0273-2289/91/2703—0251$2.00

Glucose Utilization by Lysine-Producing Fluoroacetate-Sensitive Mutants of *Corynebacterium Glutamicum*

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Received September 27, 1989; Accepted March 22, 1990

ABSTRACT

A fluoroacetate-sensitive mutant was isolated from *Corynebacterium glutamicum*, ATCC 21513, following mutagenesis with NTG. Batch fermentations show that in terms of growth kinetics, glucose utilization, and lysine formation, there are significant differences between the mutant and the parent. The mutant's specific growth rate (0.22/h) is lower than that for the parent (0.34/h). Also, the yield expressed as lysine/glucose consumed does not alter as a function of the glucose concentration for the mutant, and is about 0.22, whereas for the parent, this coefficient decreases with increasing glucose concentration. The maximum specific rate of lysine production for the mutant is 1.3 g/L/h that is about two-fold higher than that for the parent.

Index Entries: *Corynebacterium glutamicum;* fluoroacetate sensitivity; L-lysine production; strain improvement.

INTRODUCTION

L-lysine is an essential amino acid that is used as an animal feed supplement in quantities exceeding 80,000 tons/yr (*1*). Only about 5% of this L-lysine is produced using enzymatic techniques (*2*), whereas the rest is produced via fermentation using mainly mutants belonging to the genus *Brevibacterium* or *Corynebacterium*.

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Whereas fluoroacetate resistance or sensitivity has been described in many systems, such as, *Escherichia coli* (*3*), as well as citrate overproducing mutants of the genus *Candida* (*4*, cited in *5*), there is a lack of information on the effect of fluoroacetate sensitive mutants on lysine production by corynebacteria.

We report studies on a mutant of *Corynebacterium glutamicum* ATCC 21513 that was isolated by virtue of its hypersensitivity to fluoroacetate. The aim of the work is to make a comparison between the parent's and mutant's growth kinetics and glucose utilization, so as to gain an insight into the basis for the mutant's improved capacity to overproduce lysine.

METHODS

Derivation of the Mutant Strain

The parent strain *C. glutamicum*, ATCC 21513, (10^9 cells/mL) was incubated in 0.1*M* sodium phosphate buffer, pH 7.0, in the presence of *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine (500 μg/mL) for 15 min at 30°C. The cells were harvested by centrifugation, washed twice, and then inoculated directly onto a medium containing nutrient agar, plus sodium acetate (2 g/L).

Colonies were allowed to grow for 48 h at 30°C, and then transferred by replica plating onto plates with either minimal medium agar or minimal medium agar, plus sodium monofluoroacetate (0.01 to 1 m*M*).

About 800 colonies were then tested for growth on minimal medium as well as growth on minimal medium, plus fluoroacetate.

Innoculum Preparation

A sterile seed medium with the following composition was prepared (g/L); glucose monohydrate, 40; KH_2PO_4, 0.5; $K_2HPO_4 \cdot 3H_2O$, 1.5; $MgSO_4 \cdot 7H_2O$, 0.5; peptone, 20; beef extract, 5; and biotin, 50 μg/L. This medium was innoculated with the appropriate strain of *C. glutamicum* and grown for 24 h at 30°C.

Fermentations

These were carried out in 250 mL Erlenmeyer flasks, and unless otherwise specified, a medium with the following composition was used (g/L): glucose monohydrate, 100; KH_2PO_4, 0.7; $K_2HPO_4 \cdot 3H_2O$, 4; $MgSO_4 \cdot 7H_2O$, 0.3; $(NH_4)_2SO_4$, 40; trypticase (BBL's tryptic digest of casein), 20; calcium carbonate, 20; thiamin, 5 mg/L and biotin, 60 μg/L, pH 7.0. Fermentations in batch were begun by innoculation with 5% of the seed medium described above. These were carried out for 48 h with orbital shaking (220 rpm) at 30°C, unless otherwise specified.

Analytical Methods

Lysine was determined using a modified acidic ninhydrin assay (*6*). Residual glucose was measured using the dinitrosalicyclic acid reagent (*7*). Cell density was determined by turbidity measurement at 660 nm and expressed as the dry wt (48 h, 105°C), determined using a dry wt conversion factor.

RESULTS

Characterization of Fluoroacetate-Sensitive (FA^S) Mutant

Out of about 800 colonies of the parent strain *C. glutamicum*, ATCC 21513, that were subjected to mutagenesis and subsequently screened for sensitivity toward fluoroacetate, the growth of about 11% was inhibited by fluoroacetate (0.05 m*M*). Within this group of fluoroacetate-sensitive mutants, about one-fourth produced significantly more lysine than the parent strain. In contrast, mutants that were not sensitive to fluoroacetate did not produce significantly more lysine than the parent strain. The results presented are representative of the group of fluoroacetate sensitive mutants that also retained the parent's key characteristics, namely, resistance to the lysine analogue S-(2'-aminoethyl)-L-cysteine, as well as resistance to penicillin G.

Growth Kinetics and Glucose Utilization

The growth curves (Fig. 1) show that whereas the lag phase for the parent was short, lasting about 4 h, that for the mutant was considerably longer, and lasted about 15 h. Maximal growth was attained after about 20 h in the case of the parent, whereas for the mutant, it took twice as long. Specific growth rate (μ) for the parent was found to be 0.34 h^{-1} is higher than that observed for the mutant, 0.22/h. The parent's growth rate occurred concommitantly with a higher rate of glucose consumption. At the end of the logarithmic growth phase, virtually 90% of the glucose added was consumed by the parent, whereas the mutant had about 35% glucose remaining at the end of its logarithmic growth phase.

Yield Coefficients

The differences in carbohydrate utilization between the parent and mutant strains was reflected primarily in the yield (g lysine/g glucose consumed) profile (Fig. 2). In the case of the parent strain, a reproducible trend was observed in which this coefficient decreases with increasing glucose concentration, whereas in the case of the mutant, it is independent of the glucose present in the fermentation medium. Within a 10-fold

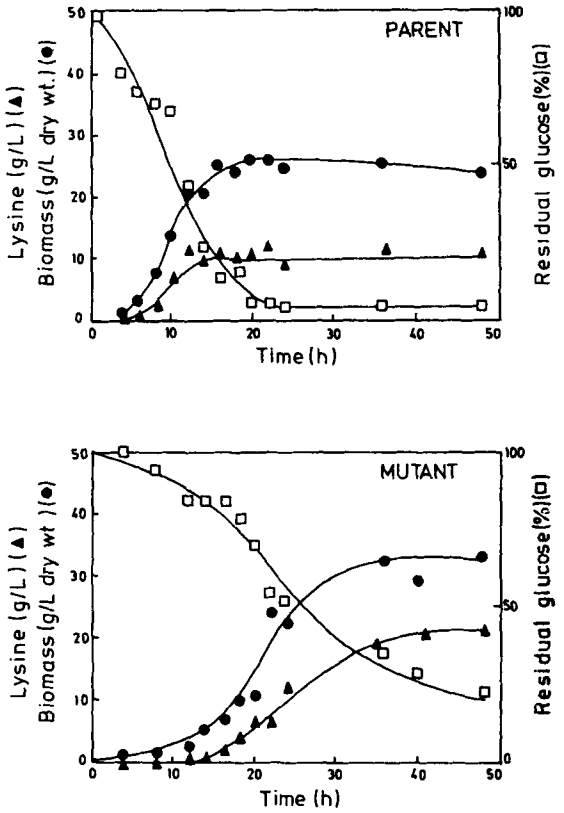

Fig. 1. Time-course of fermentation for the parent strain *Corynebacterium glutamicum*, ATCC 21513, and for the FA-sensitive mutant. Fermentations were carried out for 48 h in complex media. For experimental details, see METHODS. Growth (g/L dry wt.), (●); residual glucose (%), (□); Lysine (g/L), (▲).

glucose concentration range of 2 to 20%, a constant value of about 0.22 was obtained. When 10% glucose was used, the $Y_{(lys/glu)}$ for the mutant was about twofold higher than that for the parent strain.

Lysine Production

When glucose (10%) was used, the maximum rate of lysine productivity for the mutant was 1.3 g lysine/L/h and occurred at about 24 h (Fig. 3). This value is about twofold higher than that observed for the parent strain. At this glucose concentration, lysine production appears to be a growth-coupled process as the production period can be roughly correlated with the corresponding exponential growth phase.

It can be seen from Table 1 that when glucose (10%) was employed as the main carbon source in the presence of a tryptic digest of casein, the mutant produced about 70% more lysine than the parent strain. In the absence of trypticase, i.e., in a chemically defined medium, the mutant is a slightly better producer of lysine than its parent. With a beetroot molasses (15%) medium, a production of about 19 g/L lysine was obtained

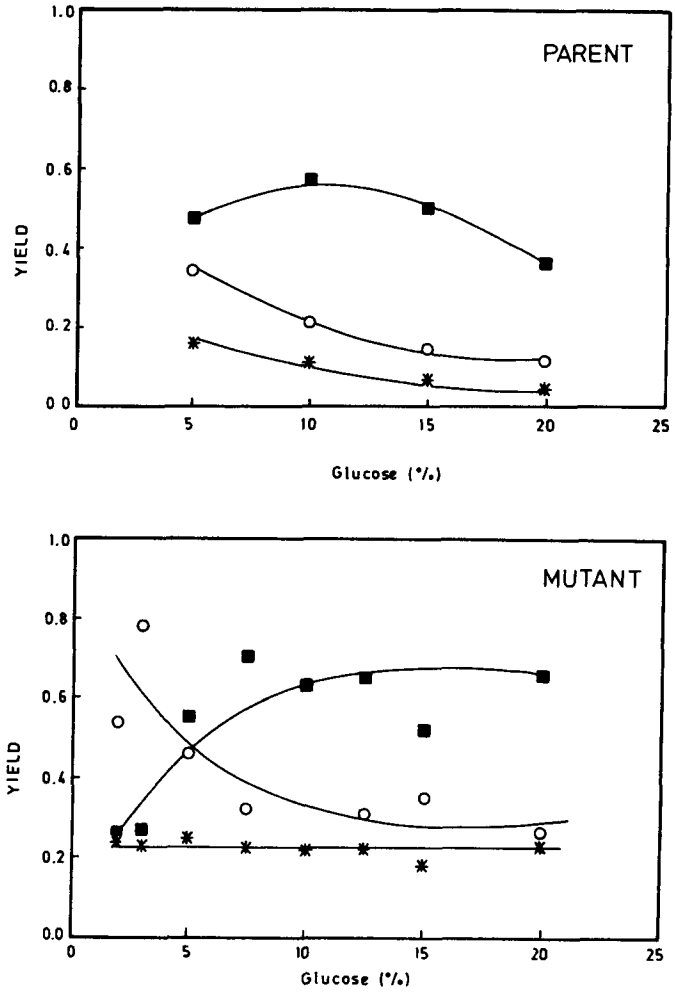

Fig. 2. Yield coefficients for the parent strain C. *glutamicum*, ATCC 21513, and for the FA-sensitive mutant, expressed as a function of increasing glucose concentration. Y(lys/biomass) (■); Y(biomass/glu) (○); and Y(lys/glu) (✳).

for the mutant, and was about 30% more than the amount produced by the parent strain. In a medium containing a combination of glucose (5%), plus acetate (0.75%), the mutant produced about 12 g/L lysine that was an increment of about 37% over the amount produced by the parent strain.

DISCUSSION

The FA-sensitive mutant described in the present work showed marked differences from the parent C. *glutamicum*, ATCC 21513, in terms of both glucose (present work) and acetate (unpublished results) utilization. It is possible that a mutation(s) has occurred in the early steps of the TCA

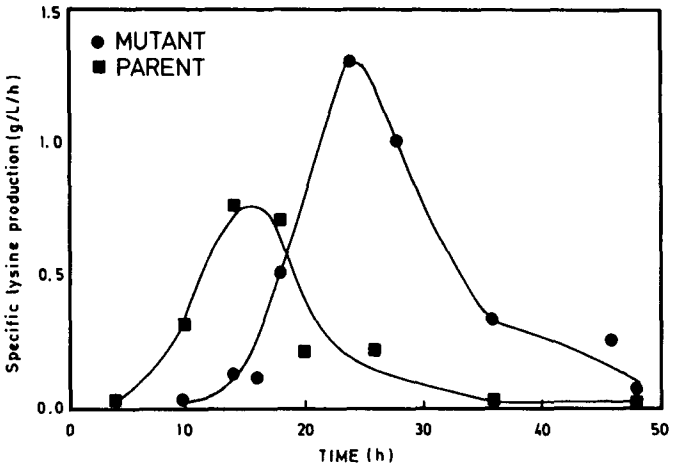

Fig. 3. Specific rate of lysine productivity in fermentation medium containing 10% glucose. Parent ATCC 21513 (■); FA-sensitive mutant (●).

Table 1
Effect of Fermentation Medium Composition on Lysine Production by Parent and Mutant Strains

FERMENTATION MEDIUM*	LYSINE (g/l) PARENT (ATCC 21513)	LYSINE (g/l) FA^S MUTANT
1. Glucose 10% (w/v)	13.3	22.1
(n = 6)	±2.7	±1.3
2. Glucose 10% (w/v) no Trypticase**	10.8	12.6
(n = 5)	±0.5	±1.8
3. Beetroot molasses 15 % (w/v)	14.5	19.1
(n = 3)	±2.9	±1.5
4. Glucose 5% (w/v) plus Acetate 0.75%	8.7	11.9
(n = 3)	±0.5	±1.7

* The basic medium and fermentation conditions are as described under METHODS.
** Determined after 96-h fermentation.
Values shown are the mean + S.E of *n* determinations.

cycle, prior to the channeling of the branch-point intermediate, isocitrate, either further along the TCA cycle or into the glyoxylate bypass. This is in agreement with studies using FA-sensitive mutants of *Candida lipolytica* that overproduced citric acid as a result of reduced aconitate hydrase activity (*4*). Accumulation of glutamate by the *C. glutamicum* parent but not by the FA-sensitive mutant could conceivably have accounted for higher lysine accumulation by the mutant. However, HPLC analysis of the amino acids in the fermentation broth showed that neither the parent nor the mutant accumulated any glutamate (results not shown).

A relevant difference between the parent and mutant strains revealed in the HPLC chromatogram was the parent's accumulation of a small amount of the pyruvate-derived amino acid, valine. The mutant did not accumulate this amino acid, that may possibly suggest an additional mutation prior to the valine or indeed, pyruvate, synthesis.

These data, taken together with the higher apparent lysine yield on glucose consumed observed for the FA-sensitive mutant, would suggest that glucose is channeled toward lysine production via a different route. It is possible that in this mutant, the involvement of the TCA cycle in lysine formation is reduced, and that the asparatate precursor, oxaloacetate, may be synthesized via the carboxylation of phosphoenolpyruvate. Previous studies with *Brevibacterium flavum* mutants defective in glycolytic (*8–10*) and/or TCA cycle (*11,12*) enzymes have shown that one of the more efficient routes for lysine formation involves minimal participation of the TCA cycle, and is one in which oxaloacetate is synthesized via phosphoenolpyruvate carboxylase.

Currently, work is in progress to determine the activities of relevant enzymes about gaining an insight into the biochemical basis for the mutant's ability to accumulate greater amounts of lysine than its parent. The ability of such mutants to utilize a wide range of carbon substrates, including glucose, molasses, as well as a combination of glucose/acetate, make them promising candidates for the production of amino acids of the aspartate family.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent technical assistance of Natalina da Gloria.

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