1), an obligately psychrophilic bacterium (TAKADA *et al.*, 1979), has two isozymes of isocitrate dehydrogenase (IDH; EC 1.1.1.42) (OCHIAI *et al.*, 1979). The two isozymes were clearly distinguished from each other in their thermostabilities, kinetic characteristics (OCHIAI *et al.*, 1979), quaternary structures (OCHIAI *et al.*, 1984), and in immunochemical properties (ISHII *et al.*, 1987). We demonstrated that one of the isozymes, IDH-I, can be specifically induced in the cells growing on acetate (ISHII *et al.*, 1987).

Furthermore we have observed that *Vibrio* ABE-1 exhibited unique, over-exponential growth in batch culture supplemented with acetate as the sole source of carbon and energy, and we obtained interesting results suggesting that the level of one IDH isozyme (IDH-1) was closely related to the growth of *Vibrio* ABE-1 on acetate. In this paper we describe the experimental evidences indicating the inhibitory effect of acetate on the growth of *Vibrio* ABE-1 and discuss about the relation between IDH-I activity and the

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bacterial growth.

## Materials and Methods

### *Organism, culture media and conditions*

*Vibrio* sp. strain ABE-1 was grown aerobically in 100 ml medium in a 500 ml flask with a temperature controlled rotary shaker ( $\pm 1^\circ\text{C}$ ). A synthetic medium contained 50 mM Tris, 10 mM KCl, 3.3 mM  $\text{K}_2\text{HPO}_4$ , 3 mM sodium citrate, 25 mM  $\text{NH}_4\text{Cl}$ , 0.1 mM  $\text{FeSO}_4$ , 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgSO}_4$ , and desired concentration of carbon source. pH was adjusted to 7.2 with HCl, and NaCl was added to 0.5 M. Nutrient medium (pH 7.2) contained 1% meat extract, 1% peptone and 0.5 M NaCl.

### *Shift of carbon source*

Cells grown in a medium containing 100 mM succinate at  $15^\circ\text{C}$  for 12 h (middle logarithmic phase) were harvested and washed with Tris-salt medium omitted carbon source, then transferred into the same volume of each one of Tris-salt medium containing acetate (20, 50, or 100 mM of acetate) and incubated with shaking at  $15^\circ\text{C}$ . At intervals of 4 to 6 h, aliquot (40 ml) of the culture was withdrawn and centrifuged at  $4^\circ\text{C}$ . The supernatant was filtered through a Millipore filter (0.22  $\mu\text{m}$  pore size) and frozen at  $-20^\circ\text{C}$  until the determination of acetate concentration, and the cell pellet was washed with 0.5 M NaCl, and frozen at  $-20^\circ\text{C}$  until enzyme assay.

### *Measurement of growth*

Growth was followed by measuring turbidity at 600 nm by a Hitachi Perkin-Elmer spectrophotometer type 139. The specific growth rate  $k$  was calculated from the formula ;

$$k = 2.303 (\log x_2 - \log x_1) / (t_2 - t_1)$$

where,  $x_1$  and  $x_2$  are optical densities at time  $t_1$  and  $t_2$ , respectively.

### *Cell-free extract*

Cells were harvested in the late logarithmic phase, washed twice with 0.5 M NaCl, and suspended in 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl, 1 mM  $\text{MnCl}_2$ , and 10 mM 2-mercaptoethanol. Optical density of the suspension was adjusted to give protein concentration 10 to 20 mg per ml of cell-free extract. Cells were then disrupted by sonic oscillation for 1.5 min and the supernatant obtained by centrifugation at  $11,000 \times g$  for 20 min were used as cell-free extract. All procedures described above were performed at  $4^\circ\text{C}$  or below.

### *Enzyme assay*

Composition of the reaction mixture to determine the activity of

isocitrate dehydrogenase (IDH: EC 1.1.1.42) was the same as described previously (OCHIAI *et al.*, 1979). Levels of two IDH isozymes in the crude extracts were estimated as follows: remaining IDH activity after heat treatment at 30°C for 10 min was represented as IDH-I, and disappeared portion after the treatment was as IDH-II. Isocitrate lyase (ICL: EC 4.1.3.1) and malate synthetase (MS: EC 4.1.3.2) were assayed by a similar method as described by DIXON and KORNBERG (1959). The activity of malate dehydrogenase (MDH: EC 1.1.1.37) was determined by measuring the oxidation of NADH. The reaction mixtures contained in 2 ml, 33 mM Tris-HCl buffer, pH 8.4, 0.12 mM NADH, 0.1 M KCl, 4 mM oxaloacetate and crude enzyme. The activity of citrate synthetase (CS: EC 4.1.3.7) was measured spectrophotometrically at 232 nm. The reaction mixture (2 ml) contained 0.3 M potassium phosphate buffer, pH 8.0, 0.1 M KCl, 0.2 mM oxaloacetate, 0.144 mM acetyl-CoA, and the crude enzyme.

All enzyme assays were carried out at 20°C unless otherwise stated, and protein concentration was determined by the method of LOWRY *et al.* (1951) with bovine serum albumin as a standard.

#### *Determination of acetate concentration*

Acetate concentration in a medium was determined by a gas chromatography (NEVA; model 1400). The frozen supernatant sample, which had been prepared from the culture as described above, was thawed and acidified by adding a small amount of conc. HCl and applied to a column (0.3 × 180 cm) of 10% PEG 6000 on Shimalite TPA. The chromatography was performed isothermally at 130°C with a carrier gas (N<sub>2</sub>). *n*-Propionic acid was used as internal standard. Peak of acetic acid was identified with comparing the retention time of authentic standard, and peak area was calculated by a chromatopack CR-3A (Shimadzu).

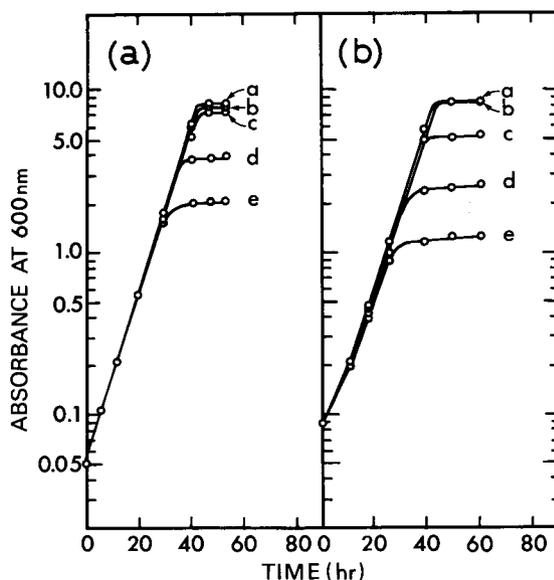
#### *Chemicals*

DL-Isocitrate (Sodium salt), glyoxylic acid (monohydrate), oxaloacetic acid, phenylhydrazine-HCl, and cystein-HCl were obtained from Nakarai Chemical Co. NADP and NADH were obtained from Boehringer Mannheim. Acetyl-Coenzyme A was a product of Sigma. All other reagents used were of reagent grade.

## **Results and Discussion**

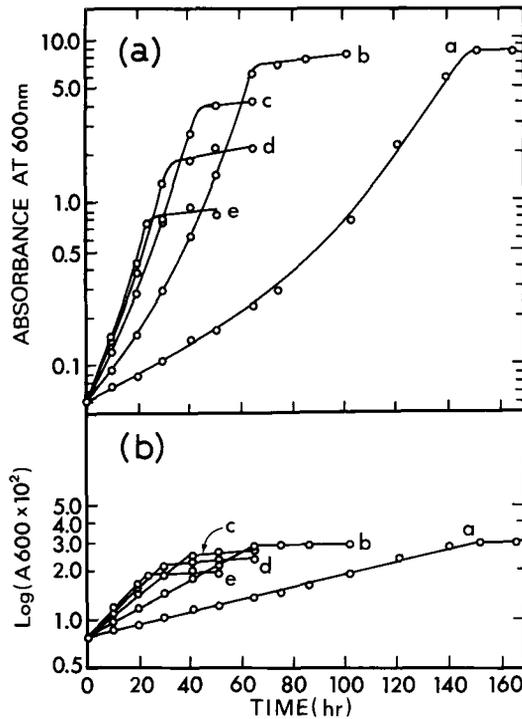
#### *Unusual growth on acetate*

Fig. 1 shows typical growth curves of *Vibrio* ABE-1 on various amounts of succinate or pyruvate. *Vibrio* ABE-1 exhibited normal logarithmic growth on succinate or pyruvate irrespective to its concentration. However,



**Fig. 1.** Growth of *Vibrio* ABE-1 in the media containing different amounts of succinate or pyruvate. (a) Growth on succinate; *Vibrio* ABE-1 was precultured on 100 mM succinate at 11°C for about 12 generations. 1 ml of precultured cells was inoculated into a fresh medium and followed the growth at the same temperature. a, b, c, d, and e indicate the growth on 12.5, 25, 50, 100, and 200 mM succinate, respectively. (b) Growth on pyruvate; culture conditions and the symbols are the same as in (a).

when the cells were inoculated on acetate, unusual growth was observed: the higher concentration of acetate was present, the longer lag phase was observed, and the specific growth rate was progressively increased beyond the usual linear increase (Fig. 2-a). As shown in Fig. 2-b, plots of duplicative logarithmic values of absorbance at 600 nm ( $A_{600}$ ) vs. culture time was almost linear. We could not find any significant difference in the ratio of dry weight of the cells to optical densities and also in the size of cells between at early and late logarithmic phases (data not shown), hence the increase in  $A_{600}$  was presumed to represent actual increase in cell mass. Further we obtained the following observations; a) change of pH in the media during the growth (7.2 in the fresh media and 8.0 at stationary phase) did not affect so much to the specific growth rate, b) ages or generations of the cells precultured on acetate did not affect the growth on acetate, c)

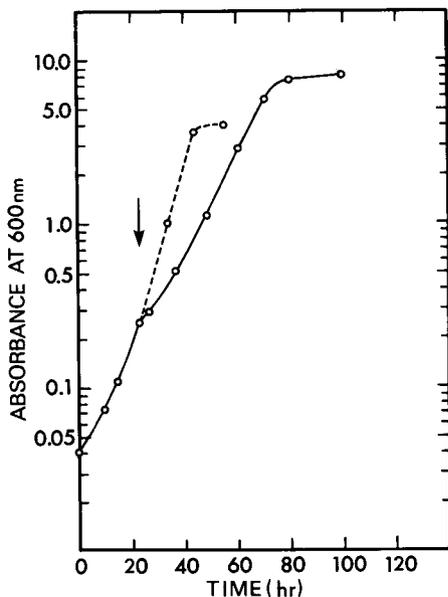


**Fig. 2.** Growth of *Vibrio* ABE-1 in the media containing different amounts of acetate. (a) *Vibrio* ABE-1 was precultured on 100 mM acetate. Precultures and growth experiment were performed at 11°C. a, b, c, d, and e indicate the same molar concentrations of acetate in a medium as described in Fig. 1. (b) Data in Fig. 2a were replotted with duplicative logarithmic values of  $A_{600}$  as ordinate.

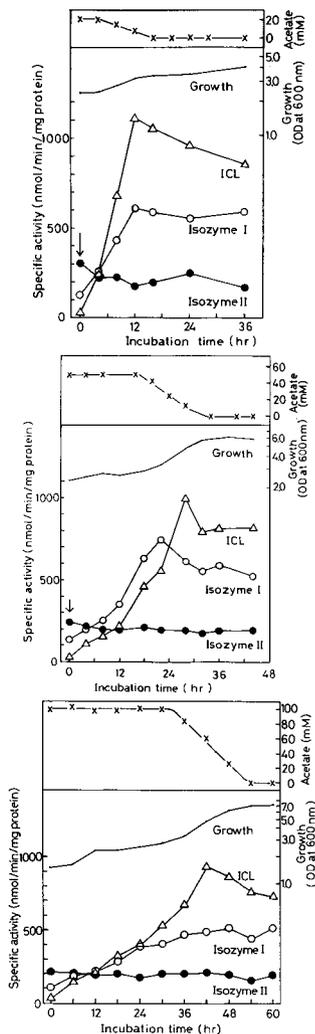
supplement of acetate to the culture in the middle logarithmic phase of growth reduced rapidly the specific growth rate (Fig. 3). These results suggest that the unusual growth of *Vibrio* ABE-1 on acetate may depend on only acetate concentration in the medium.

#### *Consumption rate of acetate in the medium*

To examine the above assumption, we attempted to determine the consumption rate of acetate in the medium. *Vibrio* ABE-1 was grown on succinate to middle logarithmic phase and the portions of the cells were transferred into fresh media supplemented with different amounts of acetate as the sole carbon source. Then we followed the bacterial growth, acetate concentration, and specific activities of ICL and IDH isozymes. These



**Fig. 3.** Growth inhibition by acetate. *Vibrio* ABE-1 was grown on 50 mM acetate at 11°C. At the time indicated by arrow, powder of sodium salt of acetate (0.82 g/100 ml medium) was added. With addition (—○—), and without addition of acetate (-----○-----).



**Fig. 4.** Consumption rate of acetate and levels of IDH isozymes and ICL. Acetate concentrations in the media were 20 (A), 50 (B), and 100 mM (C). Upper panels; consumption of acetate. Arrows indicate the points that cells grown on succinate were transferred to a fresh acetate medium. Activities of IDH-I and -II were determined at 40° and 20°C, respectively. Other experimental conditions were described in **Materials and Methods**.

results are shown in Fig. 4. The bacteria began to grow in the media containing 20 mM acetate after about 6 hr of the shift, and to consume acetate linearly with the time. In the medium containing 50 or 100 mM acetate, however, the bacteria exhibited biphasic growth, slow in the first phase and fast in the second phase. Length of the first phase in 100 mM acetate medium was longer than that in 50 mM acetate. Acetate concentration in the medium remained unchanged in the first phase, but it began to decrease linearly after about 6, 15, and 30 h of the shift to 20, 50, and 100 mM acetate, respectively. Specific activities of ICL and IDH-I, which are induced by acetate, increased soon after carbon source was shifted from succinate to acetate. However, the rates of the enzymes induction were inverse proportion to the initial concentration of acetate in the medium. For instance, the maximum activities of ICL with 20, 50, and 100 mM acetate were obtained after 12, 28, and 42 h of the shift, respectively. Thus apparent inhibitory effect by high concentration of acetate on the growth of *Vibrio* ABE-1 is possibly attributed to slow down efficient induction of the enzymes, e. g. ICL and IDH, which concerned in metabolism of acetate. On the other hand, acetate may also inhibit the acetate uptake systems or the enzymes of

**Table 1.** Specific growth rate as a function of temperature.

Carbon source	Specific growth rate (hr <sup>-1</sup> )						
	0°	5°	6°	at 11°	15°	20°	21(°C)
Succinate (0.1M)	0.042 (0.024)		0.084 (0.47)	0.142 (0.80)	0.177 (1.00)	0.110 (0.62)	0.025 (0.14)
Pyruvate (0.1M)	0.051 (0.28)	0.095 (0.52)		0.148 (0.81)	0.183 (1.00)	0.101 (0.55)	—
Acetate (0.1M)	0.049 (0.38)	0.082 (0.64)		0.108 (0.84)	0.129 (1.00)	±	—
Meat ex. (1%) + Peptone (1%)	0.063		0.119	0.186	0.262	ND	ND

*Vibrio* ABE-1 was precultured on respective carbon source for about 12 generations. One ml of the preculture was inoculated into a fresh medium. Precultures and growth experiments were carried out at the same temperature except in the case at 20° and 21°C. In these cases, the cells were precultured at 11°C. Specific growth rate on acetate was estimated near the end of the growth, where the highest rate was obtained. In other cultural conditions, growth rate was estimated at the middle logarithmic phase. Relative growth rates to that at 15°C were indicated in parentheses. —, Cells did not grow; ±, cells grew scarcely; ND, not determined.

the initial metabolism of acetate, since addition of considerable amount of acetate showed the rapid inhibitory effect on the growth (Fig. 3).

It is well known that levels of bacterial intracellular enzymes are influenced by the concentrations of carbon substrate in their environments. HARDER and DIJKHUIZEN (1983) described that responses of enzyme synthesis most frequently observed is increase of the specific activity of enzymes involved in initial metabolism of substrate with decreasing substrate concentrations. Although the above conclusion was drawn from the results by continuous culture experiments, our results of the acetate batch culture seem to accord with the above category.

#### *Effect of temperature on acetate inhibition*

Next, we examined the effect of temperature on the growth in a synthetic medium. As shown in Table 1, the maximum growth rate was obtained at 15°C irrespective to carbon sources tested. However, at 20°C, the bacterium could scarcely grow on acetate, but well on the other carbon sources. Although it remains unclear why the bacterium showed lower maximum temperature for growth on acetate, our results are in conflict with the speculative conclusion by RATKOWSKY *et al.* (1983) that a bacterium exhibits the same maximum growth temperature irrespective to various nutrient conditions.

#### *Intracellular levels of glyoxylate shunt- and related enzymes*

Among the carbon sources tested, only acetate exhibited inhibitory effect on the growth of *Vibrio* ABE-1, hence the growth inhibition by acetate may be due to the metabolism of acetate. Glyoxylate shunt is an anaplerotic pathway to supply tricarboxylic acid (TCA) cycle intermediates those are withdrawn from the cycle for net synthesis of cell constituents of microorganisms under the conditions utilizing C2 compound such as acetate (KORNBERG, 1966). So, we investigated the levels of the enzymes in glyoxylate shunt and of the levels of the two IDH isozymes in the bacteria grown on various amounts of acetate.

As shown in Table 2, the levels of key enzymes (ICL and MS) of glyoxylate shunt were not so varied by changing the concentration of acetate. However, high concentration of acetate (0.2 M) greatly depressed the levels of both IDHs. IDH is the enzyme operating at the metabolic branch point between TCA cycle and glyoxylate shunt, and its unique regulatory system in enteric bacteria by IDH-kinase/phosphatase, has become of interest in recent years (GARNAK *et al.*, 1979; WANG *et al.*, 1982; NIMMO *et al.*, 1984; ELMANSI *et al.*, 1986). Our results with *Vibrio* ABE-1 suggest that IDH isozymes, at least IDH-I, may be associated with the growth capacity on

acetate. However, the details of the intracellular function of IDH isozymes is still unknown and is remained as future subject.

**Table 2.** Levels of glyoxylate shunt and related enzymes.

Carbon source	Activity (nmole <sup>-1</sup> min <sup>-1</sup> mg <sup>-1</sup> protein)						
	IDH-I	IDH-II	I+II	ICL	MS	MDH	CS
Succinate (0.1M)	52	293	345	10	42	ND	ND
Pyruvate (0.1M)	93	285	378	48	113	4134	352
Acetate (0.025M)	162	250	412	294	ND	ND	ND
(0.05M)	132	268	400	283	260	7633	457
(0.1M)	132	258	390	256	ND	ND	ND
(0.2M)	57	114	171	270	200	7344	527
Meat ex. (1%) + peptone (1%)	43	234	277	12	ND	ND	ND

Cells were grown on respective carbon source at 10°C and harvested at the late logarithmic phase. Crude extract was prepared and activities of the enzymes were determined as described in **Materials and Methods**. Values are the average of at least two separate experiments. ND, not determined.

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