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# Functional Analysis of the Proteins Involved in the Aromatic Amino Acids Metabolism in Bacteria

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## Purpose

The aromatic amino acids are compounds of great importance in industry, as they are precursors of many drugs and food additives. But, the efficient fermentation methods for them have not been developed yet. Our works were focused on (I) functional analysis of the transporters of aromatic amino acids and (II) functional analysis of the regulatory protein TyrR (a chief regulator of the genes involved in the aromatic amino acid metabolism), the results of which will significantly contribute to establishment of the basis for aromatic amino acid production using bacteria.

### 1) Aromatic amino acids transporter

a) A novel phenylalanine (Phe) transport system in *Escherichia coli*[1)]

It has so far been considered that, in *E. coli*, the active transport of aromatic amino acids is mediated by five distinct permeases, AroP, Mtr, PheP, TnaB, and TyrP, and that the AroP and PheP systems are responsible for the accumulation of Phe in cells. However, we found a low level of accumulation of Phe in the strain TK1170 (*ΔaroP ΔpheP Δmtr Δtna ΔtyrP*). Growth of the aromatic transporter-negative $Phe^-$ strain (*ΔaroP ΔpheP Δmtr Δtna ΔtyrP pheA18*::Tn*10*) in minimal medium supplemented with Phe was severely inhibited in the presence of Ile and Leu (Fig. 1(A)). Moreover, the addition of Val and Leu severely decreased the Phe uptake activity of the cells, while the presence of Glu did not affect it (Fig. 1(B)). These results strongly suggested that the branched-chain amino acids transporter could function as the third Phe transporter.

Branched-chain amino acids are transported by the LIV-I/LS and BrnQ systems. Whereas BrnQ is a single membrane protein, the LIV-I/LS system comprises two substrate-binding proteins (BP), LIV-BP and LS-BP, located in the periplasm, and four inner membrane proteins LivHMGF. In order to determine whether BrnQ or LIV-I/LS carries out the uptake of Phe, a series of *E. coli* strains expressing individual transport systems were constructed and assayed for transport; AroP-expressing strain (*ΔbrnQ ΔlivHMGF ΔpheP*), BrnQ-expressing strain (*ΔaroP ΔlivHMGF ΔpheP*), LIV-I/LS-expressing strain (*ΔaroP ΔbrnQ ΔpheP*), and PheP-expressing strain (*ΔaroP ΔbrnQ ΔlivHMGF*). As shown in Fig. 2 (A), LIV-I/LS-expressing strain (*ΔaroP ΔbrnQ ΔpheP*) was able to accumulate Phe in its cells, demonstrating the involvement of the LIV-I/LS system in Phe transport. Although the initial rate and the steady-state level were lower than those in the strains expressing AroP and PheP, the LIV-I/LS-expressing cells exhibited the highest Phe uptake activity when cells were grown in the presence of tyrosine and then assayed for transport (Fig. 2 (B)), indicating the physiological significance of this system.

b) LIV-I/LS system as a determinant of azaserine sensitivity of *E. coli*[2)]

Azaserine, which was discovered in a culture filtrate of *Streptomyces fragilis*, is an antibiotic compound. It has been shown that inhibitory effect of azaserine on *E. coli* is alleviated effectively in the presence of aromatic amino

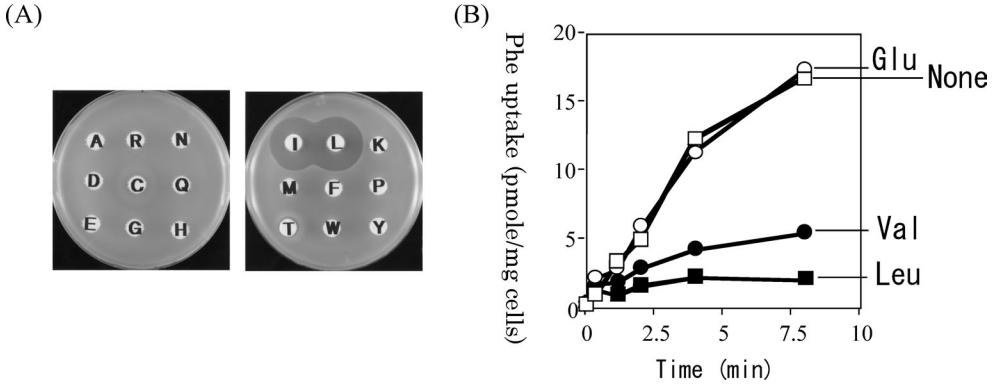


**Fig. 1.** Inhibition of Phe uptake by branched-chain amino acids. (A) Growth inhibition of Phe-auxotrophic *E. coli* strain (*ΔaroP Δmtr ΔpheP Δtna ΔtyrP pheA18*::Tn*10*) in the presence of branched-chain amino acids. Disks were impregnated with 1 mM various amino acids. (B) Phe uptake activity of *E. coli* strain TK1170 (*ΔaroP Δmtr ΔpheP Δtna ΔtyrP*). Phe was added to the final concentration of 50 $\mu$M in either the absence or presence of 5 $\mu$M Glu, Leu, or Val.

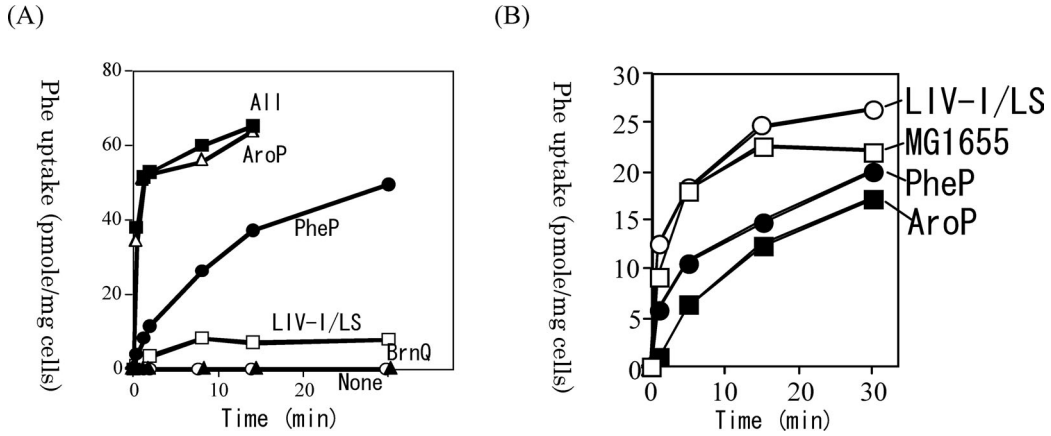


**Fig. 2.** The LIV-I/LS system as the third Phe transporter in *E. coli*. (A) Phe uptake was measured in various *E. coli* cells [BrnQ-(*ΔaroP ΔlivHMGF ΔpheP*), LIV-I/LS-(*ΔaroP ΔbrnQ ΔpheP*), PheP-(*ΔaroP ΔbrnQ ΔlivHMGF*), AroP-(*ΔbrnQ ΔlivHMGF ΔpheP*), and None-(*ΔaroP ΔbrnQ ΔlivHMGF ΔpheP*) expressing cells] in the presence of 1 $\mu$M L-(U-$^{14}$C)-Phe. (B) Accumulation of Phe in *E. coli* cells expressing individual Phe transport systems was measured in the presence of 1 mM tyrosine.

acids, especially Phe, and partially in the presence of branched-chain amino acids. The alleviating effect of aromatic amino acids could be explained by competition for the uptake of substrates by AroP (a general aromatic permease) because all azaserine-resistant mutants isolated so far have lesions in the *aroP* gene. However, why Phe is the most effective among the aromatic amino acids and how branched-chain amino acids affect azaserine sensitivity have not been elucidated. Recently, we found that the LIV-I/LS system, initially assumed to be a branched-chain amino acid-specific transporter, is the third Phe transporter. This finding led us to envisage the possible linkage between the alleviating effects of Phe and branched-chain amino acids on azaserine toxicity.

As shown in Fig. 3 (A), the disruption of *liv* was effective for increased azaserine resistance of *E. coli* cells only when *ΔaroP* co-existed. Whereas the *ΔaroP* strain was resistant to 0.1 mM azaserine at maximum, the strain carrying *ΔaroP Δliv* exhibited resistance to 0.2 mM azaserine, but no significant resistance was observed for the *Δliv* strain. Fig. 3 (B) shows the results of complementation analysis. Introduction of the *liv* genes into the *ΔaroP Δliv* strain made the cells more sensitive to azaserine as compared to the *ΔaroP* strain, maybe due to the increased gene dosage. The $K_i$ value for azaserine in the inhibition of Phe uptake was estimated to be 0.6 mM (data not shown). These results revealed that the LIV-I/LS system is one of the major determinants of azaserine sensitivity of *E. coli*.

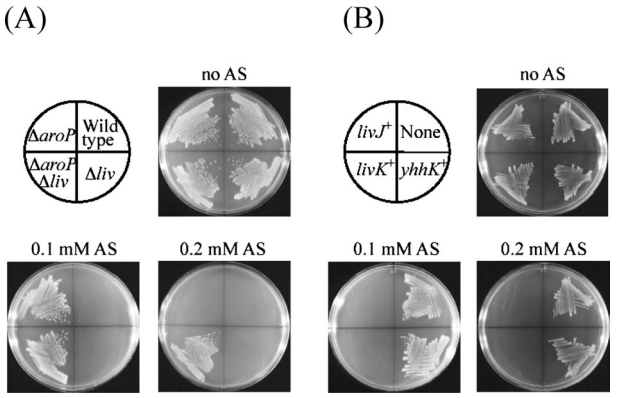


**Fig. 3.** The LIV-I/LS system as a determinant of azaserine sensitivity. (A) MG1655, its *ΔaroP*, *ΔlivJ-yhhK-livKHMGF*, and *ΔaroP ΔlivJ-yhhK-livKHMGF* derivatives were streaked on M63-glucose minimal solid medium containing azaserine (AS) at a final concentration of 0–0.2 mM. (B) The LIV-I and LS systems are both involved in azaserine sensitivity, as revealed by complementation analysis.

**2) TyrR, a chief regulator of the genes involved in the aromatic amino acid metabolism**

3,4-Dihydroxyphenyl-L-alanine (L-DOPA) is an aromatic amino acid used in the treatment of Parkinson's disease, and can be produced by the reverse reaction of tyrosine phenol-lyase (Tpl). In order to construct a high-Tpl-expressing strain, we tried to elucidate the mechanism underlying expression of Tpl. First, we identified TyrR as a positive regulator of *tpl*, and revealed its action on the *tpl* promoter where TyrR changes its conformation from a dimer to a hexamer upon the binding of L-tyrosine so that it interacts with RNA polymerase. TyrR is known to be a chief transcriptional regulator of the genes involved in the aromatic amino acid metabolism and regulate expression of the genes positively and/or negatively by altering its conformation. Thus, elucidation of the mechanism underlying the dimer-hexamer conformational change of TyrR is of great importance not only for creating a high-Tpl-expressing strain but also for figuring out the metabolic flow of the aromatic amino acids in cells.

a) Identification of an amino acid residue involved in hexamer formation of TyrR

Wilson *et al.* reported that E275Q amino acid substitution severely impairs the ability of TyrR to form a hexamer. Indeed, a mutant TyrR$^{E275Q}$ was incapable of activating *tpl* even in the presence of tyrosine (data not shown). Using the *tpl-lac* reporter system, we obtained several revertants of this mutant. Sequence analysis showed that all revertants carry the common amino acid replacements in the central domain of TyrR. We are now trying to determine the properties of these mutant TyrRs using the purified proteins.

b) Efficient production of L-DOPA using cells carrying a mutant TyrR[3)]

Using the *lac* reporter system, we obtained several mu-

tant TyrR proteins with enhanced ability to activate *tpl*. The mutations dispersed in the N-terminal (NT), central (Cen), and C-terminal (CT) domains of TyrR were accumulated by genetic manipulations, and these *tyrR* alleles were introduced into the *tyrR*-deficient cells, and then the recombinant strains were evaluated as to L-DOPA productivity. The highest value ($33\,g\,l^{-1}\,h^{-1}$) was obtained for the cells carrying the $tyrR^{NT\,Cen\,CT}$ allele, and was 18-fold higher than the heretofore achieved level ($1.8\,g\,l^{-1}\,h^{-1}$) with the use of wild-type cells. Our work could be a good example demonstrating that the exploitation of a transcriptional regulator is also one of the promising ways for improving the quality and quantity of a final product in microbial biotechnology.

## References


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3) Koyanagi, T., Katayama, T., Suzuki, H., and Kumagai, H. (2004). Effective production of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) with *Erwinia herbicola* cells carrying a mutant transcriptional regulator TyrR. *Journal of Biotechnology*. in press.