Development of a novel fermentation technology using mechanosensitive channels

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Research aims

Inosinate and guanylate are commonly used worldwide as seasonings because they evoke umami, one of the five basic tastes. Inosinate is an umami component of the taste of katsuobushi, and guanylate is an umami component of the taste of shiitake mushrooms; these are two of the most traditional seasonings in Japan. These compounds are produced by microorganisms; however, the mechanisms by which these compounds are exported out of cells have not yet been found.

When some compounds are produced as a result of direct fermentation, it is essential to know how the compound is exported; however, the analysis and development of procedures to study membrane proteins are challenging. The NCgl1221 channel is a glutamate exporter, first described in *Corynebacterium glutamicum*—a microorganism used for industrial glutamate fermentation.

We have previously discovered that aspartate, phenylpropionate, and lysine can also pass through the NCgl1221 channel. We hypothesized that the yield of a substance can be improved by using this channel as a novel exporter in the substance-producing cell. In this study, we focused on inosinate and guanylate production, because it is still unknown how these substances are exported by cells. NCgl1221 channels were expressed in a strain of *Escherichia coli* with enhanced biosynthesis of inosinate and guanylate[(1)]; we analyzed the effects of NCgl1221 on the production of inosinate and guanylate, and on the gating properties of NCgl1221, for the

development of a versatile exporter using this mechanosensitive channel.

Methods

1. **Effects of NCgl1221 gene transfer on inosinate and guanylate production**

   C. glutamicum does not produce glutamate in normal growth conditions; however, glutamate overproduction can be induced in this strain by biotin limitation, Tween 40 supplementation, or penicillin supplementation. Studies have shown that these culture conditions induce NCgl1221 channel opening; this is essential for NCgl1221 to be used as an exporter of inosinate and guanylate. Two mutations of NCgl1221 (A111V and W15CSLW) were identified, which induce glutamate overproduction in normal growth conditions[(2)]; these mutant proteins were expected to form the channel opening. These mutants of the NCgl1221 gene were expressed in a strain of *E. coli* with enhanced biosynthesis of inosinate and guanylate (*E. coli* FADR *add edd yicP pgi xapA ushA aphA*/ pMWKQ); culture experiments were performed with this strain.

2. **Analysis of the gating properties of NCgl1221**

   A detailed analysis of the gating properties of NCgl1221 was needed for the development of the novel, versatile exporter. The influence of membrane conditions on the gating properties of NCgl1221 was considered, because the channel is opened by alterations of membrane tension. In this study, we analyzed the gating properties of NCgl1221 present in the membrane of *C. glutamicum* or *E. coli*.

Results

1. **Effect of NCgl1221 gene transfer on inosinate and guanylate production**

   The A111V or W15CSLW mutant gene was introduced into inosinate- and guanylate-producing *E. coli*, using a plasmid vector. As shown in Fig. 1, the data suggest that this channel forms a path for the excretion of inosinate and guanylate across the cytoplasmic membrane in *E. coli*. The data also suggest that the excretion of inosinate and guanylate is a rate-limiting step in inosinate and guanylate production in this strain, and that intracellular accumulation of inosinate and guanylate inhibits

growth.

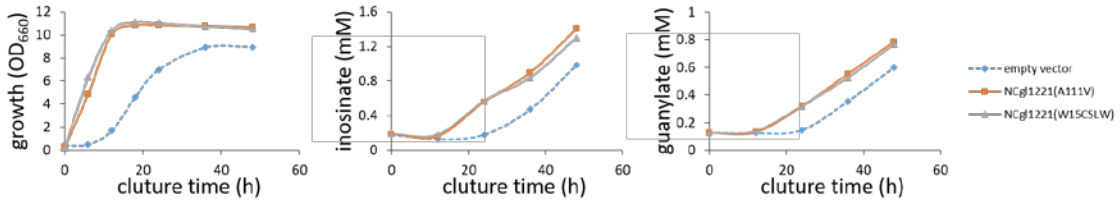

Fig. 1. Effects of introducing mutant NCgl1221 genes into inosinate- and guanylate-producing *E. coli*

2. Analysis of the gating properties of NCgl1221

We analyzed the gating properties of NCgl1221 using the patch clamp technique for prokaryotic cells. This method requires the preparation of giant spheroplasts, because normal prokaryotic cells are too small to be accessed by the patch pipette. We have previously been able to prepare giant spheroplasts of *E. coli* and *Bacillus subtilis*, but not of *C. glutamicum*. We developed a method to prepare giant spheroplasts of *C. glutamicum* using the SI method[3], with supplementation of ethambutol.

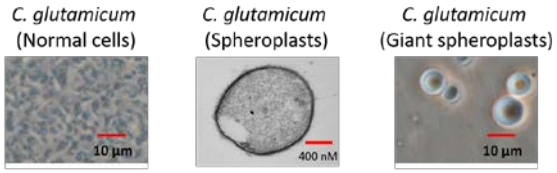

Fig. 2 Giant spheroplast of *C. glutamicum*

We analyzed the gating properties of NCgl1221 by comparing the giant spheroplasts of *C. glutamicum* to those of NCgl1221-expressing *E. coli*. When a pipette voltage of +80 mV was used, NCgl1221 in the native membrane of *C. glutamicum* was maintained in the open state for 0.5 s without membrane tension, after the channel was opened once.

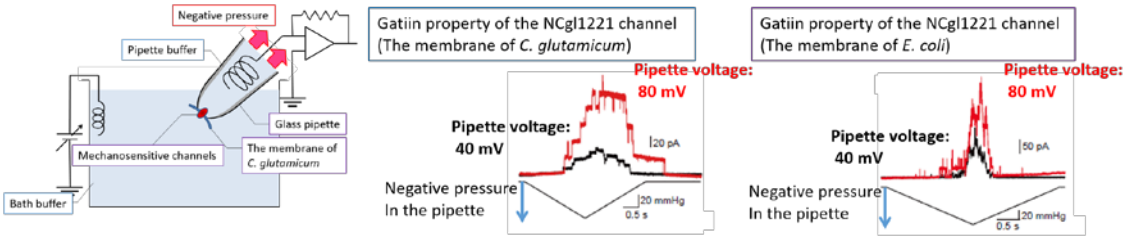

Fig. 3. Gating property of the NCgl1221 channel in the membrane of *C. glutamicum* or *E. coli*

Conclusion

In this study, we developed improvements in inosinate and guanylate production by the introduction of genes encoding mutant forms of NCgl1221. This study suggests that NCgl1221 can function as a novel exporter of nucleotides.

However, the amount of inosinate and guanylate obtained was lower than the amount of glutamate obtained by the cultivation of *C. glutamicum*. Our patch clamp data showed that NCgl1221 was maintained in the open state for 0.5 s without membrane tension, after the channel was opened once in the *C. glutamicum* membrane; this suggests that NCgl1221 is difficult to close in this membrane. Because membrane conditions vary among microorganisms, we believe that 1) the improvement of NCgl1221 for each host strain, and 2) the optimization of culture conditions for altering membrane conditions of host strains are important goals for improving productivity.

References

1) Kakehi, M., Usuda, Y., Tabira, Y., and Sugimoto, S. (2007) Complete deficiency of 5'-nucleotidase activity in *Escherichia coli* leads to loss of growth on purine nucleotides but not of their excretion. *J. Mol. Microbiol. Biotechnol.* **13:**96–104.

2) Nakamura, J., Hirano, S., Ito H., and Wachi, M. (2007) Mutations of the *Corynebacterium glutamicum NCgl1221* gene, encoding a mechanosensitive channel homolog, induce L-glutamic acid production. *Appl. Environ. Microbiol.* **73:**4491–4498.

3) Kuroda, T., *et al.* (1998) *J. Biol. Chem.* **273:**16897–16904.