Generation of Energy Coupled with Membrane-bound Glucose Dehydrogenase in *Acinetobacter calcoaceticus*

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Acinetobacter calcoaceticus, which is unable to grow in the presence of glucose because it lacks hexokinase and gluconokinase activities, grows well in the presence of permeable intermediates of the tricarboxylic acid cycle. However, addition of glucose led to increased rates of growth with other carbon sources, although it is unable to grow in the presence of glucose as the sole carbon source. This species possesses pyrrolo-quinoline quinone-dependent glucose dehydrogenase on the outside of the membrane, and through coupling with this enzyme, ATP was synthesized in the presence of glucose. The newly biosynthesized ATP with the addition of glucose led to increases in the initial rate of the active transport system and the growth rate of the cells. The supplemental energy due to glucose oxidation may be obtained through a cytochrome-linked reaction with the glucose dehydrogenase.

Pseudomonas, *Serratia* and *Klebsiella* species possess an NAD(P)⁺-independent glucose dehydrogenase.^{1,2)} This enzyme belongs to a novel class of dehydrogenases, viz. the quinoproteins.^{3,4)} A wide range of sugars are oxidized and the corresponding aldric acids are excreted into the growth medium. The glucose dehydrogenase is believed to be localized on the outside of the membrane and appears to be linked to the electron transport system of the membrane.^{5~7)} Thus, it was interesting to investigate the physiological significance of this enzyme.

A. calcoaceticus differs from the above-mentioned bacteria,⁸⁾ which are able to grow in the presence of glucose and gluconate, because it lacks hexokinase and gluconokinase activities.⁹⁾ But it is able to oxidize several sugars via glucose dehydrogenase.¹⁰⁾ The oxidation of glucose by the glucose dehydrogenase greatly stimulates the active transport of various substrates in the cells. Furthermore,

addition of glucose to a growth medium results in a greater rate of growth.¹¹⁾ Based on this evidence, we have assumed that glucose dehydrogenase plays a role in the coupling of energy to active transport. This stimulation is certainly caused by supplementally generated ATP with the addition of glucose.

In this paper, we report that supplemental energy obtained on oxidation of glucose by *A. calcoaceticus* was correlated with the primary glucose dehydrogenase activity.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *A. calcoaceticus* K-104 was obtained from the Institute of Microbiology, Kyoto. *A. calcoaceticus* K-104M, obtained through mutation of K-104, was deficient in glucose dehydrogenase activity. These cells were grown on Stanier's minimum medium¹²⁾ containing 50 mM proline or succinate at 30°C with good aeration.

Preparation of right-side out and inside out membranes.

Cells were harvested by continuous centrifugation at $10,000 \times g$ and then washed with 10 mM Tris-HCl (pH 7.2). Right-side out and inside out membranes were prepared by the methods of Yamato *et al.*¹³⁾ and Futai,¹⁴⁾ respectively, with some modification. The harvested cells were suspended in Tris-HCl containing 3 mM EDTA-lysozyme as the right-side out membrane fraction or in 0.5 mM $MgCl_2$ as the inside out membrane fraction. After the cell suspension had been disrupted in a French press at 400 kg, it was centrifuged for 40 min at $100,000 \times g$ and then washed twice with Tris-HCl containing 0.5 mM $MgCl_2$. These fractions were used as the respective membrane fractions. All the procedures described above were carried out at 4°C.

Enzyme assays. Glucose dehydrogenase activity was determined spectrometrically by measuring the rate of decoloration of 2,6-dichlorophenol-indophenol at 605 nm. The reaction mixture contained 10 mM glucose, 200 μ M 2,6-dichlorophenol-indophenol, membrane fraction (1.5 mg protein) and 50 mM phosphate buffer (pH 6.8) in a total volume of 3.0 ml.

Starvation of cells. Washed, fresh cells were suspended in 33 mM Tris-HCl (pH 7.2) containing 5 mM $MgCl_2$ and sodium arsenate at the concentration of 1×10^{10} cells/ml.¹⁵⁾ The suspension was incubated for 60 min at 30°C with gentle stirring and then the cells were collected by centrifugation.

Measurement of the ATP level. The cellular concentrations of ATP in unstarved and starved cells on the addition of glucose were determined after incubation for 15 min, the reactions being terminated by the addition of 1 ml of ice-cold 20% perchloric acid. The extraction was allowed to proceed for 30 min at 4°C and then the denaturated protein was removed by centrifugation. The supernatant (1 ml) was mixed with 1 ml of 1.5 N KOH, and the 1 ml of $KHCO_3$.¹⁶⁾ The precipitated $KClO_4$ was removed by filtration. The ATP contents of extracts were determined by the luciferin-luciferase assay. To a scintillation vial containing 2 ml of 50 mM sodium arsenate, 5 mM potassium phosphate and 0.5 mM $MgCl_2$, pH 7.0, was added 0.5 ml of the experimental perchlorate extract. Firefly lantern extract (0.1 ml) was added and then the chemiluminescence was determined by counting for 0.2 min at ambient temperature with a scintillation counter.¹⁷⁾ The results were expressed as nmol of ATP/mg protein.

Assays of proline and succinate uptake. The reaction mixture contained 50 mM phosphate buffer (pH 6.8) including 0.5 mM $MgCl_2$, 100 μ M chloramphenicol and cells (5×10^8 cells/ml), at 20°C. After preincubation for 5 min with 0.1 μ M glucose, 1 μ M ^{14}C -substrate was added. The reaction was stopped by the addition of 10 ml of an ice-cold solution of 50 mM phosphate buffer (pH 6.8) containing 0.5 mM $MgCl_2$. The mixture was applied to a

membrane filter (0.45 μ m pore size) and then the radioisotope retained on the filter was determined with a scintillation counter. The initial rates of uptake were defined as the amount of ^{14}C -substrate taken up in 20 sec under the above-mentioned conditions, as pmol/20 sec/ 5×10^8 cells.

Protein assay. The protein contents of cells and membrane fractions were determined by the method of Markwell *et al.*¹⁸⁾

Chemicals. ^{14}C -Proline and ^{14}C -succinate were purchased from New England Co., Inc. Firefly lanterns and 2,6-dichlorophenol-indophenol were purchased from Sigma Chemical Co., Inc. All other chemicals were obtained from commercial sources and were either of reagent grade or the highest purity available.

RESULTS

Glucose dehydrogenase assay

The glucose dehydrogenase activities of K-104 and K-104M were compared (Table I). The glucose dehydrogenase activity in whole cells of K-104M was one-twentieth in comparison with that in the case of K-104. Membrane fraction 1 comprised inside out membranes, which were prepared by the method of Futai, and membrane fraction 2 comprised right-side out membranes, which were prepared by the method of Yamato *et al.* The glucose dehydrogenase activity of membrane fraction 2 was higher than that of membrane fraction 1. These results indicated that the glucose dehydrogenase was located on the outside of the membrane of the cells.

Effect of glucose on growth

Stainer's minimum medium containing 50 mM proline, the same medium supplemented with 10 mM glucose and the medium containing 10 mM glucose alone were inoculated with 5×10^5 cells/ml. The growth curves are shown in Fig. 1A for K-104, and in Fig. 1B for K-104M. The results show that K-104 shows greater rate of growth with the addition of glucose. The same phenomenon was seen when other carbon sources were used. In the case of K-104M, growth was scarcely affected by the addition of glucose. When the medium contained gluconate instead of glucose, the

TABLE I. GLUCOSE DEHYDROGENASE ACTIVITIES IN WHOLE CELLS AND MEMBRANE FRACTIONS OF *A. calcoaceticus* K-104 AND K-104M

Strains	Fractions		
	Whole cells ^a (units/mg protein)	Membrane fraction 1 ^b (units/mg protein)	Membrane fraction 2 ^c (units/mg protein)
K-104	4.41	0.13	3.83
K-104M	0.20	0.05	0.11

^a Glucose dehydrogenase activities of whole cells were measured as the decrease in absorbance of 2,6-dichlorophenol-indophenol at 605 nm per 10 min. The activity was calculated using an extinction coefficient of 10 mM^{-1} for 2,6-dichlorophenol-indophenol.

^b Membrane fraction 1, which is almost exclusively the inside out membrane fraction, was prepared by disruption of the cells by the method of Futai¹⁴⁾ with some modification, and the enzyme activity was measured as the decrease in absorbance of 2,6-dichlorophenol-indophenol.

^c Membrane fraction 2, which is almost exclusively the right-side out membrane fraction, was prepared by disruption of the cells by the method of Yamato *et al.*¹³⁾ with some modification, and the enzyme activity was measured as the decrease in absorbance of 2,6-dichlorophenol-indophenol.

FIG. 1. The Growth Rates of *A. calcoaceticus* K-104 and K-104M.

Comparative growth, expressed as the number of viable cells, of *A. calcoaceticus* K-104 (A) and K-104M (B) growing in the presence of 50 mM proline (●), 50 mM proline plus 10 mM glucose (○), and 10 mM glucose alone (▲)

supplemental effect on growth was not seen (data not shown).

Measurement of the ATP level

Table II shows the cellular concentrations of ATP biosynthesized on the addition of glucose. The endogenous ATP concentrations in unstarved cells and starved cells were deter-

mined. The results showed a change in ATP level after incubation with glucose for 15 min at 25°C, especially in the case of starved cells. With this starvation, the cellular concentration of ATP was reduced to about one-fifth compared with in the case of unstarved cells. It was found that K-104 cells showed potent ability to synthesize ATP on the addition of glucose. On

the other hand, K-104M did not show such potent ability. These phenomena were remarkable in the starved cells. In the case of K-104, about 1.5-fold ATP was biosynthesized in unstarved cells on the addition of glucose, and 8.0-fold ATP in starved cells. But the concentration of biosynthesized ATP with the addition of glucose was not greater than the cellular ADP plus inorganic phosphate concentration before the glucose addition (data not shown). However, both strains were unable to biosynthesize ATP on the addition of gluconate.

TABLE II. THE CONCENTRATIONS OF ATP IN STARVED AND UNSTARVED CELLS ON THE ADDITION OF GLUCOSE AND GLUCONATE IN *A. calcoaceticus* K-104 AND K-104M

Condition of cells	Addition	Cellular ATP concentration (nmol/mg protein)	
		K-104	K-104M
Unstarved	None	1.57	1.32
	Glucose (10 mM)	2.25	1.46
	Gluconate (10 mM)	1.46	1.32
Starved	None	0.18	0.28
	Glucose (10 mM)	1.49	0.41
	Gluconate (10 mM)	0.16	0.26

TABLE III. INITIAL RATES OF PROLINE AND SUCCINATE UPTAKE WITH THE ADDITION OF GLUCOSE AND GLUCONATE IN *A. calcoaceticus* K-104 AND K-104M

Condition of cells	Addition	Proline uptake (pmol/20 sec/5 × 10 ⁸ cells)		Succinate uptake (pmol/20 sec/5 × 10 ⁸ cells)	
		K-104	K-104M	K-104	K-104M
Unstarved	None	12.5	13.2	25.0	20.1
	Glucose (0.1 μM)	23.4	13.9	41.1	20.3
	Gluconate (0.1 μM)	11.6	12.1	20.3	18.3
Starved	None	3.4	4.2	6.8	6.1
	Glucose (0.1 μM)	14.4	4.8	23.6	6.5
	Gluconate (0.1 μM)	2.8	3.9	5.9	5.0

Assays of proline and succinate uptake

Table III shows the effect of the addition of glucose or gluconate on proline and succinate uptake. For the transport of proline or succinate across the cell membrane, a driving force is necessary. The initial rate of proline or succinate uptake of unstarved cells of K-104 was increased about 2-fold on the addition of glucose but that of K-104M scarcely increased. After starvation, the initial rates of proline and succinate uptake were reduced markedly in both strains and those in K-104 increased about 4-fold on the addition of glucose but in the case of K-104M they scarcely increased.

DISCUSSION

Because *A. calcoaceticus* species lacks hexokinase and gluconokinase activities,⁹⁾ it is unable to assimilate glucose and gluconate. On the other hand, it is able to oxidize several sugars to their lactons *via* glucose dehydrogenase. In 1979, it was concluded that the prosthetic group of glucose dehydrogenase was pyrrolo-quinoline quinone.⁴⁾

On comparison of inside out and right-side out membranes, the location of the glucose dehydrogenase on the membrane was assumed to be outside of the membrane in *A. calcoaceticus*. In *Pseudomonas*, *Serratia* and *Klebsiella* species, the primary dehydrogenase of the usual system is believed to exist on the outside of the membrane. But its physiological function is uncertain.

The growth rate of *A. calcoaceticus* which shows high glucose dehydrogenase activity becomes higher on the addition of glucose,¹¹⁾ but strains deficient in the glucose dehydrogenase activity are not affected by the addition of glucose. Therefore, it can be expected that energy due to glucose oxidation *via* glucose dehydrogenase become available for the cell growth.

Some of the problems as to oxidative phosphorylation in the bacterial cell membrane may be solved by measuring proton extrusion in intact cells. According to the chemiosmotic hypothesis theory, proton translocation *via* the electron transport system generates both ΔpH and electrical potentials, which can provide ATP *via* proton translocating ATPase. As we reported in this paper, *A. calcoaceticus* K-104 could biosynthesize ATP on the addition of glucose. On the other hand, K-104M could not biosynthesize ATP on the addition of glucose. This may support the hypothesis of a glucose-oxidizing energy production system.^{5~7,19)}

Active transport is good marker for investigating the glucose oxidizing-energy production system energetically. Even if the driving forces are $\Delta\psi$ or/and ΔpH or/and ATP, this system needs energy. The initial rates of substrate transport were increased by the addition of glucose in K-104. As a result of activation of active transport, the growth rates of the cells may be increased by the energy due to glucose oxidation. This assumption is based on the results of a comparative study on K-104 and K-104M.

A similar energy production system may exist in at least several glucose oxidation bacteria, for example, *Pseudomonas*, *Serratia*, *Klebsiella*, etc. But these species are able to grow in the presence of glucose, and thus it is difficult to detect and investigate a glucose oxidizing-energy production system. From this

point of view, *A. calcoaceticus* may be very attractive bacterium for studying this energy production system.

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