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学　位　論　文　題　目	Improvement of organic solvent tolerance in *Escherichia coli* by gene mutations （和訳：遺伝子変異による大腸菌の有機溶媒耐性の向上）
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【論文審査】Review of the thesis

Hydrophobic organic solvents are known to be toxic to organisms. Organic solvents accumulate in and disrupt the cell membrane because they can bind to the cell membrane, thereby affecting its integrity. Disruption of membrane functions implies loss of the permeability barrier and the energy transducer, and this thereby leads to growth inhibition and cell death. Whole-cell biocatalysts are beneficial in the bioconversions involving in their internal cofactor regeneration and requiring multi-step metabolic pathways. Bioconversions of hydrophobic compounds using whole-cell biocatalysts have been studied in aqueous-organic solvent two-phase systems. Organic solvent tolerant bacteria can expand the usability for bioconversion in the presence of a wide range of the solvents and enhance the productivity levels. Various mechanisms underlying microbial tolerance and responses to solvents have been revealed by the genetic, physiological and biochemical characterization of organic solvent tolerant bacteria. Until now, more is known about how cells respond to organic solvents, but less about how to develop tolerant strains.

This thesis entitled “Improvement of organic solvent tolerance in *Escherichia coli* by gene mutations” has been divided into 4 chapters, “Chapter 1: Introduction”, “Chapter 2: Contributions of mutations in *acrR* and *marR* genes to organic solvent tolerance in *Escherichia coli*”, “Chapter 3: Improvement of organic solvent tolerance by disruption of the *lon* gene in *Escherichia coli*”, and “Chapter 4: Conclusion”. This thesis mainly deals

with the isolation of organic solvent tolerant *E. coli* strains and genetic analysis of isolated mutants.

Chapter 1 Introduction

The discovery of organic solvent tolerant bacteria is discussed in the first part. Mr. R. Watanabe described various organic solvent tolerant bacteria that have been reported so far. In addition, the toxicity of organic solvents and organic solvent tolerance of bacteria are discussed. Mr. Watanabe summarized an evaluation method to measure the toxicity of an aqueous-organic solvent two-phase system. Bacterial solvent tolerance mechanisms are discussed in detail. These mechanisms include changes of the energetic status, changes of the membrane's fluidity, changes in the cell wall and outer membrane, modification of surface properties, changes of metabolic flux, and active transport of solvents from the membrane into the environment by efflux systems, and modification of membrane proteins. The applications of organic solvent tolerant bacteria in a two-phase bioconversion system such as steroid bioconversion, production of textile dye, 3-Methylcatechol production from toluene, and phenol bioproduction from glucose, are summarised. The application of efflux pump in nano device is also summarized.

Chapter 2 Contributions of mutations in *acrR* and *marR* genes to organic solvent tolerance in *Escherichia coli*

Involvement of *acrR* and *marR* genes in organic solvent tolerance of *E. coli* is discussed in chapter 2. In *E. coli*, the AcrAB-TolC efflux pump belonging to the RND family has been shown to provide intrinsic tolerance to organic solvents. This pump enhances the release of solvents intracellularly accumulated in *E. coli* cells. *acrAB* and *tolC* are *marA/soxS/rob* regulon genes. MarA and SoxS proteins are transcriptional activators belonging to the AraC/XylS family. These activators control the expression of *marA/soxS/rob* regulon genes. MarA and SoxS are transcriptionally regulated. Transcription of the *marRAB* is repressed by MarR, whereas it is autoactivated by MarA. *soxS* transcription is repressed by SoxR and enhanced by the activated form of SoxR after exposure to superoxides or nitric oxide. Mutations in *marR* or *soxR* were suggested to enhance the expression level of the AcrAB-TolC efflux pump. In addition, *acrAB* expression is modulated locally by the repressor AcrR. Thus, mutations in *acrR* can lead to the enhanced expression of AcrAB.

Mutations conferring a multidrug resistance phenotype have been found in the genes *marR*, *soxR*, and *acrR* among clinical and veterinary *E. coli* isolates. Some of those studies suggested that organic solvent tolerance is correlated with these mutations in these isolates. However, the extent to which these mutations contribute to organic solvent tolerance has not been clarified because *E. coli* isolates used in these studies had a variety of genetic backgrounds. In addition, the synergistic effects of these mutations on organic solvent tolerance were ambiguous. To clarify the effects of mutations on the tolerance phenotype, it is necessary to reconstruct selected mutations in one type of strains in various combinations. In this study, Mr. R. Watanabe identified mutations in the genes *marR*, *soxR*, and *acrR* in organic solvent tolerant *E. coli* mutants. Eight cyclohexane-tolerant *E. coli* JA300 mutants were isolated, and mutations in *marR*, *soxR*, and *acrR* in these mutants were examined. Every mutant carried a mutation in either *marR* or *acrR*. Among all mutants, strain CH7 carrying a nonsense mutation in *marR* (named *marR109*) and an insertion of IS5 in *acrR*, exhibited the highest organic solvent tolerance levels. To examine the involvement of these mutations in improving organic solvent tolerance, they were introduced into the *E. coli* JA300 chromosome by site-directed mutagenesis using λ red-mediated homologous recombination. Consequently, JA300 mutants carrying *acrR*::IS5, *marR109*, or both were constructed and named JA300 *acrR*IS, JA300 *marR*, or JA300 *acrR*IS *marR*, respectively. The organic solvent tolerance levels of these mutants were increased in the following order: JA300 < JA300 *acrR*IS < JA300 *marR* < JA300 *acrR*IS *marR*. JA300 *acrR*IS *marR* formed colonies on an agar plate overlaid with cyclohexane and *p*-xylene (6:4 vol/vol mixture). The organic solvent tolerance level and AcrAB-TolC efflux pump-expression level in JA300 *acrR*IS *marR* were similar to those in CH7. Thus, it was shown that the synergistic effects of mutations in only two regulatory genes (*acrR* and *marR*) can remarkably elevate the level of organic solvent tolerance in *E. coli*. Organic solvent-tolerance levels of various mutants and recombinants from strain JA300 have been investigated by measuring the colony-forming efficiencies of mutants on an LBGMg agar plate overlaid with organic solvents. Overexpression of the *marA* gene has been shown to raise the organic solvent tolerance of *E. coli*. JA300 overexpressing the *marA* gene formed colonies in spots containing more than 10^6 cells in the presence of cyclohexane. It was previously reported that the organic solvent tolerance of strain JA300 significantly improved the double disruptions of *marR* and *proV*. JA300*ΔproVΔmarR* formed colonies in spots containing more than 10^5 cells in the presence of cyclohexane and thus exhibited higher organic solvent-tolerance levels than JA300 overexpressing the *marA* gene. In the

present study, JA300 *acrR*IS *marR* showed 10^4-fold higher colony-forming efficiencies in the presence of cyclohexane than JA300*ΔproVΔmarR*. Owing to the wealth of genetic and metabolic knowledge associated with *E. coli*, organic solvent-tolerant *E. coli* can be a convenient and efficient catalyst when it is used as a host expressing enzymes that are useful for producing valuable chemicals in two-phase systems employing organic solvents. These findings are expected to provide valuable knowledge for increasing organic solvent-tolerance levels in *E. coli* to improve the usability of whole-cell biocatalysts in two-phase systems.

Chapter 3 Improvement of organic solvent tolerance by disruption of the *lon* gene in *Escherichia coli*

Involvement of *lon* gene in organic solvent tolerance of *E. coli* is discussed in chapter 3. The Lon is an ATP-dependent protease belonging to the AAA^+ (ATPases associated with a variety of cellular activities) superfamily of enzymes. The *E. coli* Lon protease has been shown to be involved in a number of biological processes, such as SOS response, capsule synthesis, DNA methylation, motility, defense against chemicals, methionine biosynthesis, acid tolerance, and nutrient stress. The Lon protease causes a rapid turnover of MarA and SoxS. Therefore, MarA and SoxS are unstable in the presence of Lon protease. *lon* mutants increase the expression level of the AcrAB-TolC pump. In addition, *lon* mutants enhance the production of a capsular polysaccharide, colanic acid, and this leads to a mucoid phenotype. The enhanced polysaccharide biosynthesis has been thought to cause increased antibiotic resistance via decreased permeability. In addition, extracellular polysaccharide is suggested to play an important role in organic solvent tolerance in several bacteria. Thus, Mr. R. Watanabe expected that overproduction of capsule polysaccharide would contribute to organic solvent tolerance in *E. coli*. The colanic acid biosynthesis requires 19 genes located on the same cluster, denoted *wca* and formerly called *cps*. WcaJ is predicted to initiate the synthesis of colanic acid by transferring α-D-glucose-1-phosphate to undecaprenyl phosphate. WcaJ is required for capsule polysaccharide synthesis, and the *wcaJ* mutant forms nonmucoid colonies. In this study, Mr. Watanabe investigated the organic solvent tolerance of a *Δlon* mutant of *E. coli* K-12 and found that the mutant showed significantly higher organic solvent tolerance than the parent strain. *Δlon* mutants are known to overproduce capsular polysaccharide and concomitantly form mucoid colonies. Thus, it was possible that this increase in capsular

polysaccharide production might be involved in the organic solvent tolerance in *E. coli*. However, this study showed that a *Δlon ΔwcaJ* double-gene mutant displaying a nonmucoid phenotype was as tolerant to organic solvents as the *Δlon* mutant. This result indicated that capsular polysaccharide is not involved in organic solvent tolerance. On the other hand, the Lon protease is known to cause rapid turnover of MarA and SoxS, which can enhance the expression level of the AcrAB-TolC efflux pump. Mr. Watanabe found that the *Δlon* mutant showed a higher expression level of AcrB than the parent strain. In addition, the *Δlon ΔacrB* double-gene mutant showed a significant decrease in organic solvent tolerance. Thus, it was indicated that organic solvent tolerance in the *Δlon* mutant depends on the AcrAB-TolC pump but not capsular polysaccharide. As described in the previous chapter, Mr. Watanabe constructed *E. coli* strain JA300 *acrR*IS *marR*. This *E. coli* mutant overexpresses the AcrAB-TolC pump and exhibits high-level solvent tolerance. In an attempt to further improve the solvent tolerance of JA300 *acrR*IS *marR*, a *lon* gene disruptant of this strain was constructed. However, the resulting mutant JA300 *acrR*IS *marR Δlon* showed lower solvent tolerance than JA300 *acrR*IS *marR*. In addition, Mr. Watanabe examined antibiotic susceptibilities of the *Δlon* mutant. The *Δlon* mutant did not exhibit a remarkable multidrug resistance-phenotype. Thus, this study showed that *lon* disruption can significantly enhance the tolerance of *E. coli* against hydrophobic organic solvents, unlike in the case of multidrug resistance.

Chapter 4　Conclusion

The concluding remarks of the thesis are discussed in the chapter 4. These findings suggest new strategy for increase of organic solvent-tolerance level in *E. coli* to improve the usability of the whole-cell biocatalysts in the two phase systems employing organic solvents.

【審査結果】Summary and decision

The thesis entitled “Improvement of organic solvent tolerance in *Escherichia coli* by gene mutations” focuses on the organic solvent tolerance of *Escherichia coli* and its tolerance mechanism. The results shown in the thesis are outstanding from an international point of view and the significant points in the present study are summarised below;

(1) In this study, to efficiently isolate mutants with high-level organic solvent tolerance, *E.*

coli parent strain was precultured in the LBGMg liquid medium overlaid with cyclohexane. Owing to this procedure, high-level organic solvent tolerant mutants were efficiently isolated from *E. coli* parent strain. Strain CH7 formed colonies even in spots containing 10^3 cells on an agar plate overlaid with a mixture of cyclohexane and *p*-xylene (7:3 vol/vol mixture). The organic solvent tolerance level of CH7 is remarkably higher than those of a number of other organic solvent tolerant *E. coli* strains reported so far.

(2) Strain JA300-based *marR* and/or *acrR* genes-mutants were successfully constructed by site-directed mutagenesis using λ red-mediated homologous recombination. By the use of these mutans, it was shown that the organic solvent-tolerance levels of the mutants correlated with their AcrAB-TolC efflux pump-expression levels. Thus, it was indicated that the synergistic effects of mutations in only two regulatory genes, *acrR* and *marR*, can significantly increase organic solvent tolerance in *E. coli*.

(3) This study revealed that disruption of the *lon* gene was able to improve organic solvent tolerance in *E. coli* for the first time.

(4) *E. coli lon* mutants overproduce the capsular polysaccharide. Extracellular polysaccharide has been reported to be involved in organic solvent tolerance in several bacteria. Thus, it was expected that the overproduction of capsule polysaccharide might improve organic solvent tolerance also in *E. coli*. However, this study clarified that the overproduction of capsular polysaccharide is not involved in the improvement of organic solvent tolerance in *E. coli*. On the other hand, this study showed that the increase in the AcrAB-TolC efflux pump was the main cause of the improved organic solvent tolerance in the *lon* mutant.

Two first-authoring papers have been published by international journals such as *AMB Express* (Springer) and *Journal of Bioscience and Bioengineering* (Elsevier).

Judging by the results shown in the thesis and the number of international papers published so far, the level of the present research results is definitely high by international standards and the present results may well make a great contribution to the construction of high-level organic solvent tolerant *E. coli* strain which is useful as the

whole-cell biocatalysts in the two phase systems employing organic solvents. The present results may also contribute to the application of AcrAB-TolC bacterial nano pump for removal of antibiotics and organic solvents from waste fluid. In conclusion, the thesis is considered as a high quality, high standard one by international standards.