The hydantoin transport protein from *Microbacterium liquefaciens*

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**Introduction**

The stereo-selective hydrolyses of D,L-5-monosubstituted hydantoin compounds have been widely studied for the production of optically pure D- or L-amino acids, which are important intermediates for production of various drugs and pharmaceuticals. In spite of intensive studies for their commercialisation, the physiological roles of these hydantoin-related enzymes still remain obscure. The hyu genes encoding the enzymes often form gene clusters, indicating coherent roles for the combination of hydantoinase, N-carbamoylase, and HRase. Amongst these genes for the three hydantoin metabolic enzymes, we found genes encoding putative transporters, for example in the hyu gene clusters from *Pseudomonas sp.* NS671, *Arthrobacter aurescens* DSM 3747, and *Microbacterium liquefaciens* AJ 3912.

In order to establish, for the first time, the function of these transporters encoded by the genes located in hyu gene clusters, we have cloned and expressed in *Escherichia coli* one of them ‘Mhp’ from *M. liquefaciens* AJ3912. This is found to transport primarily 5-indolyl-methyl-hydantoin (IMH) and 5-benzyl-hydantoin (BH). The (His)$_6$-tagged protein, MhpH$_6$, was purified to an homogeneity sufficient for crystallisation trials.

**Cloning and heterologous expression of transport activity associated with the hyuP gene**

A general strategy for the amplified expression in *Escherichia coli* of membrane transport proteins from other bacteria has been devised in the Henderson laboratory, and Shun’Ichi Suzuki of Ajinmoto Inc. in Japan spent two years in the Astbury Centre to exploit this. The hyuP gene from *M. liquefaciens* was ligated into the *Eco*RI+PstI-cut vector pTTQ18. The resulting plasmid designated pSHP11 containing hyuP downstream of the IPTG-inducible tac promoter was transformed into the *E. coli* BLR host, which was grown plus or minus IPTG. The washed cells of the recombinant strain (*E. coli*/pSHP11) were examined for the uptake of radiolabelled L-BH or L-IMH made with the help of Simon Patching and Richard Herbert.

When using either L-BH or L-IMH as the substrate, higher uptake was observed in the cells of the IPTG-induced culture than in those of the uninduced culture (Fig. 1). Using induced cells of *E. coli* BLR/pTTQ18 without any inserts, the uptake of substrates was less than that observed in uninduced cells of *E. coli*BLR/pSHP11 (data not shown), demonstrating that the host strain did not significantly accumulate these hydantoins. The extents of uptake kept increasing even after 300 sec, but the rates of uptake for both the substrates progressively decreased as the reactions proceeded. Judging from both the initial uptake rates and the extents of uptake in 300 sec, L-IMH was a better substrate for MhpH$_6$ than L-BH.

![Figure 1. Time course of uptakes of 5-substituted-hydantoins by *E. coli*/pSHP11.](image)

**Figure 1.** Time course of uptakes of 5-substituted-hydantoins by *E. coli*/pSHP11. $^3$H-L-IMH (circles) and $^3$H-L-BH (triangles) were used as the substrates. The washed cells of *E. coli*/pSHP11 obtained by growth on M9 casamino acid medium with (closed symbols) or without (open symbols) induction by IPTG were used for the uptake assay carried out in standard conditions (5 mM MES buffer (pH 6.6), 150 mM KCl, and 25 M substrate, at 25°C).
Amplified expression and identification of the MhpH6 protein.
The Mhp protein containing a His6 tag at its C-terminus, was heterologously expressed by the construct pTTQ18 in an *E. coli* host. The level of expression was sufficient for the extra protein to stain with Coomassie Blue in membranes from induced, but not uninduced cells (Fig 2A, lanes 2, 3). The protein was not observed in the soluble fraction (Fig 2A, lane 4). After further separation of inner and outer membrane fractions from total membrane fraction, the localisation of MhpH6 was mainly observed in the inner membrane fraction (Fig 2A, lane 5, 6). The apparent molecular mass of the produced protein was approximately 36 kDa on SDS-PAGE, although its predicted molecular mass is 54, 580, an anomaly common for membrane transport proteins. Its identification was authenticated by the Western blot to the RGSH6 epitope (Fig. 2B), in which a positive reaction correlated with the molecular mass and appearance/disappearance of the putative MhpH6 in the Coomassie-stained gel (Fig. 2A).

Solubilization and purification of MhpH6.
The solubilization of MhpH6 was efficiently promoted by treatment with 1% (w/v) DDM (Fig 2A, B, lanes 7, 8). Finally, the purification of MhpH6 to electrophoretic homogeneity was achieved by using Ni-NTA resin (Fig 2A, lane 9). Through all the purification steps, the molecular mass of MhpH6 was observed at 36 kDa (Fig 2A and B, lanes 5, 7, 8, 9) and no other proteins were observed in the Western-blot analysis (Fig. 2B).

The N-terminal amino acid sequence of the purified MhpH6 was determined for ten residues as MNSTPIEEAR, corresponding exactly to what was expected from the nucleotide sequence in the constructed plasmid. Coupled with the integrity of the RGSH6 C-terminal end of the protein shown by the Western blot this verifies that the purified polypeptide chain of MhpH6 is intact.

Conclusions  The *hyu* gene of *M. liquefaciens* was shown to encode a protein for transport of hydantoin. This is sufficiently important for Ajinomoto to protect the IPR by patenting the discovery. The purified protein yields diffracting crystals.

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