Cell Surface Engineering as a Tool to Transform the Moderately Halophilic *Halomonas elongata* into a Nutritious Single-Cell Eco-Feed

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ABSTRACT

Cell surface engineering is a technology used to generate versatile microbial cell factories for various applications. We previously developed a cell-surface display system for *Halomonas elongata* OUT30018, a moderately halophilic bacterium that can be used as a cell factory to produce valuable amino acids and their derivatives. In this system, Lipoprotein 5 (LP5), an outer membrane protein encoded by *H. elongata* OUT30018's *Helipop5* gene, is used as an anchor to attach peptides of interest to the outer membrane. The attached peptides are, therefore, displayed on the cell surface. This work aims to use this system to generate a recombinant *H. elongata* that displays nutrient-rich peptides on the cell surface. Because Methionine (M), Lysine (K), and Tryptophan (W) serve as essential nutrients for fish, we first screened the genome of *H. elongata* OUT30018 for fragments that encode M-, K-, and W-rich peptides. As a result, a sequence encoding a short M, K, and W-rich peptide was found and was named a nutrient peptide (NP). Subsequently, the DNA sequence that encodes NP was cloned, and plasmids for surface-displaying one to eight repeats of the NP peptide as LP5-NP x1 to x8-HA fusion proteins were constructed. After the correct expression of these fusion proteins were confirmed in *E. coli* by immunoblot of crude protein extracts with anti-HA antibody, further plasmid constructs were prepared for expressing the LP5-NPx1 or LP5-NPx8-HA fusion proteins in *H. elongata* OUT30018. Although the constructs were successfully integrated into the genome of *H. elongata* OUT30018, generating recombinant *H. elongata* SN1 and SN8 strains, HPLC analysis did not detect any increase in the M, K, and W concentrations in their cell extracts. As the expression constructs were placed under the control of the relatively weak *Helipop5* promoter, we are currently working to express this construct under a more potent promoter in the proline-overexpressing *H. elongata* HN10 previously developed in our laboratory. Proline is an essential amino acid that also functions as a feed stimulant for fish. Therefore, Proline-overexpressing *H. elongata* equipped with surface-display NP could be used as a single-cell eco-feed for aquaculture. Successful implementation of this strategy will establish a proof-of-concept for a versatile platform technology for displaying peptides on the cell surface of *H. elongata* for diverse biotechnological applications.

Keyword: Cell surface engineering/ Feed additives/ Essential amino acids/ Self-cloning/ *Halomonas elongata*

1. INTRODUCTION

Fishmeal has been used as the main feed for the aquaculture industry due to its high protein content and well-balanced amino acid composition [1]. Due to a decrease in supply, the cost of fishmeal is increasing, making it essential to find a fishmeal alternative [2]. Plant proteins are considered the alternative [3]. Still, they do not contain sufficient amino acids essential for aquaculture feed, such as M, K, and W. Therefore, there is an urgent need to develop inexpensive and sustainable alternative feeds that contain enough essential amino acids.

Cell surface engineering is a technology used to generate microbial cells that can display various proteins on their outer membrane [4]. Due to the ability of a moderately halophilic bacterium, *H. elongata* OUT30018, to thrive in a medium derived from high-salinity biomass waste [5], here, we use cell surface engineering to transform *H. elongata* OUT30018 into cells that display M, K, and W-rich NP on their surface (Figure 1). While our findings indicate that the initial quantity of the displayed NP was low, we are currently devising a potential strategy to address this issue.

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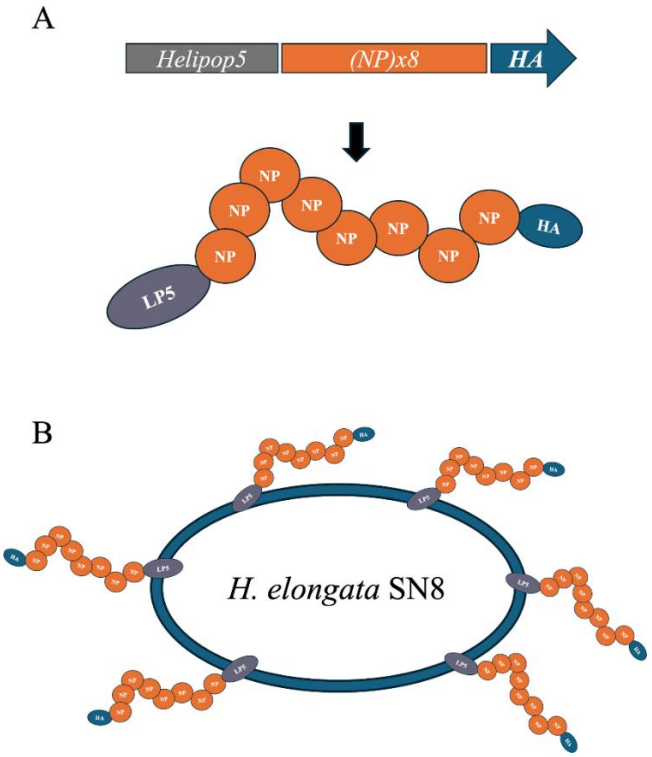

Figure 1. Generation of *H. elongata* displaying nutrient peptide (NP) on the cell surface. A. Structure of synthetic gene (*Helipop5*::*NP*x*8*::*HA*) for expressing eight repeats of NP on the surface of *H. elongata* SN8 cell. B. Schematic diagram of recombinant *H. elongata* SN8 displaying NP peptide on the cell surface. The NP peptides were anchored to the outer membrane through LP5 proteins. *Helipop5*, a gene encoding *H. elongata*'s lipoprotein 5 (LP5); (*NP*)x*8*, a synthetic gene encoding eight repeats of the nutrient peptide (NP); *HA*, a gene encoding the HA (hemagglutinin) tag, which is derived from the human influenza virus HA protein.

2. METHODOLOGY

2.1 Plasmid construction and protein verification

The DNA fragments encoding LP5, one to eight repeats of NP, and an HA tag (LP5-NPx1 to x8-HA) fusion proteins were assembled in pET vectors. The constructs were introduced into *E. coli* BL21-DE3 for protein expression. The correct expressions of the fusion proteins were verified by immunoblot with an anti-HA antibody.

2.2 Generation of recombinant Halomonas elongata

The constructs expressing LP5-NPx1 or x8-HA were assembled in the pK18mobsacB vector that will be used in the transformation of the *H. elongata* OUT30018 via the tri-parental mating method. After double homologous recombination, the expression cassettes encoding LP5-NPx1 or x8-HA would replace the coding region of the *Helipop5* gene, which encodes the native LP5 protein, on the *H. elongata* OUT30018 genome. Correct transgenes integrations in the transformants were confirmed by genomic PCR using specific primers designed to amplify the fragment between the 5' and 3'- flanking regions of the *Helipop5* coding sequence (CDS). The forward primer sequence was 5'-TACAACCAGCGCCTTTCCGAGCGTC-3', and the reverse primer sequence was 5'-CCGGAATGGGCAAGAAAGGGTGGACAAG-3'. Correct expressions of the fusion proteins were also confirmed by immunoblot with an anti-HA antibody.

2.2 Preparation of the microsomal protein fraction

The microsomal fraction was isolated from the crude protein extract by centrifugation at 12,000 × g for 15 min. After the centrifugation, the supernatant was collected as a soluble protein fraction, and

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the pellet was dissolved in phosphate-buffered saline (PBS) to the volume equal to that of the soluble fraction and used as a microsomal protein fraction.

2.3 High-Performance Liquid Chromatography (HPLC) analysis of dabsyl amino acids

Dabsylation of amino acids was performed based on a previously described method [6]. Briefly, the dabsyl amino acids solution of each sample was collected and filtered through a filter vial with 0.2 μm pore-size Polytetrafluoroethylene (PTFE) membrane (SEPARA® Syringeless filter, GVS Japan K.K., Tokyo, Japan) before being used as an HPLC sample. The quantification of dabsyl amino acids was carried out using an HPLC system (Shimadzu, Kyoto, Japan) equipped with a UV/VIS detector (SPD-10 A VP), an autosampler (SIL-10 AD VP), two pumps (LC-10 AD VP), degasser (DGU-14A), system controller (SCL-10A Vp), and column oven (CTO-10AC VP). The LabSolutions LC software (Shimadzu, Kyoto, Japan) was used to control the system and collect data. Dabsyl amino acids were separated through an analytical C18 column (Poroshell 120, 2.7 μm, EC-C18, 4.6 × 75 mm, Agilent Technologies Inc.) equipped with C18 guard column (Poroshell 120, 2.7 μm Fast Guard, EC-C18, 4.6 × 5 mm, Agilent Technologies Inc.) using a mobile phase gradient system consisting of 15% acetonitrile in 20 mM sodium acetate (pH 6.0) (mobile phase A) and 100% acetonitrile (mobile phase B). Dabsyl amino acids were detected by the UV/VIS detector at 468 nm. The injection volume was 10 μL, the flow rate was 0.5 mL/min, and the column temperature was maintained at 25°C. The eluent gradient was set as previously described [6].

3. RESULTS AND DISCUSSION

3.1 Expression of LP5-NPx1 to x8-HA in E. coli

To express the NP peptide on the cell surface, we designed a construct to fuse the NP-coding sequence to the *Helipop5* gene, which encodes Lipoprotein5 (LP5), a native outer-membrane protein of *H. elongata* OUT30018. A sequence encoding HA-tag was also added to the 3' end of these constructs, generating a series of pET plasmids containing *Helipop5*::*NPx*n::HA (*n*=1 to 8) expression cassettes. These constructs were introduced into *E. coli* BL21-DE3 for immunoblot verification of correct protein expressions from the constructs. As shown in Figure 2, all constructs were correctly translated. As indicated by the strength of the detection signal, proteins containing lower numbers of repeats (one to three repeats, Lanes 1 to 6) of NP are translated more efficiently, probably because they are smaller in size. Lower signals in Lanes 7 to 16 reflect the lower expression level of the longer peptide sequences (four to eight repeats).

Figure 2. Immunoblot of crude proteins extracted from *E. coli* cells expressing LP5-NPx1 to x8-HA fusion proteins. The HA-tagged proteins were detected by immunoblotting with anti-HA antibody. Lanes 1 and 2, LP5-NPx1-HA; Lanes 3 and 4, LP5-NPx2-HA; Lanes 5 and 6, LP5-NPx3-HA; Lanes 7 and 8, LP5-NPx4-HA; Lanes 9 and 10, LP5-NPx5-HA; Lanes 11 and 12, LP5-NPx6-HA; Lanes 13 and 14, LP5-NPx7-HA; Lanes 15 and 16, LP5-NPx8-HA; M, molecular weight marker; kDa, kilodalton.

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3.2 Expression of LP5-NPx1 and x8-HA in H. elongata

To generate *H. elongata* cells with surface-display NP, we further transferred the *Helipop5*::*NP*x1 or x8::HA constructs to pK18mobsacB *H. elongata* OUT30018 transformations. After confirming the correct integration of the transgenes by genomic PCR, crude proteins extracted from *H. elongata* SN1 and SN8, which contain expression cassettes of LP5-NPx1-HA and LP5-NPx8-HA fusion proteins on their genome, were also subjected to immunoblot analysis using anti-HA antibodies. The result shown in Figure 3 indicates that the strains correctly expressed LP5-NPx1 and LP5-NPx8-HA fusion proteins. The result also shows that most fusion proteins are correctly targeted to the cell membrane, as the detection signals were more substantial in the sample derived from the microsomal fractions. Further *in situ* immunolabelling with anti-HA antibodies would further confirm the presence of NP peptides on *H. elongata* SN1 and SN8's cell surface.

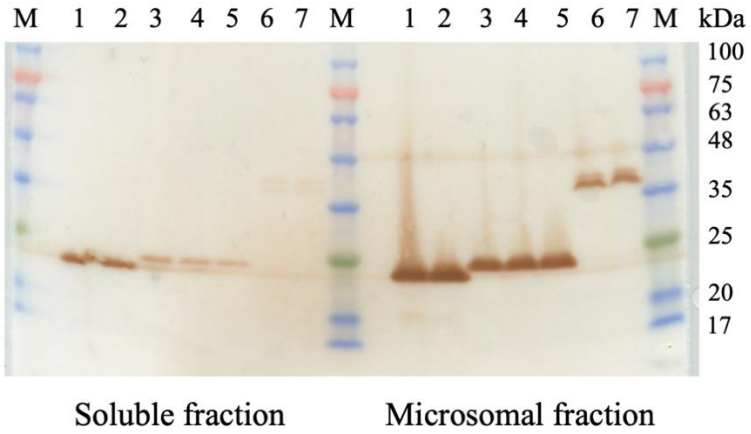

Figure 3. Immunoblot of soluble and microsomal fractions of the proteins extracted from *H. elongata* SN1 and SN8, which express LP5-NPx1-HA or LP5-NPx8-HA fusion proteins, respectively. The HA-tagged fusion proteins were detected by immunoblotting with anti-HA antibody. Lanes 1 and 2, control LP5-HA; Lanes 3, 4, and 5, LP5-NPx1-HA; Lanes 6 and 7, LP5-NPx8-HA; M, molecular weight marker; kDa, kilodalton.

4. CONCLUSIONS

We successfully expressed one to eight repeats of NP fused to the outer membrane LP5 protein in both *E. coli* and *H. elongata* cells. By separating the microsome fraction from crude protein extracted from *H. elongata* SN1 and SN8, we also confirm that the LP5-NPx1 or x8-HA are associated with the microsomal fraction. However, HPLC analysis of the amino acids content of the microsomal fractions found no difference in M, K, and W concentration between the extracts from *H. elongata* SN1, SN8, and the wild-type OUT30018 (data not shown). The weak activity of the *Helipop5* promoter may cause this. Therefore, our next step is to express *Helipop5*::*NP*x8::*HA* construct under a more potent promoter in the proline-overexpressing *H. elongata* HN10 previously developed in our laboratory [6]. Proline is an essential amino acid that also functions as a feed stimulant for fish. Therefore, Proline-overexpressing *H. elongata* equipped with surface-display NP could be used as a single-cell eco-feed for aquaculture. Due to the ability of *H. elongata* OUT30018 to thrive in a medium derived from high-salinity biomass waste, the development of a single-cell eco-feed with the genetic background of *H. elongata* OUT30018 has the potential to enhance the sustainability of the aquaculture industry.

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References

[1] Tacon, A. G. J. & Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. Aquaculture, 285 (1–4), 146–158.

[2] Olsen, R. L. & Hasan, M. R. (2012). A limited supply of fishmeal: Impact on future increases in global aquaculture production. Trends in Food Science & Technology, 27 (2),120–128.

[3] Jannathulla, R., Rajaram, V., Kalanjiam, R., Ambasankar, K., Muralidhar, M., & Dayal, J.S (2019). Fishmeal availability in the scenarios of climate change: Inevitability of fishmeal replacement in aquafeeds and approaches for the utilization of plant protein sources. Aquaculture Research, 50, 3493–3506.

[4] Nakayama, H. (2015). Cell-surface engineering of *Halomonas elongata* as an element recycling biotechnology in high salinity environments. In Asia Pacific Confederation of Chemical Engineering Congress 2015: APCChE 2015, incorporating CHEMECA 2015. Engineers Australia.

[5] Tanimura, K., Nakayama, H., Tanaka, T., & Kondo, A. (2013). Ectoine production from lignocellulosic biomass-derived sugars by engineered *Halomonas elongata*. Bioresource Technology, 142, 523–529.

[6] Khanh, H. C., Kaothien-Nakayama, P., Zou, Z., & Nakayama, H. (2024). Expression of an engineered salt-inducible proline biosynthetic operon in a glutamic acid over-producing mutant, *Halomonas elongata* GOP, confers increased proline yield due to enhanced growth under high-salinity conditions. Bioscience, Biotechnology, and Biochemistry, 88(10), 1233–1241.

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