BMC Biotechnology

Research article

# L-Glutamate production by lysozyme-sensitive *Corynebacterium glutamicum ltsA* mutant strains

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Published: 16 October 2001

*BMC Biotechnology* 2001, **1**:9

Received: 28 June 2001

Accepted: 16 October 2001

This article is available from: http://www.biomedcentral.com/1472-6750/1/9



## Abstract

**Background:** A non-pathogenic species of coryneform bacteria, *Corynebacterium glutamicum,* was originally isolated as an L-glutamate producing bacterium and is now used for fermentative production of various amino acids. A mutation in the *C. glutamicum ltsA* gene caused susceptibility to lysozyme, temperature-sensitive growth, and L-glutamate production.

**Results:** The characteristics of eight lysozyme-sensitive mutants which had been isolated after *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine mutagenesis were examined. Complementation analysis with the cloned wild-type *ltsA* gene and DNA sequencing of the *ltsA* region revealed that four mutants had a mutation in the *ltsA* gene. Among them, two mutants showed temperature-sensitive growth and overproduced L-glutamate at higher temperatures, as well as the previously reported *ltsA* mutant. Other two showed temperature-resistant growth: one missense mutant produced L-glutamate to some extent but the other nonsense mutant did not. These two mutants remained temperature-resistant in spite of introduction of *ltsA::kan* mutation that causes temperature-sensitive growth in the wild-type background.

**Conclusions:** These results indicate that a defect caused by the *ltsA* mutations is responsible for temperature-sensitive growth and L-glutamate overproduction by *C. glutamicum*. The two temperature-resistant mutants seem to carry suppressor mutations that rendered cells temperature-resistance and abolished L-glutamate overproduction.

## Background

Coryneform bacteria are rod-shaped, non-sporulating Gram-positive bacteria that are widely distributed in nature. One of the non-pathogenic species of coryneform bacteria, *Corynebacterium glutamicum,* is now used for industrial production of amino acids. L-Glutamic acid [1,2], L-lysine [3], L-ornithine [4] and L-threonine [5], which are used as flavor enhancers in food and animal feed supplements, are now produced by direct fermentation using this bacterium.

*C. glutamicum* was originally isolated as an L-glutamate producing bacterium [1,2]. L-Glutamate excretion by *C. glutamicum* is induced by biotin limitation [6], treatment with penicillin [7], or by addition of fatty acid ester surfactants [8]. Since these treatments correlate with alterations in cell surface structure, it had been thought

until the 1980s that glutamate leaks passively through a membrane. Recently, several findings that do not agree with the leakage model were reported [9–11]. At present, the mechanism of L-glutamate excretion of *C. glutamicum* is remained to be elucidated.

*C. glutamicum* shows high tolerance against lysis by lytic enzymes such as egg white lysozyme that catalyzes hydrolysis of β-1,4 glycoside bond between *N*-acetylglucosamine and *N*-acetymuramic acid of peptidoglycan. This is probably because the outer layer consisting mainly of mycolic acid affords enough resistance to cell lysis and/or functions as a permeability barrier against the enzymes. In order to analyze the cell wall structure of *C. glutamicum* and the relationship between L-glutamate production and the treatments that affect cell surface integrity, we have analyzed lysozyme-sensitive mutants of *C. glutamicum*.

In a previous paper, we isolated a novel gene, *ltsA,* that complements the mutation of a *C. glutamicum* lysozyme-sensitive mutant KY9714 [12]. DNA sequencing of the *ltsA* gene suggested that it encodes a *purF*-type glutamine-dependent amidotransferase [13]. The *ltsA* gene product shows high sequence homology with glutamine-dependent asparagine synthetases from various organisms, but the *ltsA* gene could not rescue the asparagine auxotrophy of an *Escherichia coli asnA asnB* double mutant. Disruption of the *ltsA* gene caused temperature-sensitive growth, susceptibility to lysozyme, and induced hyperproduction of L-glutamate at elevated growth temperatures. To study the *ltsA* gene function in *C. glutamicum,* we have screened lysozyme-sensitive mutants of *C. glutamicum* whose sensitivities were complemented by *ltsA* gene and examined L-glutamate production by these mutants.

## Results and Discussion

### *Complementation analysis of C. glutamicum lysozyme-sensitive mutants by the ltsA gene*

*C. glutamicum* lysozyme-sensitive mutants were isolated after *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) mutagenesis by a research group of Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). The method for isolation of such mutants was described by Katsumata et al. [14]. Among the eight mutants examined, four showed temperature-sensitive growth and lysozyme-sensitivity similar to those of the previously reported *ltsA* mutant strain KY9714 [12]. The other four showed only lysozyme-sensitivity.

First, we carried out complementation analyses of these mutants using the *ltsA* gene. We introduced by electroporation pHLS4 [12] which carries an approximately 4 kb *Eco*RI fragment containing the *ltsA* gene on the *E. coli-C. glutamicum* shuttle vector pC2 [15], and the transformants were checked for temperature-sensitivity and lysozyme-sensitivity. Strains KY9704 and KY9706, which showed both lysozyme-sensitivity and temperature-sensitive growth, were complemented for lysozyme-sensitivity and temperature-sensitivity by pHLS4 like KY9714. Strains KY9713 and KY11939, which showed only lysozyme-sensitivity, were also complemented by pHLS4. The other 4 strains were not complemented by pHLS4. These results are summarized in Table 1.

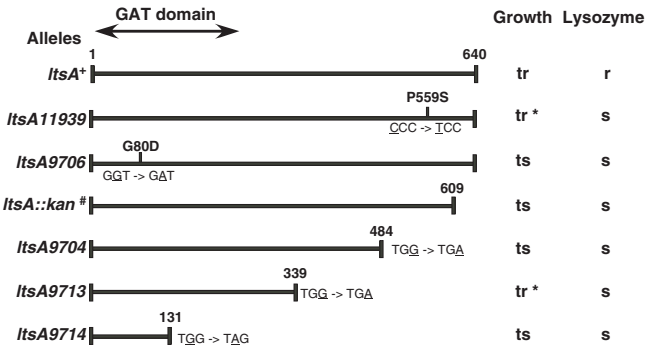


**Figure 1**
Summary of mutation sites of the *ltsA* mutant strains. The length of the horizontal lines corresponds to that of the LtsA proteins produced by the *ltsA* mutants. Double-headed arrow indicates probable glutamine amido transfer (GAT) domain of the LtsA protein. tr: temperature-resistant, ts: temperature-sensitive, r: resistant to lysozyme, s: sensitive to lysozyme.*These two strains probably carry suppressor mutations that suppress the temperature-sensitive growth of *ltsA* mutants as described in the text. #The *ltsA*::*kan* disruptant strain carries the 5'-truncated *ltsA* and the 3'-truncated *ltsA*. However, a product expressed from only the 3'-truncated one is shown because the 5'-truncated gene is not transcribed due to the absence of its promoter.

### *Determination of mutation sites of the mutants complemented by the ltsA gene*

To localize mutation sites of the mutants that were complemented by pHLS4, we carried out recombinational complementation tests as described in Materials and Methods section, using plasmids composed of parts of the *ltsA* gene DNA fragment and pC2. Judging from the results (data not shown), the mutation(s) of KY9704 and KY9713 should be carried by the 0.9 kb *Age*I-*Sal*I fragment and that of KY11939 in the 0.2 kb *Kpn*I-*Sal*I fragment. Both fragments encode the C-terminal regions of the LtsA protein. KY9706 should have a mutation(s) in the 1.1 kb *Eco*RI-*Sac*I fragment which encodes the N-terminal portion of the LtsA.

The corresponding regions of the mutant genes were sequenced after amplification by polymerase chain reac-

**Table 1: Summary of temperature- and lysozyme-sensitivity of *C. glutamicum* lysozyme-sensitive mutants and complementation by the *ltsA* gene**

| Strains | Parent strains | Growth[a] | Lysozyme-sensitivity[b] | Complementation by *ltsA* gene [c] |
|---|---|---|---|---|
| KY9703 | ATCC 13032 | tr | s | - |
| KY9704 | ATCC 13032 | ts | s | + |
| KY9705 | ATCC 13032 | tr | s | - |
| KY9706 | ATCC 13032 | ts | s | + |
| KY9707 | ATCC 13032 | ts | s | - |
| KY9708 | ATCC 13032 | ts | s | - |
| KY9713 | KY9611 | tr | s | + |
| KY9714[d] | KY9611 | ts | s | + |
| KY11939 | KY9611 | tr | s | + |

[a] tr, temperature-resistant; ts, temperature-sensitive. [b]s, sensitive to lysozyme.[c] +, complemented; -, not complemented.[d] KY9714 was described in the previous paper [12].

tion (PCR). The results of sequencing are summarized in Fig. 1. All mutations found were G:C → A:T transitions, as expected from the mutation spectrum caused by NTG mutagenesis. A temperature-sensitive mutant KY9704 had a nonsense mutation causing substitution of the 485th tryptophan residue of the LtsA protein for a stop codon (W485opal; this mutant allele was designated as *ltsA9704*). Another temperature-sensitive mutant KY9706 had a missense mutation (G80D; *ltsA9706*) at the conserved residue in the N-terminal possible glutamine amido transfer (GAT) domain of the *ltsA* gene product. A temperature-resistant mutant KY11939 had a missense mutation (P559S; *ltsA11939*) at the C-terminal non-conserved residue of the LtsA protein. Another temperature-resistant mutant KY9713 had a nonsense mutation (W340opal; *ltsA9713*) in the *ltsA* locus.

### *Suppressor mutations that render ltsA mutants temperature-resistance*

Among six *ltsA* mutants (including *ltsA::kan* mutant [12]) isolated so far, the two mutant strains carrying the alleles *ltsA9713* and *ltsA11939,* respectively, did not exhibit temperature-sensitive growth. Since even the *ltsA::kan* mutation which produces a longer LtsApeptide (609 amino acid residues) than the *ltsA9713* (W340opal, 339 amino acid residues) causes temperature-sensitive growth (see Fig. 1), it was curious that the *ltsA9713* mutant KY9713 was temperature-resistant. In order to confirm whether these two alleles, *ltsA9713* and *ltsA11939,* really confer temperature-resistant growth on the mutant cells, disruption of the *ltsA* locus in these two mutant strains was carried out by homologous recombination between the *ltsA* locus on the chromosome and the internal fragment of the *ltsA* gene (1.2 kb *Pst*I-*Sal*I fragment) on *E. coli* vector plasmid pHSG298 as reported previously [12]. The chromosomal disruption of *ltsA* was checked by PCR (data not shown). A 5'-truncated *ltsA* and a 3'-truncated *ltsA* should be generated by a single recombinational event, but only the 3'-truncated one is expressed because the 5'-truncated gene is not transcribed due to the absence of its promoter. The resultant KY9713 *ltsA::kan* and KY11939 *ltsA::kan* disruptant strains produced the same truncated LtsA protein as that produced in KY9611 *ltsA::kan* strain [12] and showed leakiness in lysozyme-sensitivity like KY9611 *ltsA::kan* as previously reported [12]. However, these disrupted strains still showed temperature-resistant growth. These results indicate that the KY9713 and KY11939 strains seem to carry suppressor mutations that render *ltsA* mutants temperature-resistance extragenically.

### *L-Glutamate production by C. glutamicum ltsAmutant strains*

In order to determine the effect of the *ltsA* mutations on L-glutamate production by *C. glutamicum,* we examined L-glutamate production of the *C. glutamicum ltsA* mutants at various temperatures (Fig. 2).

Wild-type strains, both ATCC 13032 and KY9611, produced very small amounts of L-glutamic acid at all temperatures examined. On the other hand, temperature-sensitive *ltsA* mutants, KY9706 and KY9704 as well as KY9714 and KY9611 *ltsA::kan* [12], produced significant amounts of L-glutamate at higher temperatures. KY9706 carrying a missense mutation in the probable GAT domain of the LtsA protein and showing temperature-sensitive growth produced a significant amount of L-glutamate at 35°C. KY9704 carrying a nonsense mutation produced large amounts of L-glutamate at 37°C, comparable to KY9611 *ltsA::kan* [12].

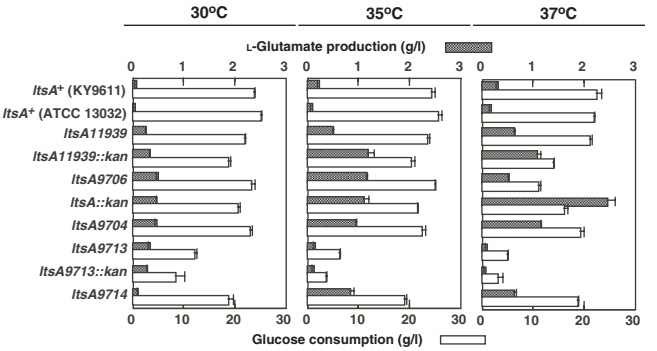


**Figure 2**
L-Glutamate production of *C. glutamicum ltsA* mutants. L-Glutamate production by the *ltsA* mutants was examined in basal salt medium at 30, 35 and 37°C. After 24 h of cultivation, concentrations of L-glutamate and glucose in the medium were measured by using glutamate oxidase or glucose oxidase sensor. Means ± standard deviations are indicated ($n$ = 3). The results of the KY9714 (*ltsA9714*) and KY9611 *ltsA::kan* were as previously reported [12].

Temperature-resistant KY9713 that appeared to carry a suppressor mutation did not overproduce L-glutamate at all temperatures examined. Interestingly, the *ltsA*-disrupted strain of the KY9713 also did not overproduce L-glutamate. On the other hand, another temperature-resistant strain KY11939 produced L-glutamate to some extent at higher temperatures and that production was further enhanced by introduction of *ltsA::kan*. These results suggest that the suppressor mutation in the KY9713 diminishes the ability of L-glutamate production by the *ltsA::kan* mutants but that in the KY11939 does not. Therefore, these two strains may carry different suppressor mutations.

## Conclusions

Considering all these results, mutations in the *ltsA* gene cause a growth defect and induce L-glutamate overproduction by this bacterium. Among nine lysozyme-sensitive mutants of *C. glutamicum* examined so far, five have a mutation in the *ltsA* gene, indicating that the *ltsA* gene plays a central role in determination of lysozyme-sensitivity of *C. glutamicum*. Further analysis of the *ltsA* gene function is necessary to clarify the relationship between cell surface structure and L-glutamate production of *C. glutamicum*.

In this study, we found two suppressor mutations that suppress temperature-sensitivity of *ltsA* mutants. The suppressor mutation in KY9713 diminished L-glutamate production by the *ltsA::kan* disruptant but the one in KY11939 did not. It is expected that a factor that interacts with the LtsA protein will be discovered through further analysis of these suppressor mutations. It is also expected that a substrate of the amido-transfer reaction catalyzed by the LtsA protein will be revealed through analysis of these mutations. Strains used for industrial production were constructed by repeated mutagenesis. Analysis of the *ltsA* locus in these strains will be interesting.

## Materials and Methods

### *Bacterial strains, media and plasmids*

*C. glutamicum* wild-type and lysozyme-sensitive mutant strains (listed in Table 1) were obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). *E. coli* K-12 strain JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1Δ(lac-proAB)*/ F'(*traD36proAB*$^+$*lacI*$^q$*lacZΔM15*)] [16] was used for recombinant DNA procedures. For preparation of plasmid DNA for transformation of *C. glutamicum, E. coli* JM110 [*dam dcm supE44 hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr tsx*Δ(*lac-proAB*)/ F'(*traD36 proAB*$^+$ *lacI*$^q$ *lacZΔM15*)] [16] was used to escape from the restriction system of *C. glutamicum* [17].

Cells were grown in Lennox (L) medium (1 % Bactopepton, 0.5 % yeast extract, 0.5 % Nad, 0.1 % glucose, pH 7.0) or basal salt medium [5 g of $(NH_4)_2SO_4$, 5 g of urea, 2 g of $KH_2PO_4$ 2 g of $K_2HPO_4$, 0.25 g of $MgSO_4{\cdot}7H_2O$, 0.01 g of $FeSO_4{\cdot}7H_2O$, 0.01 g of $MnSO_4{\cdot}4{-}5H_2O$, 0.01 g of $CaCl_2{\cdot}2H_2O$, 0.03 mg of $ZnSO_4{\cdot}7H_2O$, 0.1 mg of $H_3BO_4$, 0.07 mg of $CoCl_2{\cdot}6H_2O$, 0.03 mg of $CuCl_2{\cdot}2H_2O$, 0.01 mg of $NiCl_2$, 0.1 mg of $NaMo_2O_4{\cdot}2H_2O$, 50 g of glucose, 200 μg of biotin, per liter, pH 7.0] [9]. L plates were solidified with addition of 1.5 % agar. If necessary, kanamycin (10 μg/ml for *C. glutamicum* and 20 μg/ml for *E. coli*) or chloramphenicol (20 μg/ml for *E. coli*) were added to the medium.

Plasmids pHSG298 and pHSG398 (Takara Shuzo Co., Ltd., Kyoto, Japan) were used for *E. coli* vectors and plasmid pC2 [15] was used for *E. coli-C. glutamicum* shuttle vector.

### *Temperature-sensitivity and lysozyme-sensitivity checks of C. glutamicum lysozyme-sensitive mutants*

For temperature-sensitivity check, 10-fold serial dilutions of cultures were spotted on L plates and the plates were incubated at 30 or 37°C for 24 h. For lysozyme-sensitivity check, L plates containing 50 μg/ml of lysozyme were used and plates were incubated at 30°C.

### *Determination of the mutation sites in the ltsA mutant strains*

For determination of mutation sites of the *ltsA* mutants by recombinational complementation tests, plasmids composed of parts of the *ltsA* gene fragment and pC2 were introduced by electroporation into the *ltsA* mutants and temperature-sensitivity and lysozyme-sensitivity of

the transformants were checked. Plasmids used are; pHLS4K carrying 2.4 kb *Eco*RI-*Kpn*I fragment, pHLS4KSC carrying 1.3 kb *Sac*I-*Kpn*I fragment, pHLS4ASC carrying 0.6 kb *Sac*I-*Age*I fragment, pHLS2SLSC carrying 1.5 kb *Sac*I-*Sal*I fragment, and pHLS2ASL carrying 0.9 kb *Age*I-*Sal*I fragment.

The corresponding regions of the mutation sites of the *ltsA* mutants were amplified by PCR and the PCR products were cloned into *E. coli* vector pHSG398. A set of primers, 5'-TTGACTCAACCGGAATTCCGCCGCT-3' and 5'-CTCCTTCAACTAAAGAGGATCCGTT-3', was used for amplification from KY9704, KY9713 and KY11939, and a set of primers, 5'-TTTGAATTCAGGAGGATTTTTCATT-3' and 5'-TCCAAGGTCGACATGATCTTAGGAA-3', for amplification from KY9706. *ltsA* genes of wild-type strains, ATCC 13032 and KY9611 were amplified using both sets of primers. PCR was carried out by using a programmable thermal cycler PTC-100 (MJ-Research, Watertown, USA) and LA PCR kit ver.2.1 (Takara Shuzo Co., Ltd.). Three independently isolated clones from each strain were sequenced by using Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech UK Ltd.) with an automated DNA sequencer DSQ-2000L (Shimadzu Co., Kyoto, Japan).

### *Measurement of L-glutamate production and glucose consumption*

Cells grown in basal salt medium at 30°C to $OD_{660}$ = 0.4 – 0.5 were shifted to 30, 35 and 37°C and incubated for another 24 h. After the cells were removed by centrifugation, the concentrations of L-glutamate and glucose in the medium were measured with a Biotech-analyzer AS-210 (Sakura Seiki, Tokyo, Japan) using glutamate oxidase sensor or glucose oxidase sensor.

## Acknowledgements

We thank M. Schaechter for critical reading of the manuscript. We also thank Kyowa Hakko Kogyo, Co. Ltd. for providing *C. glutamicum* strains. This work was partly supported by Grant-in-Aid for Scientific Research (A) (11356003), Grant-in-Aid for Scientific Research of Priority Areas (B) (11227203) and Grant-in-Aid for Scientific Research (C) (12660069) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

## References

1. Udaka S: **Screening method for microorganisms accumulating metabolites and its use in the isolation of Micrococcus glutamicus.** *J Bacteriol* 1960, **79**:754-755
2. Kinoshita S, Udaka S, Shimono M: **Studies on the amino acid fermentation. Part I. Production of L-glutamic acid by various microorganisms.** *J Gen Appl Microbiol* 1957, **3**:193-205
3. Nakayama K, Kitada S, Kinoshita S: **Studies on lysine fermentation I. The control mechanism on lysine accumulation by homoserine and threonine.** *J Gen Appl Microbiol* 1961, **7**:145-154
4. Udaka S, Kinoshita S: **Studies on L-ornithine fermentation. I. The biosynthetic pathway of L-ornithine in Micrococcus glutamicus.** *J Gen Appl Microbiol* 1958, **4**:272-282
5. Shiio I, Nakamori S: **Microbial production of L-threonine. Part II. Production by α-amino-β-hydroxyvaleric acid resistant mutants of glutamate producing bacteria.** *Agric Biol Chem* 1970, **34**:448-456
6. Shiio I, Otsuka S, Takahashi M: **Effect of biotin on the bacterial formation of glutamic acid. I. Glutamate formation and cellular permeability of amino acids.** *J Biochem* 1962, **51**:56-62
7. Nunheimer TD, Birnbaum J, Ihnen ED, Demain AL: **Product inhibition of the fermentative formation of glutamic acid.** *Appl Microbiol* 1970, **20**:215-217
8. Takinami K, Yoshii H, Tsuri H, Okada H: **Biochemical effects of fatty acid and its derivatives on L-glutamic acid fermentation. Part III. Biotin-Tween 60 relationship in the accumulation of L-glutamic acid and the growth of Brevibacterium lactofermentum.** *Agric Biol Chem* 1965, **29**:351-359
9. Hoischen C, Krämer R: **Membrane alteration is necessary but not sufficient for effective glutamate secretion in Corynebacterium glutamicum.** *J Bacteriol* 1990, **172**:3409-3416
10. Gutmann M, Hoischen C, Krämer R: **Carrier-mediated glutamate secretion by Corynebacterium glutamicum under biotin limitation.** *Biochim Biophys Acta* 1992, **1112**:115-123
11. Kimura E, Yagoshi C, Kawahara Y, Ohsumi T, Nakamatsu T, Tokuda H: **Glutamate overproduction in Corynebacterium glutamicum triggered by a decrease in the level of a complex comprising DtsR and a biotin-containing subunit.** *Biosci Biotechnol Biochem* 1999, **63**:1274-1278
12. Hirasawa T, Wachi M, Nagai K: **A mutation in the Corynebacterium glutamicum ltsA gene causes susceptibility to lysozyme, temperature-sensitive growth, and L-glutamate production.** *J Bacteriol* 2000, **182**:2696-2701
13. Zalkin H: **The amidotransferases.** *Adv Enzymol Relat Areas Mol Biol* 1993, **66**:203-309
14. Katsumata R, Oka T, Fruya A: **Japan patent HOI-3475.** 1991
15. Goyal D, Wachi M, Kijima N, Kobayashi M, Yukawa H, Nagai K: **A cryptic plasmid pBLI from Brevibacterium lactofermentum causes growth inhibition and filamentation in Escherichia coli.** *Plasmid* 1996, **36**:62-66
16. Yanisch-Perron C, Vieira J, Messing J: **Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpl8 and pUC19 vectors.** *Gene* 1985, **33**:103-119
17. Vertes AA, Inui M, Kobayashi M, Kurusu Y, Yukawa H: **Presence of mrr- and mcr-like restriction system in coryneform bacteria.** *Res Microbiol* 1993, **144**:181-185