L-Glutamate production by lysozyme-sensitive Corynebacterium glutamicum ItsA mutant strains
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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 itsA 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 itsA gene and DNA sequencing of the ItsA region revealed that four mutants had a mutation in the ItsA gene. Among them, two mutants showed temperature-sensitive growth and overproduced L-glutamate at higher temperatures, as well as the previously reported itsA 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 itsA:kan mutation that causes temperature-sensitive growth in the wild-type background.

Conclusions: These results indicate that a defect caused by the itsA 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 \textit{C. glutamicum} is remained to be elucidated.

\textit{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-acetylgulosamine and N-acetylmuramic 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 \textit{C. glutamicum} and the relationship between L-glutamate production and the treatments that affect cell surface integrity, we have analyzed lysozyme-sensitive mutants of \textit{C. glutamicum}.

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

\textbf{Results and Discussion}

\textbf{Complementation analysis of \textit{C. glutamicum} lysozyme-sensitive mutants by the \textit{ltsA} gene}

\textit{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 \textit{ltsA} mutant strain KY9714 [12]. The other four showed only lysozyme-sensitivity.

First, we carried out complementation analyses of these mutants using the \textit{ltsA} gene. We introduced by electroporation pHLS4 [12] which carries an approximately 4 kb \textit{EcoRI} fragment containing the \textit{ltsA} gene on the \textit{E. coli}-\textit{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.

\textbf{Determination of mutation sites of the \textit{ltsA} mutant strains}

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 \textit{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 \textit{AgeI}-\textit{SalI} fragment and that of KY11939 in the 0.2 kb \textit{KpnI}-\textit{SalI} fragment. Both fragments encode the C-terminal regions of the \textit{ltsA} protein. KY9706 should have a mutation(s) in the 1.1 kb \textit{EcoRI}-\textit{SacI} fragment which encodes the N-terminal portion of the \textit{ltsA}.

The corresponding regions of the mutant genes were sequenced after amplification by polymerase chain reac-
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 KY9706 had a missense mutation (G80D; ltsA9706) at the conserved residue in the N-terminal possible glutamine amido transfer (GAT) domain 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-resistant

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 PstI-SacI 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 KY1939 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 KY1939 strains seem to carry suppressor mutations that render ltsA mutants temperature-resistance extragenically.

L-Glutamate production by C. glutamicum ltsA mutant 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].

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

| Strains  | Parent strains | Growtha | Lysozyme-sensitivityb | 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                     | +                             |
| KY9714d  | KY9611         | ts      | s                     | +                             |
| KY11939  | KY9611         | tr      | s                     | +                             |

a tr, temperature-resistant; ts, temperature-sensitive. bs, sensitive to lysozyme. c +, complemented; -, not complemented. d KY9714 was described in the previous paper [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 relA1(lac-proAB)/ F’(traD36proAB lacIq lacZM15)] [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 ltsA(lac-proAB)/ F’(traD36 proAB+ lacFI lacZM15)] [16] was used to escape from the restriction system of C. glutamicum [17].

Cells were grown in Lennox (L) medium (1% Bactopeptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, pH 7.0) or basal salt medium [5 g of (NH4)2SO4, 5 g of urea, 2 g of KH2PO4, 2 g of K2HPO4, 0.25 g of MgSO4·7H2O, 0.01 g of FeSO4·7H2O, 0.01 g of MnSO4·4H2O, 0.01 g of CaCl2·2H2O, 0.03 mg of ZnSO4·7H2O, 0.1 mg of H3BO4, 0.07 mg of CoCl2·6H2O, 0.03 mg of CuCl2·2H2O, 0.01 mg of NiCl2, 0.1 mg of NaMoO4·2H2O, 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

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 (°C) | L-Glutamate production (g/l) | Glucose consumption (g/l) |
|----------------|-------------------------------|--------------------------|
| 30°C           |                               |                          |
| ltsA+ (KY9611) |                                |                          |
| ltsA- (ATCC 13032) |                             |                          |
| ltsA11939::kan |                                |                          |
| ltsA::kan      |                                |                          |
| ltsA9704       |                                |                          |
| ltsA9713::kan  |                                |                          |
| ltsA9713       |                                |                          |
| ltsA9714       |                                |                          |

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

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the transformants were checked. Plasmids used are; pHLS4K carrying 2.4 kb EcoRI-KpnI fragment, pHLS4KSC carrying 1.3 kb SacI-KpnI fragment, pHLS4ASC carrying 0.6 kb SacI-AgeI fragment, pHLS2SLSC carrying 1.5 kb SacI-SalI fragment, and pHLS2ASL carrying 0.9 kb AgeI-SalI 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’-TTGACTCAACCGGAATTCGCCCGCT-3’ and 5’-CTCTTCAACTAAAGAGGATCCGTT-3’, was used for amplification from KY9704, KY9713 and KY11939, and a set of primers, 5’-TTTGAATTCAGGAGGATTTTTCATT-3’ and 5’-TCCAAGTTGACATGATCTTAGGAA-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) (1260069) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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