Development of a method to discover exporters for valuable compounds, and exploration and improvement of exporters

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Research objective

Microbial cell factories (MCFs), which use renewable resources to produce valuable compounds, are gaining attention for their role in building a sustainable society. Excreting target compounds synthesized within MCF cells is often advantageous. However, “exporter engineering” which involves searching for, selecting, designing exporters that transport these compounds synthesized within MCF cells remains underdeveloped. While many genes encoding exporters in various bacterial genomes, but there is limited understanding of the specific compounds they export. Moreover, no method currently exists to semi-comprehensively investigate or estimate the substrates of these exporters at a realistic throughput. Additionally, membrane transport proteins, which are responsible for exporting compounds, operate through significant structural changes influenced by surrounding lipid molecules and membrane potential. Despite this, the mechanisms behind their function are not well understood, sufficient details regarding their operating mechanisms is not available, and no general strategies to improve their activity have been established. Therefore, this study aimed to (1) develop innovative technology to search for exporter proteins, (2) identify and discover membrane transport proteins that export compounds not previously known to be excreted from cells, and (3) improve the activity of exporter proteins using digital biotechnology.

Methods

To analyze exporters using solid-supported membrane-based electrophysiology (SSM) technology[1)] with *E. coli*-derived cell membranes, several steps were taken. These included investigating the genotype of the *E. coli* used, developing a method to remove cell debris generated during the preparation of inverted membrane vesicles, and devising a method to concentrate these vesicles. The SURFE^{2}R 1N (Nanion Technologies) was employed for analysis.

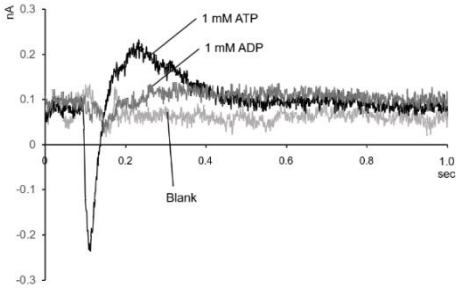
Amino acid substitutions were predicted to strengthen interactions between transmembrane helices (TMs) in the 5′-inosinic acid (IMP) exporter, Cs0286 for *Corynebacterium stationis*, a bacterium that directly ferments IMP. The effects of these substitutions were evaluated by

measuring IMP production in *E. coli* with enhanced IMP biosynthesis. The interaction energies between TMs for the beneficial substitutions that showed a positive effect were calculated using all-atom molecular dynamics simulations.

Results

Development of innovative technology to search for exporter

Generally, measuring exporter activity is challenging because the substrate's chemical structure remains unchanged by the transporter, and preparing a sample with sufficiently high density for the transport rate by the transporter. We decided to use a device that made it possible to measure the transporter activity by overcoming this difficulty using electrophysiological principles. To measure the activity of exporters using this device, we employed a method for preparing inverted membrane vesicles with a diameter of several micrometers[2)], which were generated using the SI method[2)]. We selected *E. coli*, considering its potential as a host for heterologous expression. The method for preparing inverted membrane vesicles was the same as that described in previous reports; however, it was reconfirmed that the gene-deficient strain of Braun lipoprotein, which links peptidoglycan and the outer membrane, was superior in terms of the frequency of inverted membrane vesicle formation. In the analysis using the patch-clamp method, the cell residue after extraction of the inverted membrane vesicles from giant cells was removed by washing after extraction. Additionally, since only one inverted membrane vesicle is used, in patch-clamp analysis, the density of the inverted membrane vesicles is not a major issue during preparation. However, because this device requires many inverted membrane vesicles, it is necessary to address these issues. As a result of repeated investigations, we succeeded in efficiently separating inverted membrane vesicles from cell residues using a Percoll density gradient, and concentrating inverted membrane vesicles. Using these inverted membrane vesicles, , we investigated whether it is possible to measure the activity of membrane transport from outside the membrane vesicles to inside the membrane vesicles (equivalent to transport from inside the cell to outside the cell) using the above-mentioned device. We successfully observed the current generated by H^+ transport via the F_oF_1-ATPase, as shown in figure.



Search for and discovery of exporter for a useful compound with unknown extracellular excretion

We searched for exporters of a useful compound X, without waiting for the completion of the search method to be developed in (1). We introduced genes encoding potential exporters of compound X into a strain with enhanced biosynthesis of X, including heterologous expression. As a result, we identified an exporter that facilitated the excretion of compound X and, for the first time, successfully achieved the fermentative

production of X, which is currently produced via extraction.

Improving activity of exporter aided by digital biotechnology

Based on the amino acid substitution (G64E), which significantly activates the IMP efflux membrane transport protein (Cs0286), it was proposed that strengthening the interaction between TM helices in the N-terminal and C-terminal lobes, which function as a single unit in MFS-type membrane transport proteins, is a general strategy for enhancing their activity[3]. To achieve a similar effect, we generated G178E, F181E, M413E, and V417D amino acid substitution variants of Cs0286 and examined their effects. Mutant genes were introduced into *E.coli* with enhanced IMP biosynthesis, *E.coli* with enhanced AMP biosynthesis, and *E.coli* with enhanced compound X biosynthesis. As a result, the introduction of the M413E substitution gene into *E.coli* with enhanced compound X biosynthesis, led to an increase in the production of the target compound. When the inter-TM interaction energy of the M413E substitution-type Cs0286 was calculated, the inter-TM interaction that comprised the C-terminal lobe was strengthened, as expected. These results indicate that enhancing TM interactions in both the N-terminal lobe, as seen in the G64E-substituted Cs0286, and the C-terminal lobe, leads to increased activity. However, whether the enhancement of TM interactions improves activity depends on the substrate.

Conclusion

(1)We successfully measured the export activity of inverted membrane vesicles using SSM technology, forming the basis for innovative technology to search for innovative technology to search for exporters in bacteria. (2) We successfully achieved the fermentative production of compound X, which is currently produced industrially through extraction. (3) Additionally, we found that strengthening the TM interactions in the C-terminal lobe improves exporter activity.

References

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