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ORIGINAL PAPER

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H^+-ATPase defect in *Corynebacterium glutamicum* abolishes glutamic acid production with enhancement of glucose consumption rate

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Abstract A mutant of *Corynebacterim glutamicum* (‘*Brevibacterium flavum*’) ATCC14067 with a reduced H^+-ATPase activity, F172–8, was obtained as a spontaneous neomycin-resistant mutant. The ATPase activity of strain F172–8 was reduced to about 25% of that of the parental strain. Strain F172–8 was cultured in a glutamic-acid fermentation medium containing 100 g/l of glucose using a jar fermentor. It was found that glucose consumption per cell during the exponential phase was higher by 70% in the mutant than in the parent. The respiration rate per cell of the mutant also increased to twice as much as that of the parent. However, the growth rate of the mutant was lower than that of the parent. Under those conditions, the parent produced more than 40 g/l glutamic acid, while the mutant hardly produced any glutamic acid. Instead the mutant produced 24.6 g/l lactic acid as the main metabolite of glucose. Remarkably, the accumulation of pyruvate and pyruvate-family amino acids, i.e., alanine and valine, was detected in the mutant. On the other hand, the parent accumulated α-ketoglutaric acid and a glutamate-family amino acid, proline, as major by-products. It was concluded that the decrease in the H^+-ATPase activity caused the above-mentioned metabolic changes in strain F172–8, because a revertant of strain F172–8, R2–1, with a H^+-ATPase activity of 70% of that of strain ATCC14067, showed a fermentation profile similar to that of the parent. Sequence analyses of the *atp* operon genes of these strains identified one point mutation in the gamma subunit in strain F172–8.

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Introduction

Various kinds of useful metabolites have been produced from sugar substrates by fermentation. Therefore, in general, enhancement of sugar metabolism of a producer is considered to be quite important for fermentation industries. Against this background, we have been studying the enhancement of glucose metabolism using a pyruvic acid-producing mutant of *Escherichia coli* K-12 as a model system (Herbert and Guest 1968; Yokota et al. 1994a). In *E. coli*, glucose metabolism via the glycolytic pathway is considered to be regulated by the energy level of the cell. That is, some enzymes of the glycolytic pathway, namely, phosphofructokinase-I (PFK-I, one of the isozymes of *E. coli* PFK), and pyruvate kinase-II (PYK-II, one of the isozymes of *E. coli* PYK), are activated allosterically by ADP and AMP, respectively (Kotlarz et al. 1975; Babul 1978). Thus, a defective gene for F_1-ATPase was introduced into the pyruvic acid producer to cut energy production by oxidative phosphorylation. As a result, the F_1-ATPase-defective mutant showed a remarkable increase in pyruvic acid productivity together with an enhancement of glucose metabolism (Yokota et al. 1994b). Further analyses of the F_1-ATPase-defective mutant revealed a higher rate of oxygen consumption per cell and higher activities of some enzymes in the glycolytic pathway, including the phosphotransferase system for glucose uptake, than those of the parent (Yokota et al. 1997). From these results, it has been suggested that the energy-deficiency mutation enhanced glucose metabolism not only with already known allosteric activation of glycolytic enzymes but also with some aforementioned qualitative changes of the cell associated with differences in the energy level of the cell (Yokota et al. 1999).

In this study, based on the results obtained with *E. coli*, the possibility of the enhancement of glucose metabolism in *Corynebacterium glutamicum* – an industrially important glutamic acid-producing bacterium – was investigated. In *C. glutamicum*, glycolysis is most likely controlled by PYK, since only this enzyme has been re-

ported to be subject to allosteric activation by AMP (Ozaki and Shiio 1969; Jetten et al. 1994). Thus an enhancement of glucose metabolism can be expected with the H^+-ATPase defect, as experienced in *E. coli* K-12. Therefore, a H^+-ATPase-defective mutant was derived for the first time from *C. glutamicum*, and the effects of the mutation on both glucose metabolism and glutamic acid production were investigated. In addition, the mutation point was determined by sequence analysis of the *atp* operon genes.

Materials and methods

Bacterial strains

A wild-type strain of *C. glutamicum* ATCC 14067 (‘*Brevibacterium flavum*’ no. 2247, designated no. 2247 in this study), strain F172–8, a spontaneous H^+-ATPase-defective mutant derived from strain no. 2247, and strain R2–1, a revertant of strain F172–8 having a H^+-ATPase activity of about 70% of that of strain no. 2247, were used.

Media

The complete medium, Medium 7 (Shiio and Ujigawa 1978), contained (1^{-1}) 10 g Polypepton (Nihon Pharmaceutical, Tokyo, Japan), 10 g yeast extract (Oriental Yeast, Tokyo, Japan), 5 g NaCl, 5 g glucose; the pH was adjusted to 7.0 with NaOH. When necessary, 20 g/l of agar was added to solidify this medium, and supplements of filter-sterilized neomycin solution were added to final concentrations of 3–5 µg/ml. Minimal medium consisted of (1^{-1}) 10 g $(NH_4)_2SO_4$, 3 g urea, 1 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 8.1 mg $MnSO_4 \cdot 4–5H_2O$, 100 µg thiamine·HCl, 300 µg biotin, an appropriate carbon source and NaOH to adjust the pH to 7.3. As the carbon source, either 20 g/l glucose, 24.88 g/l sodium lactate or 44.98 g/l disodium succinate hexahydrate was used. The seed medium for glutamic acid production by jar fermentor, Medium S2, contained (1^{-1}) 40 g glucose, 4 g urea, 1 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 4–5H_2O$, 200 µg thiamine·HCl, 60 µg biotin, 0.02 ml antifoaming agent, Adekanol LG-109 (Asahi Denka Kogyo, Tokyo, Japan), 7 ml soy bean-meal hydrolysate (total nitrogen 69.2 g/l) and KOH to adjust the pH to 7.0. The fermentation medium for glutamic acid production by jar fermentor, Medium F3, contained (1^{-1}) 100 g glucose, 1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 4–5H_2O$, 200 µg thiamine·HCl, 3 µg biotin, 0.02 ml Adekanol LG-109, 7 ml soy bean-meal hydrolysate. Glucose and KH_2PO_4 were prepared separately, and mixed with the other components after sterilization. The pH was adjusted to 7.0 with ammonia solution just before seeding.

Derivation of a H^+-ATPase-defective mutant from strain no. 2247

In order to derive the H^+-ATPase-defective mutant from *C. glutamicum*, neomycin-resistant mutants were derived spontaneously from strain no. 2247. Cells cultured overnight in Medium 7 were spread on Medium 7 agar plates containing 3–5 µg/ml neomycin at 10^6–10^7 cells per plate, and cultured at 30°C for 7 days. Colonies appearing on the plates were picked as neomycin-resistant mutants. From among those colonies, strains that could utilize glucose but neither lactate nor succinate as the energy source were selected (Butlin et al. 1971; Rosen 1973); the obtained neomycin-resistant mutants were checked for their growth on minimal agar plates containing glucose, lactate or succinate as the sole carbon source. The strains showing the expected growth response were selected, single-colonies were isolated, and their H^+-ATPase activities were measured. As a result, strain F172–8, with a H^+-ATPase activity of about 25% of that of the parental strain was identified, and kept as a H^+-ATPase-defective mutant.

Derivation of a revertant from strain F172–8

The washed cell suspension prepared from an overnight culture of strain F172–8 in Medium 7 was spread on minimal agar plates containing lactate as a sole carbon source at approximately 10^8 cells per plate. After incubation at 30°C for 7 days, the colonies formed were picked, single colonies isolated, and their H^+-ATPase activities measured. As a result, strain R2–1, with a H^+-ATPase activity of about 70% of that of strain no. 2247, was selected as a revertant of strain F172–8.

Assay of ATPase activity

A suitable method for H^+-ATPase measurement was developed for *C. glutamicum* in which permeabilized cells were used as the crude enzyme. The H^+-ATPase activity was quantified by determining the amount of phosphate released from ATP by the enzyme.

Preparation of the crude enzyme

Cells were cultured in 50 ml of Medium 7 in a 500-ml Sakaguchi flask with shaking at 31.5°C for 12 h until the stationary phase. The cells were harvested by centrifugation, and washed twice with 100 mM Tris-HCl buffer (pH 7.5) containing 2.5 mM $MgCl_2$, and a concentrated cell suspension was prepared in the same buffer to an OD_{660} of 120–150.

Chloroform was added to this cell suspension to a final concentration of 1% (v/v). The mixture was vortexed for 1 min, and incubated at 37°C for 20 min. Then the cells were collected by centrifugation (6,000 *g*; 4°C), washed once with the same buffer, and resuspended in the same buffer to an OD_{660} of 120–150. This suspension was used as the crude enzyme.

ATPase assay

In the course of experiments to establish the assay conditions, the optimum pH for the measurement of the ATPase activity was found to be between 6 and 7 (data not shown). Thus, the reaction mixture contained 20 mM bis-tris propane (pH 6.5), 2.5 mM Na_2ATP, 1 mM $MgCl_2$, 10 µg bovine serum albumin (BSA), 50 µ1 ethanol [final concentration, 8.3% (v/v)], 50 µl crude enzyme, in a total volume of 600 µl. The reaction mixture without Na_2ATP was incubated at 37°C for 20 min, and then Na_2ATP was added to start the reaction. After 15 min at 37°C, 300 µl of 0.1 N HCl was added to stop the reaction, and the mixture was put on ice. The mixture was centrifuged at 5,000 *g* at room temperature for 10 min, and the amount of phosphate in the supernatant was determined. The determination of phosphate was carried out by mixing 0.7 ml of the supernatant with 1.63 ml of coloring reagent, followed by incubation at 18°C for 15 min, and immediate measurement of optical density at 660 nm. The coloring reagent was prepared by mixing 1 part 5N H_2SO_4, 1 part 25 g/l ammonium molybdate, 1 part mixture of 10 g/l *p*-(methylamino)phenol sulfate and 30 g/l $NaHSO_3$, and 4 parts water just prior to use. Potassium phosphate solution was used as the standard. When necessary, *N*,*N*′-dicyclohexylcarbodiimide (DCCD) or NaN_3 was added to the reaction mixture. DCCD was included in the 50 µl ethanol portion of the reaction mixture. The specific activity was defined as nmol of phosphate formed·min^{-1}·mg $protein^{-1}$ of the crude enzyme.

Protein assay

The protein concentration of the crude enzyme was determined by DC Protein Assay kit (Bio-Rad, Calif., USA) with BSA as the

standard. For the determination of the protein in the crude enzyme, 90 µl of the cell suspension before chloroform treatment was mixed with 5 µl of 10% SDS and 5 µl of 10 N NaOH, and the mixture was boiled for 10 min. Then the mixture was centrifuged and the supernatant obtained was used as the sample.

Glutamic acid fermentation

Cells were precultured in 20 ml of Medium S2 in 500-ml Sakaguchi flasks with shaking at 31.5°C for about 12 h until early stationary phase. The cells were harvested by centrifugation, washed once and resuspended in 0.85% NaCl. They were inoculated into 3 l of Medium F3 in a 5-1 jar fermentor (KMJ-5, Mitsuwa Bio Systems, Osaka, Japan) to give an OD_{660} of approximately 2.0. The culture was incubated at 31.5°C with aeration at 3 l min^{-1} and stirring at 600 rpm. The pH was controlled at 7.0 with ammonia solution.

Fermentation analysis

Growth was measured by OD_{660} of the culture broth after appropriate dilution with distilled water. The supernatant of the culture broth obtained by centrifugation was used in the following analysis. L-Glutamic acid was determined enzymatically with L-glutamate oxidase using a YAMASA L-Glu Assay Kit (Yamasa, Choshi, Japan). Glucose was determined by the glucose oxidase method using Glucose-AR II (Wako Pure Chemical Industries, Osaka, Japan). Pyruvic acid, α-ketoglutaric acid and lactic acid were analyzed by HPLC (column: AMINEX HPX-87H, Bio-Rad; mobile phase: 0.01 N H_2SO_4; flow rate: 0.6 ml min^{-1}; detection: absorbance at 2l0 nm). Amino acids in the supernatant were analyzed by amino acid analyzer (Hitachi L-8500, Hitachi, Tokyo, Japan). For the determination of alanine, the supernatant was developed by paper chromatography using the solvent system of 1-butanol;acetic acid;water at 4:1:2 (by vol.), respectively. The paper was dried, then soaked in acetone containing 0.2% ninhydrin, dried again in the dark, incubated at 60°C for 10 min for coloration. The spots corresponding to alanine were excised, extracted with methanol containing 0.004% $Cu(NO_3)_2$ for 30 min, and then the absorbance at 506 nm was measured.

Measurement of respiration rate

During glutamic acid fermentation, the culture broth was withdrawn at appropriate intervals and the respiration rate was measured. The respiration rate of the culture broth was analyzed using a dissolved oxygen analyzer (model MD-1000, Iijima Electronics, Japan) equipped with a Clark-type oxygen electrode. The measurement was done at 30°C in the air-tight chamber with stirring, within the range giving linearity between cell concentration and respiration rate. The oxygen solubility at 30°C was taken to be 0.23 mM in the calculation. The results were expressed as µmol O_2 consumed·min^{-1}·(g dry cell weight)$^{-1}$. The dry cell weight of strains no. 2247 and F172–8 was calculated from the correspondence of one optical density unit at 660 nm to 0.204 mg and 0.248 mg dry cell weight per ml, respectively.

Sequencing and analysis of the *atp* operon genes

The DNA sequence of the *atp* operon genes was determined in order to examine the possible mutation points in the *atp* operon genes of strain F172–8. Genomic DNA of *C. glutamicum* was prepared basically by the method of Nakamori et al. (1987). The primers were designed from the sequence data of the *atp* operon genes of *C. glutamicum* ATCC13060 (DDBJ/EMBL/GenBank database accession number: AB046112), which had been deposited by the authors (H. Sekine, A. Yokota and F.Tomita). The DNA sequencing was carried out using an ABI PRISM 377 DNA Sequencing System (Applied Biosystems, Foster City, Calif.) with a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems). As the thermal cycler, the GeneAmp PCR system 2400 or 9600 (Applied Biosystems) was used. Sequence compilation and computer analysis were performed with the software GENETYX-MAC (Software Development, Japan). The alignment of amino acid sequences was performed on the BLAST server at the National Center for Biotechnology Information (NCBI) and by using the software GENETYX-MAC.

Results

Some characteristics of the H^+-ATPase of strain no. 2247

The assay method for the H^+-ATPase activity of strain no. 2247 was established as described in Materials and methods. Here, some important characteristics of the enzyme are described.

Effect of ethanol on H^+-ATPase activity

When the ATPase activity of strain no. 2247 was assayed in the presence of ethanol, a marked increase and stabilization of the activity was observed compared to that measured in its absence. Therefore, the effect of ethanol concentration on the activity of ATPase was investigated. As shown in Fig. 1A, the ATPase activity increased with increasing ethanol concentrations, and reached 19.5 nmol min^{-1} mg^{-1} in the presence of 15% (v/v) ethanol. This value was about three times that measured in the absence of ethanol. However, addition of ethanol to 20% (v/v) was found to be excessive. From these results, ethanol was added to the reaction mixture at a concentration of 8.3%. Under these conditions, ATPase activity of about 15 nmol min^{-1} mg^{-1} was obtained.

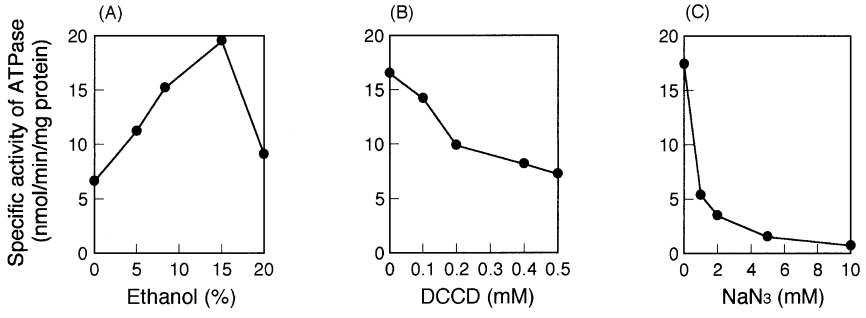

Fig. 1A–C The effects of the addition of **A** ethanol, **B** *N,N537′*-dicyclohexylcarbodiimide (DCCD), **C** NaN_3, on the ATPase activity of strain no. 2247. The activity was measured as described in Materials and methods except that the indicated additions were made

Effects of DCCD and NaN_3 on H^+-ATPase activity

The effects of DCCD and NaN_3, typical inhibitors of F_1F_o-ATPase, on the activity were investigated. As shown in Fig. 1, the activity of F_1F_o-ATPase was reduced in the presence of increasing concentrations of these inhibitors. DCCD inhibited the activity about 50% at 0.5 mM (Fig. 1B). The inhibition by NaN_3 was 91% and 96% at 0.1 mM and 0.5 mM, respectively (Fig. 1C). These results led to the conclusion that the measured ATPase activity can be considered as that of F_1F_o-ATPase.

Derivation of a H^+-ATPase-defective mutant

Neomycin-resistant mutants were obtained on Medium 7 plates containing 3–5 µg/ml neomycin at frequencies of 1.9×10^{-8} to 5.8×10^{-6}. Of 593 resistant mutants, 22 strains that grew on glucose but on neither lactate nor succinate were selected. Measurements of the ATPase activity of these strains revealed that two strains possessed reduced ATPase activity. One of these, strain F172–8, was finally selected for its stability. The ATPase activity of the mutant is presented in Fig. 2. In the mutant, DCCD-sensitive ATPase activity was found to be reduced to less than 25% of that of the parental strain. However, a mutant completely lacking ATPase activity was not obtained.

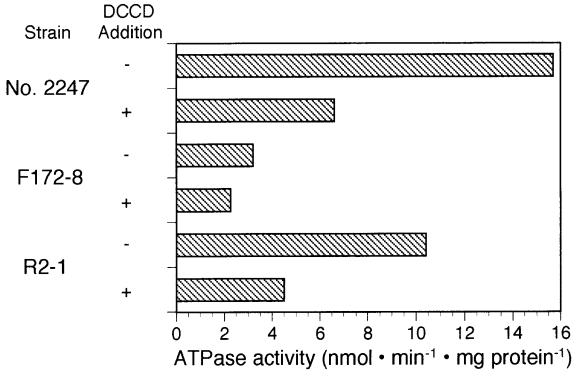

Fig. 2 ATPase activities of the strains no. 2247, F172–8 and R2–1, measured in the presence or absence of 0.5 mM DCCD

Glucose metabolism of strains no. 2247 and F172–8

The H^+-ATPase-defective mutant, F172–8, was cultured in a jar fermentor as described in Materials and methods, and the glutamic acid productivity was compared with that of strain no. 2247, the parental strain. Typical fermentation results are shown in Fig. 3. The rate and level of growth of strain F172–8 in the early stage of the culture were lower than those of strain no. 2247 (Fig. 3A). However, at the end of the culture period, both strains showed similar growth levels. Despite these differences, the rate of disappearance of glucose from the culture medium in both strains was found to be almost the same (Fig. 3B). The times required for the complete consumption of glucose for parental strain no. 2247 and for the mutant, F172–8, were 21 h and 18 h, respectively. As for glutamic acid production, strain F172–8 showed very low productivity at 1.8 g/l, whereas strain no. 2247 produced 41.6 g/l glutamic acid after 21 h of culture (Fig. 3C, Table 1). Table 1 shows the fermentation profiles. After 7 h, the growth level of strain F172–8 was about half that of strain no. 2247, while both strains consumed similar amounts of glucose. Thus, glucose consumption per cell [(B)/(A) Table 1], was found to be higher by a factor of 1.7 in strain F172–8 than in strain

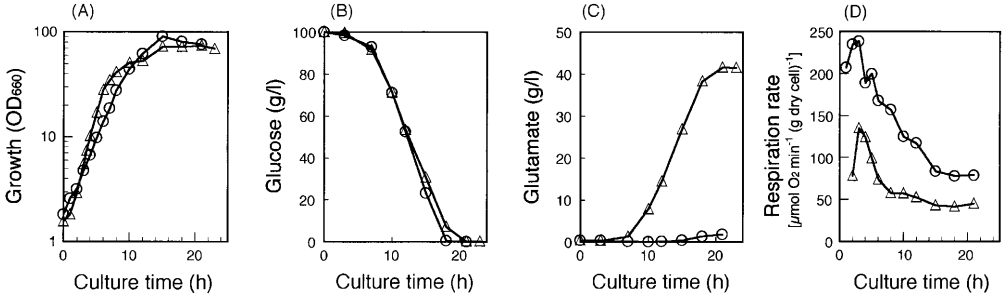

Fig. 3 **A** Growth curves, **B** glucose consumption, **C** glutamic acid production, **D** respiration rate, in a jar fermentor culture. *Triangles* Strain no. 2247, *circles* strain F172–8

Table 1 The fermentation profiles of strains no. 2247, F172-8 and R2–1

	Strain		
	no. 2247	F172-8	R2–1
(A) Increase of cells (OD_{660})[a]	33.2	16.9	38.8
(B) Decrease of glucose (g/l)[a]	9.5	8.5	13.8
(B)/(A)	0.29	0.50	0.36
Glutamic acid produced (g/l)[b]	41.6	1.8	20.7

[a] The fermentation data between 0–7 h were used
[b] The fermentation data after 21 h were used

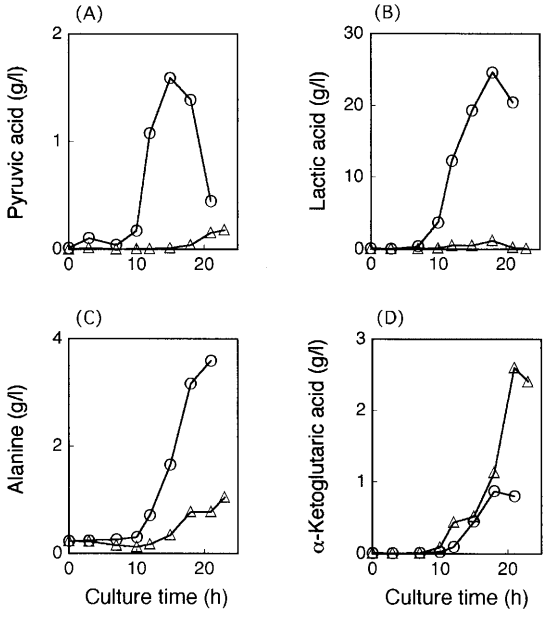

Fig. 4 Production of **A** pyruvic acid, **B** lactic acid, **C** alanine, **D** α-ketoglutaric acid, in a jar fermentor. *Triangles* Strain no. 2247, *circles* strain F172–8

Table 2 The amounts of amino acids produced by strains no. 2247, F172–8, and R2–1. The fermentation medium, Medium F3, originally contained 0.40 g/l L-glutamic acid, 161 mg/l L-proline, 0.17 g/l L-alanine and 107 mg/l L-valine. The fermentation data after 21 h were used

	No. 2247	F172–8	R2–1
Glutamic acid (g/1)	36.2	1.27	24.3
Proline (mg/l)	758.4	48.5	621.7
Alanine (g/l)	0.92	4.36	1.7
Valine (mg/l)	25.5	369.6	88.9

no. 2247. Moreover, in the mutant, respiration rates increased to about twice as much as those of the parental strain during the fermentation (Fig. 3D). Fermentation products other than glutamic acid are shown in Fig. 4. Increased formation of pyruvic acid (Fig. 4A), lactic acid (Fig. 4B), and alanine (Fig. 4C) were observed in strain F172–8. Notably, lactic acid, which is hardly produced by the parental strain, was produced by strain F172–8 to a level as high as 25 g/l. In contrast, lower amounts of α-ketoglutaric acid (Fig. 4D) were observed in strain F172–8. Table 2 shows the results of amino acid analysis of the culture broth. Four amino acids that showed differences between strains no. 2247 and F172–8 are described. Besides alanine, a marked increase in the formation of the pyruvate-family amino acid valine was found in strain F172–8. In contrast, in strain F172–8, formation of a glutamate-family amino acid, proline, decreased to 1/15 of the level produced by strain no. 2247. To summarise these data, changes in metabolism in the mutant leading to increases in glucose consumption per cell, respiration rate, formation of pyruvic acid and the pyruvate-family amino acids alanine and valine, and decreases in glutamic acid production and formation of the glutamate-family amino acid proline, were observed.

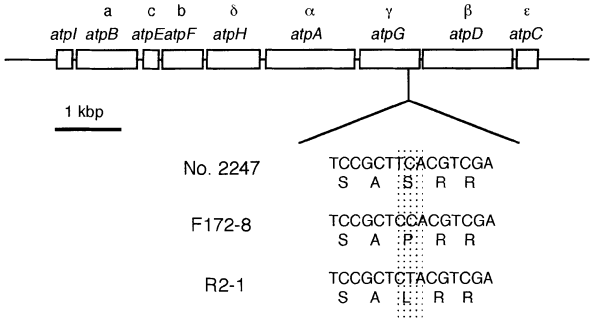

Fig. 5 The *atp* operon structure of strain no. 2247 and the mutation points in strains F172–8 and R2–1

Glucose metabolism by the revertant, R2–1

To check if the changes in the fermentation profile observed in strain F172–8 were due to the defect in H^+-ATPase activity, a spontaneous revertant, R2–1, with a level of H^+-ATPase activity comparable to that of strain no. 2247, was derived from strain F172–8 as described in Materials and methods. As shown in Fig. 2, strain R2–1 restored DCCD-sensitive ATPase activity to 70% of that of the original strain, no. 2247. The results of the jar fermentation in glutamic acid-production medium are summarized in Tables 1 and 2. It was found that the fermentation profile of strain R2–1 was similar to that of the original strain, no. 2247, in terms of growth, glucose consumption, production of glutamic acid and amino acid by-products. The amounts of lactic acid and pyruvic acid detected in the culture broth were also similar to those of strain no. 2247 (data not shown). From these results, it was concluded that the changes in glucose metabolism observed in strain F172–8 were due to the defect in H^+-ATPase activity.

Analysis of the *atp* operon genes and the mutation point

The DNA sequence of the *atp* operon genes of strain no. 2247 was determined (DDBJ/EMBL/GenBank database accession number: AB048368). It revealed a general bacterial-type operon structure (Fig. 5). Operon size, from putative promoter to terminator, was about 7,800 bp and the order of the genes was *atpI*, *atpB*, *atpE*, *atpF*, *atpH*, *atpA*, *atpG*, *atpD*, and *atpC*, encoding an unknown protein, and the subunits a, c, b, δ, α, γ, β, and ε, respectively.

Comparison of the DNA sequence of the *atp* operon genes of strains no. 2247 and F172–8 revealed only one difference. The T at nucleotide position 817 in the γ subunit gene in strain no. 2247 has been changed to a C in strain F172–8. This results in one amino acid change

from serine (amino acid position 273) to proline (Fig. 5). The DNA sequence around this mutation point was examined in the revertant, R2–1. Interestingly, C^{817} in strain F172–8 had not reverted but the next base, C^{818}, was changed to T in strain R2–1. As a result, $proline^{273}$ in the γ subunit of strain F172–8 had been changed to leucine in the revertant (Fig. 5).

Discussion

In this study, an appropriate method for the measurement of H^+-ATPase activity of *C. glutamicum* was developed using permeabilized cells as the enzyme preparation. This ATPase activity was activated and stabilized in the presence of ethanol (Fig. 1A), resulting in a level of activity sufficient for measurement. The activity was strongly inhibited by DCCD (Fig. 1B) and NaN_3 (Fig. 1C), indicating that the activities detected represented those of H^+-ATPase (Roisin and Kepes 1972, 1973).

In this report, we have shown that the isolation of a H^+-ATPase-defective mutant from a coryneform glutamic acid-producing bacterium, *C. glutamicum* ATCC14067 (strain no. 2247), is possible by using such selection markers as neomycin resistance, assimilation of glucose and non-assimilation of succinic acid and lactic acid. In *E. coli*, a correlation between resistance to neomycin (Kanner and Gutnick 1972; Rosen 1973) or to gentamicin (Humbert and Altendorf 1989) and defects in ATPase activity has been reported. Some of these independently obtained aminoglycoside antibiotic-resistant ATPase mutants of *E. coli* appeared to have membranes leaky to protons, and a decrease in proton gradient across such membranes was demonstrated (Tsuchiya and Rosen 1975; Humbert and Altendorf 1989). Genetic analyses of these mutants have located the mutation to the γ subunit of F_1-ATPase (Kanazawa et al. 1985; Humbert and Altendorf 1989). It has been elucidated that the assembly of the F_1 complex is incomplete with the defective γ subunit. Under these conditions, a defective proton pathway is formed by F_1F_o-ATPase, and the leakage of protons would occur (Kanazawa et al. 1983). The main driving force for the uptake of neomycin into an *E. coli* cell has been presumed to be the membrane potential (Δφ, interior negative) component of proton motive force (Humbert and Altendorf 1989). Thus in cells with this kind of defective H^+-ATPase, the decrease in Δφ due to proton leakage would result in the reduced uptake of neomycin into the cells, thereby leading to resistance to the antibiotic (Humbert and Altendorf 1989). Whether or not this mechanism also functions in *C. glutamicum* is not clear. It has also been shown that ATPase-defective mutants are not able to utilize such unfermentable carbon sources as succinic acid or lactic acid (Butlin et al. 1971; Rosen 1973). This is because of the fact that these carbon sources yield ATP mainly via oxidative phosphorylation, which is defective in the mutants. On the other hand, ATPase-defective mutants are able to grow on glucose, which yields ATP not only via oxidative phosphorylation but also via substrate-level phosphorylation. Similar characteristics, i.e, neomycin resistance, ATPase defect, utilization of glucose and non-utilization of succinate, have also been reported in *Bacillus subtilis* (Santana et al. 1994).

The mutant obtained, F172–8, still exhibited DCCD-sensitive ATPase activity at a level of about 25% of that of the parental strain under the assay conditions (Fig. 2). No mutant completely lacking ATPase activity was obtained. Despite the detectable ATPase activity, strain F172–8 did not assimilate lactic acid nor succinic acid, and a correlation between the recovery of lactic acid assimilation and the restoration of ATPase activity was observed in a revertant, R2–1, derived from strain F172–8. Thus it was assumed that the H^+-ATPase complex of strain F172–8 either lacks ATP synthesizing activity in vivo or has only very low levels, and does not produce enough ATP to support growth. Therefore, this strain was judged to be suitable for the present purpose.

From the sequence analyses of the *atp* operon genes of the strains no. 2247 and F172–8, the mutation was localized to the γ subunit as a point mutation (Fig. 5). Since the γ subunit works as the main shaft for the rotation of the H^+-ATPase rotor comprising the c, γ and ε subunits (Abrahams et al. 1994; Engelbrecht and Junge 1997; Noji et al. 1997), this mutation might reduce the ATP synthesizing function of the H^+-ATPase. The finding that strain R2–1 has a pseudo-wild-type γ subunit well explained the partial restoration of its ATPase activity (Figs. 2, 5).

From the results of the jar fermentation of strains no. 2247 and F172–8 in glutamic acid production medium (Fig. 3A, B), it appeared that although the growth rate of strain F172–8 was lower than that of strain no. 2247 in the early stage of the culture, the amount of glucose consumed per cell was higher in the former by 70% (Table 1). This increased activity of glucose metabolism was considered to be a response of the *C. glutamicum* cell to compensate for the shortage of intracellular ATP due to the defect in oxidative phosphorylation through enhanced substrate-level phosphorylation. Under these conditions, the increased amounts of NADH formed along with glycolysis must be recycled by reoxidation through the respiratory chain. The observation that the respiration rate per cell of strain F172–8 was increased about twofold seems to support this explanation (Fig. 3D). Similar results were also obtained with *E. coli* (Yokota et al. 1997) and *B. subtilis* (Santana et al. 1994).

Glutamic acid production was hardly detected in strain F172–8, while strain no. 2247 produced more than 40 g/l of glutamic acid (Fig. 3C). Instead, strain F172–8 produced large amounts of lactic acid (Fig. 4B). A decrease in the formation of α-ketoglutaric acid (Fig. 4D), a precursor of glutamic acid, and proline (Table 2), a glutamate-family amino acid, was also observed. In contrast, formation of pyruvic acid (Fig. 4A), and its related amino acids, alanine (Fig. 4C, Table 2) and valine

(Table 2), was increased. The increase in pyruvic acid and related compounds seemed to reflect the high intracellular pyruvic acid concentration due to the enhanced glucose metabolism. These observations suggest that remarkable changes in the glucose metabolism have been brought about in strain F172–8 by the H^+-ATPase defect. Why glutamic acid production was decreased in strain F172–8 is not clearly understood. However, it seemed that the intracellular concentrations of NADH and pyruvic acid in strain F172–8 were increased by the enhanced rate of glycolysis. Under these conditions, the formation of lactic acid by lactate dehydrogenase must be enhanced as evidenced by the increased excretion of lactic acid into the medium (Fig. 4B). The carbon flow into the TCA cycle seems to be decreased in strain F172–8, judging from its decreased α-ketoglutaric acid excretion (Fig. 4D). The mechanism for this change in the metabolic flow is not known, but some regulation that prevents excess formation of NADH through TCA cycle reactions might be working. These points need to be clarified in future investigations.

This study clearly demonstrated that glucose metabolism is enhanced in a H^+-ATPase-defective mutant of *C. glutamicum* as in the case of the F_1-ATPase-defective mutant of *E. coli* (Yokota et al. 1994b, 1997, 1999). This finding is significant considering the industrial importance of *Corynebacterium* in the production of amino acids and nucleic acids. The characterization of strain F172–8 will contribute not only to the study of energy metabolism in coryneform–glutamic acid producers, but to the more effective utilization of this group of bacteria in the fermentation industry.

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