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Received: 29 December 2025

Accepted: 26 February 2026

Published online: 07 March 2026

Cite this article as: Lee T.H., Do S., Lee H. *et al.* Adaptive laboratory evolution with ethionine identifies novel genetic determinants for enhanced protein and methionine accumulation in *Saccharomyces cerevisiae*. *J Biol Eng* (2026). <https://doi.org/10.1186/s13036-026-00652-x>

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Adaptive laboratory evolution with ethionine identifies novel genetic determinants for enhanced protein and methionine accumulation in *Saccharomyces cerevisiae*

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Abstract

Enhancing methionine and protein content in *Saccharomyces cerevisiae* is essential for its use as single-cell protein. Here, we applied ethionine resistance-mediated adaptive laboratory evolution (ALE) to generate strains with improved resistance to this toxic methionine analog. Stepwise adaptation enabled growth at ethionine concentrations of up to 0.50 mM and yielded strains with progressively higher intracellular methionine levels and improved protein production efficiency. Whole-genome sequencing identified nonsynonymous SNPs in 32 genes, of which nine candidates were functionally validated. CRISPR/Cas9-based editing demonstrated that mutations in *MDE1* and *JJJ1* directly elevated free methionine levels, whereas most other mutations increased overall protein accumulation. Functional annotations linked these genes to RNA processing, protein degradation, methionine salvage, and amino acid uptake, highlighting RNA processing as a major target for global protein enhancement. These findings reveal that ethionine resistance-mediated ALE induces multifactorial adaptations. They also provide new insights into protein biosynthesis regulation and lay a foundation for future engineering of high-performance yeast strains.

Keywords: *Saccharomyces cerevisiae*, single-cell protein, adaptive laboratory evolution, ethionine resistance, single nucleotide polymorphism

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Introduction

Single-cell protein (SCP) is gaining recognition as a sustainable alternative source of protein that can contribute to addressing pressing global challenges such as population growth, climate change, and food security [1, 2]. Given its microbial origin, SCP offers distinct advantages over conventional agriculture and livestock production, including rapid production, reduced land and water requirements, and lower greenhouse gas emissions [3, 4]. Recent scenario-based modeling has further indicated that substituting even 20% of the global ruminant meat consumption with microbial protein by 2050 could substantially ease environmental pressures, halving projected deforestation and associated CO₂ emissions, while also lowering methane emissions [5].

Saccharomyces cerevisiae stands out among microbial SCP sources for its substantial protein content (30–50%) and generally recognized as safe (GRAS) status [6]. *S. cerevisiae* biomass is also a rich source of functional nutrients such as β -glucan, glutathione, and B-complex vitamins, which further enhance its overall nutritional value [7, 8, 9]. Nonetheless, compared to bacterial SCP, *S. cerevisiae*-derived SCP has a low overall protein yield and less favorable essential amino acid profile, particularly limited levels of sulfur-containing essential amino acids such as methionine [7, 10].

Given the vast number of genes implicated in regulating protein accumulation in yeast, targeted genetic engineering remains highly

challenging [11, 12]. Consequently, some studies have employed random mutagenesis to generate mutants with enhanced protein content, but this approach has been found to be limited by low reproducibility and the difficulty of identifying desired variants within large mutant libraries [13, 14]. In light of these limitations, the present study employed adaptive laboratory evolution (ALE) as an alternative strategy to systematically obtain *S. cerevisiae* strains with improved protein and methionine content [15, 16, 17]. In this approach, ethionine, a toxic methionine analog, was used as a selective pressure to enrich for mutants with enhanced protein metabolism (Fig. 1A). This strategy dates back to early studies in the 1970s aimed at enhancing methionine and S-adenosylmethionine (SAM) production [17, 18, 19]. Building on this established strategy, ethionine selection has since been widely applied in metabolic engineering studies targeting methionine-related pathways. Ethionine resistance can arise from diverse underlying mechanisms and may include alterations in amino acid metabolism, protein synthesis, or stress-response pathways [20, 21]. Ethionine toxicity mainly arises from its competitive inhibition of S-adenosylmethionine (SAM) synthetase, which interferes with SAM biosynthesis and methylation-dependent processes [22]. Under prolonged ethionine stress, cells may acquire adaptive mutations that alleviate this inhibition, thereby enhancing methionine availability and protein production (Fig. 1B).

As expected, the evolved ethionine-resistant strains exhibited higher levels of both methionine and total protein. Furthermore, to uncover the genetic

determinants associated with this enhanced protein accumulation, whole-genome sequencing was performed using next-generation sequencing (NGS). From the single nucleotide polymorphisms (SNPs) identified by NGS, select mutations were individually introduced into the parental strain using CRISPR-Cas9 genome editing to assess their effects. This inverse metabolic engineering approach enabled easy identification and validation of genetic targets linked to increased protein production, thereby paving the way for rational design of *S. cerevisiae* strains with enhanced protein content for SCP production.

Methods

Strains and plasmids

Escherichia coli TOP10 (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) was used for plasmid construction, and *S. cerevisiae* D452-2 (*MAT α* , *leu2*, *his3*, *ura3*, and *can1*) [23], an auxotrophic strain with an S288C-related background, was used for SCP production due to its genetic stability and suitability for CRISPR/Cas9-based genome editing. All strains and plasmids used in this study are listed in Table S2.

Ethionine resistance-mediated ALE

To determine the growth-inhibiting concentration of ethionine, cells were cultivated under batch culture conditions in yeast synthetic complete (YSC)

medium lacking methionine (6.7 g/L yeast nitrogen base without amino acids, 1.92 g/L complete supplement mixture without methionine, and 20 g/L glucose) and supplemented with varying concentrations of ethionine (0.005–0.2 mM). Cultures were incubated at 30°C with shaking at 250 rpm, and cell growth was monitored.

Passage cultures were performed in methionine-free ethionine-containing YSC medium. At each passage, cultures were allowed to reach maximum cell density, after which approximately 1.7×10^8 colony-forming units from the previous passage were transferred into fresh medium (1% v/v) to initiate the next passage. Ethionine concentrations in the medium were increased stepwise from 0.15 mM to 0.5 mM in 0.05 mM or 0.1 mM increments depending on the observed growth rate. Cultures were incubated at 30°C with agitation at 250 rpm, and aliquots were preserved as glycerol stocks at -80 °C at the end of each passage.

Genetic manipulation

Gene cloning and gRNA plasmid construction for the CRISPR/Cas9 system were performed using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. The primer sets used to amplify guide RNA (gRNA) plasmids are listed in Table S3.

S. cerevisiae strains carrying SNPs were generated using a CRISPR/Cas9-based genome editing system as described previously [24]. In brief, gRNA

plasmids and repair DNA fragments (amplified with primers listed in Table S3) were cotransformed into the D452-2 strain harboring pCas9_AUR [24] to yield the recombinant strains (Table S2).

Media and culture conditions

E. coli strains were cultivated in Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) containing 50 µg/mL ampicillin. *S. cerevisiae* strains were precultured in YP20D medium at 30°C and 250 rpm for 48 h. The precultured cells were harvested and inoculated into baffled flasks containing 100 mL of YP50D medium at an initial OD₆₀₀ of 1.0. Batch fermentations were performed at 30°C and 250 rpm for 48 h.

Genome sequencing

The genomes of *S. cerevisiae* strains were sequenced and analyzed following the procedures described in a previous study [14]. In brief, genomic DNA (gDNA) was extracted from the parental *S. cerevisiae* D452-2 strain and from the evolved fifth-passage strain, using a gDNA extraction kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The purified gDNA samples were submitted to Macrogen (Seoul, Republic of Korea) for library preparation and whole-genome resequencing. Sequencing libraries were generated and analyzed using the HiSeq 4000 platform (Illumina, San Diego, CA, USA). Raw sequencing

reads were quality-filtered ($Q30 > 94\%$), yielding approximately 1.88 Gb of clean data. According to the service report, reads were mapped to the *S. cerevisiae* reference genome using BWA-MEM, followed by duplicate removal with Sambamba and variant calling with SAMTools, achieving a mapping rate of 97.28% and a mean alignment depth of $142.89\times$.

For mutation identification and comparative analysis between the parental and evolved strains, sequencing reads were additionally mapped to the *S. cerevisiae* S288C reference genome (assembly R64-1-1) using the CLC Genomics Workbench (originally by CLC bio, Aarhus, Denmark; now part of QIAGEN Aarhus) and genetic variations were extracted for downstream analysis. Identified nonsynonymous mutations were further validated by targeted Sanger sequencing where applicable.

Analytical methods

Cell growth was measured at OD_{600} using a spectrophotometer (OPTIZEN POP, Mecasys Co., Ltd., Daejeon, Republic of Korea). Dry cell weight (DCW) was estimated by multiplying the OD_{600} value by a conversion factor of 0.3 g/L/ OD_{600} . Amino acid profiling was performed using an HPLC system (UltiMate 3000, Thermo Fisher Scientific, USA) equipped with an INNO C18 column (YoungJin Biochrom Co., Ltd., Republic of Korea). Cell lysates were prepared and subjected to precolumn derivatization with 9-fluorenylmethoxycarbonyl chloride and *o*-phthalaldehyde, followed by fluorescence detection under previously described conditions [13, 14].

Methionine content was determined following a precolumn derivatization method reported previously [25]. In brief, the cell lysates were allowed to react with 2,4-dinitrofluorobenzene (DNFB, 10 g/L) and NaHCO₃ (0.5 M, pH 9.0) at 60°C for 60 min in the dark. The mixtures were subsequently neutralized with KH₂PO₄ buffer (0.01 M, pH 7.0) and subjected to HPLC analysis using an UltiMate 3000 HPLC system (Thermo Fisher Scientific, USA) equipped with an Inertsil ODS-3 column (Shimadzu, Kyoto, Japan) under the chromatographic conditions as previously reported [13, 26]. In this study, concentration, expressed as mg/L, refers to the volumetric concentration of amino acids, protein, or free methionine. In contrast, content or level was calculated by normalizing the corresponding concentration (mg/L) to cell biomass, expressed as dry cell weight (g DCW/L), and is presented as a percentage (%). This normalization was applied to all comparisons of intracellular amino acid, protein, and methionine levels to account for differences in biomass among strains.

Statistical analysis

Statistical analyses were performed using SPSS Statistics v.28.0 (IBM Corp., Armonk, NY, USA). Data are presented as mean $\pm$ standard deviation. One-way analysis of variance was performed, and statistical significance was assessed using Tukey's honestly significant difference (HSD) test at a significance level of $p < 0.05$. In addition, an independent samples Student's *t*-test was performed to evaluate differences between two groups with statistical significance defined as $p < 0.05$.

Results and Discussion

Ethionine resistance-mediated ALE for selection of evolved strains with high methionine content

To establish the selection pressure for ethionine resistance-based ALE, we first determined the ethionine concentration that severely inhibited the growth of the parental *S. cerevisiae* D452-2 strain. Cells were cultivated in YSC medium lacking methionine and supplemented with varying concentrations of ethionine (0.005–0.2 mM). As shown in Fig. 2A, no detectable growth of D452-2 was observed at 0.2 mM ethionine; however, at a lower ethionine concentration of 0.15 mM, growth was suppressed, with an extended lag phase of approximately 60 h. Based on these observations, 0.15 mM ethionine was selected as the initial concentration for subsequent ALE experiments.

ALE proceeded via stepwise increases in ethionine concentration. In passage 1, *S. cerevisiae* cells were grown in YSC medium supplemented with 0.15 mM ethionine. To establish passage 2, these cultures were transferred into fresh YSC medium containing 0.20 mM ethionine. Notably, despite the increased ethionine concentration, the lag phase in passage 2 was shorter than that in the initial cultivation (Fig. 2B), indicating that repeated culturing in defined medium with ethionine allowed resistant mutants to emerge and dominate. Through seven successive passages with progressively increasing ethionine concentrations, *S. cerevisiae* adapted

to growing in YSC medium containing 0.50 mM ethionine, with a lag phase of approximately 30 h by the final passage. However, the maximum dry cell weight at this concentration was lower than that observed at lower ethionine concentrations.

From passages 3 to 7, cultures were first precultured in YP20D medium (10 g/L yeast extract, 20 g/L Bacto™ Peptone, and 20 g/L glucose) and then transferred to YP50D medium (10 g/L yeast extract, 20 g/L Bacto™ Peptone, and 50 g/L glucose). For each passage, intracellular free methionine levels were quantified. These levels increased steadily over successive ALE passages (Fig. 3A), peaking after passage 6 at 0.57%—a 60% increase relative to the parental D452-2 strain.

Enhanced protein production efficiency following ethionine resistance-mediated ALE

We next hypothesized that the increased intracellular methionine observed as a consequence of ethionine-mediated ALE would result in a concomitant increase in the overall protein content in the evolved strains, given methionine's role as both the initiating amino acid in protein synthesis and a key metabolite in sulfur amino acid metabolism [27, 28]. In addition, the continuous selective pressure applied during ALE was likely to have remodeled the translational machinery and amino acid sensing pathways. Potential adaptations could include increased specificity of methionyl-tRNA synthetase, upregulation of ribosomal

proteins to restore translational fidelity, and reprogramming of TORC1- and GCN4-mediated signaling to promote the expression of genes involved in translation, tRNA charging, and amino acid metabolism [29, 30, 31]. Together, these adaptations were expected to enhance protein synthesis, a possibility we verified by quantifying the total protein content in the evolved strains.

The relative protein content and concentration across evolved strains were measured using the Bradford assay, revealing increased protein levels relative to the parental strain (Fig. 3B and Fig. S1). In particular, the passage-4 strain exhibited a 2.5-fold higher protein content and 2.0-fold higher protein concentration than the parental D452-2 strain, whereas the passage-5 strain showed 2.4-fold and 2.0-fold increases, respectively. The Bradford assay was used solely to compare relative protein levels across strains; the exact protein concentrations were determined using an HPLC-based amino acid profiling method. Relative to the parental strain, passage-4 and passage-5 strains exhibited elevated levels of most amino acids (Fig. 4A). Consequently, the total protein content of these strains was 55% and 53%, corresponding to 43% and 40% increases compared to the parental strain, respectively (Fig. 4B).

It should be noted, however, that the apparently similar increases in total protein and free methionine contents should be interpreted with caution due to methodological limitations. Amino acids exist in two forms in cells: free amino acids, which are present as individual molecules, and constituent amino acids, which are bound within proteins. Accordingly,

total amino acids represent the sum of both forms. Amino acid profiling, which was conducted for total amino acid analysis using conventional sulfuric acid hydrolysis [32], causes degradation of sulfur-containing residues such as methionine, resulting in the absence of detectable methionine (Fig. 4A). Accordingly, in this study, only free methionine was quantified separately by a dedicated HPLC-based method, which accurately measures intact methionine molecules (Fig. 3A). Therefore, if alternative hydrolysis methods that better preserve methionine (e.g., methane sulfonic acid hydrolysis) [33, 34] had been applied for compositional amino acid analysis, the measured increase in total methionine content could have been substantially greater than that of the overall protein content. Nevertheless, despite these methodological limitations, the evolved populations clearly exhibited substantial increases in both intracellular methionine and total protein content, thereby validating our initial hypothesis that methionine-mediated adaptive evolution enhanced global protein synthesis through metabolic and translational remodeling.

Identification and functional validation of candidate SNPs driving adaptation under methionine selection

To test the hypothesis that methionine-mediated evolution introduced genetic variations enhancing protein production, we performed a comparative whole-genome analysis of the parental D452-2 strain and the fifth-passage strain using next-generation sequencing. SNP variations

causing amino acid substitutions were identified in 32 genes distributed across various chromosomes of the fifth-passage strain (Table S1). Because analyzing all the 32 SNP-containing genes for associations with the observed protein and methionine increases would be labor-intensive and time consuming, we focused on nine candidate genes most strongly associated with these traits, namely, *POP4*, *RRP3*, *SWT21*, *GRC3*, *JJJ1*, *DDI1*, *ATG34*, *MDE1*, and *TAT2* (Table 1). The SNPs identified in these genes produced the following missense mutations: (1) K14T and R105K in Pop4, (2) V203I in Rrp3, (3) V194M in Swt21, (4) D171N in Grc3, (5) P541S in Jjj1, (6) R61K in Ddi1, (7) V385M in Atg34, (8) D101N and V119L in Mde1, and (9) H50N and D509G in Tat2. Functional annotations revealed that these genes participate in RNA processing (*POP4*, *RRP3*, *GRC3*, *SWT21*, and *JJJ1*), protein degradation (*DDI1* and *ATG34*), the methionine salvage pathway (*MDE1*), and amino acid uptake (*TAT2*).

Finally, we tested whether the SNPs in the nine candidate genes indeed contributed to the observed increases in methionine and protein content: each SNP was introduced individually into the parental strain using CRISPR/Cas9-based genome editing, except in *MDE1*, where two closely located SNPs were introduced together (Table S1). As seen in Fig. 5, all mutations except for the K14T substitution in Pop4 led to an increase in protein content in *S. cerevisiae*. Notably, the MDE1* and JJJ1* strains, into which the *MDE1* and *JJJ1* SNPs had been introduced, exhibited 11% and 10% higher free methionine levels, respectively, compared with the parental strain. These findings suggests that, among the nine candidate

genes, only *MDE1* and *JJJ1* directly increased free methionine levels; the other mutations predominantly enhanced protein accumulation. Nonetheless, these other mutations may also have contributed to elevated total methionine content because free amino acid assays cannot distinguish between pre-existing free methionine and protein-derived methionine. The larger increase in protein levels observed in the evolved strains relative to the individual SNP mutants indicates that the phenotype results from the combined effects of multiple mutations rather than a single genetic change. This illustrates that adaptation to ethionine is multifactorial, with some mutations enhancing free methionine pools and others promoting protein biosynthesis, which indirectly increases methionine levels. To further examine the relationship between individual SNPs and ethionine resistance, the parental D452-2 strain and strains harboring select SNPs (*SWT21**, *JJJ1**, *MDE1**, *DDI1**, and *ATG34**; Table S2) were cultivated in medium supplemented with 0.15 mM ethionine. All tested SNP strains exhibited greater cell growth than the parental strain under ethionine stress (Fig. S2), indicating that the introduced mutations contribute to improved ethionine tolerance. Notably, some SNP strains showed enhanced growth despite not displaying increased free methionine levels relative to the parental strain. This observation suggests that ethionine resistance is not solely determined by intracellular methionine abundance but may also involve broader cellular adaptations, such as improved translational efficiency or proteostasis. Collectively, these findings further support the multifactorial nature of adaptation to ethionine stress.

These findings suggest that ethionine-mediated adaptive evolution imposed selective pressure not only on genes directly associated with methionine metabolism but also on genes involved in other cellular processes. Mutations in RNA processing genes (*POP4*, *RRP3*, *GRC3*, *SWT21*, and *JJJ1*) are particularly noteworthy because they affect ribosome biogenesis and RNA stability, thereby influencing translational efficiency and global protein synthesis [35]. Similarly, mutations in protein degradation genes (*DDI1* and *ATG34*) may have reduced protein degradation through changes in ubiquitin-proteasome activity or autophagic turnover. Furthermore, mutations in Mde1, a key enzyme of the methionine salvage pathway, indicate adaptive rewiring of sulfur metabolism [36]. By maintaining methionine availability under conditions of ethionine stress, these mutations may have indirectly contributed to both higher free methionine levels and increased protein biosynthesis. Finally, mutations in Tat2, a high-affinity tryptophan permease involved in amino acid uptake, point to another mechanism that supports growth under ethionine stress [37]. Enhanced uptake of specific amino acids may have relieved metabolic bottlenecks and helped meet anabolic demand, indirectly supporting methionine utilization and protein synthesis. Although 32 nonsynonymous SNPs were identified in the evolved strain, functional validation in this study was limited to 9 genes selected on the basis of their established or predicted involvement in protein- and amino-acid-related processes. The remaining SNPs occurred in genes with unknown or poorly characterized functions. Functional validation of these mutations could in turn shed light on previously unrecognized regulatory

factors contributing to methionine availability or global protein accumulation under ethionine stress and therefore warrants further investigation. In sum, ethionine-mediated ALE did not simply enrich for improved methionine biosynthesis but instead promoted global adaptations across RNA processing, protein degradation, sulfur metabolism, and amino acid uptake. Together, these changes likely adjusted proteostasis and acted synergistically to increase both methionine and total protein levels. Some experiments (Figs. 4 and 5) were conducted with two biological replicates ($n = 2$), which may limit statistical power. Therefore, the quantitative differences should be interpreted with caution.

Conclusion

This study demonstrates that ethionine supplementation not only boosts methionine production but also enhances protein production in *S. cerevisiae*. The study also uncovered molecular mechanisms linking between ethionine resistance with increased protein biosynthesis. Comparative genomic and functional analyses revealed that genes involved in RNA processing, protein degradation, the methionine salvage pathway, and amino acid uptake play central roles, with RNA processing-related genes particularly showing frequent mutations. These findings suggest that regulatory control of RNA processing may be key to enhancing protein production in yeast. Future studies will focus on

examining the effects of the identified SNPs on protein activity and testing optimal SNP combinations to further increase protein yields. Collectively, the results of this study lay a foundation for the development of high-performance yeast strains with improved protein output and offer new insights into the molecular mechanisms governing protein biosynthesis in eukaryotic cells.

Data Availability

Data are available from the corresponding author upon request.

Funding Declaration

This study was financially supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) (RS-2025-02218354) and by the R&D program of MOTIE/KEIT (RS-2025-02307907). This research was also supported by a grant from the National Research Foundation of Korea (RS-2024-00440975), funded by the Korean Ministry of Science, ICT, and Future Planning.

Author contributions

T.H.L.: Methodology, Investigation. S.D.: Methodology, Investigation. H.L.: Writing – original draft preparation, Data Curation. K.L.: Formal analysis.

J.S.: Formal analysis. Y.P.: Conceptualization. S.K.: Supervision, Writing – original draft preparation.

Competing Interests

The authors declare no competing interests.

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Table Captions

Table 1. Key candidate gene mutations associated with increased protein content in the fifth-passage strain.

Figure Captions

Fig. 1. Adaptive laboratory evolution (ALE) under ethionine stress and mechanistic model of S-adenosylmethionine (SAM) synthetase inhibition. (A) Schematic of ethionine resistance-mediated ALE, SNP identification by whole-genome sequencing, and functional validation using CRISPR/Cas9 editing. (B) Model of competitive inhibition of SAM synthetase by ethionine (Eth). In wild-type cells, SAM synthetase is competitively inhibited by Eth, leading to S-adenosylethionine formation and growth inhibition. In contrast, in evolved strains with enhanced methionine (Met) producing capability, this inhibition is alleviated, enabling SAM synthesis and restoring growth.

Fig. 2. Growth of *Saccharomyces cerevisiae* D452-2 under ethionine stress and during ALE. (A) Growth curves of the parental D452-2 strain cultivated in YSC medium lacking methionine and supplemented with varying concentrations of ethionine (0–0.2 mM). (B) Growth curves of evolved strains obtained from the first passage to the seventh passage under stepwise increments of ethionine concentrations (0.15–0.50 mM). Error bars represent standard deviations from biological replicates ($n \geq$

2).

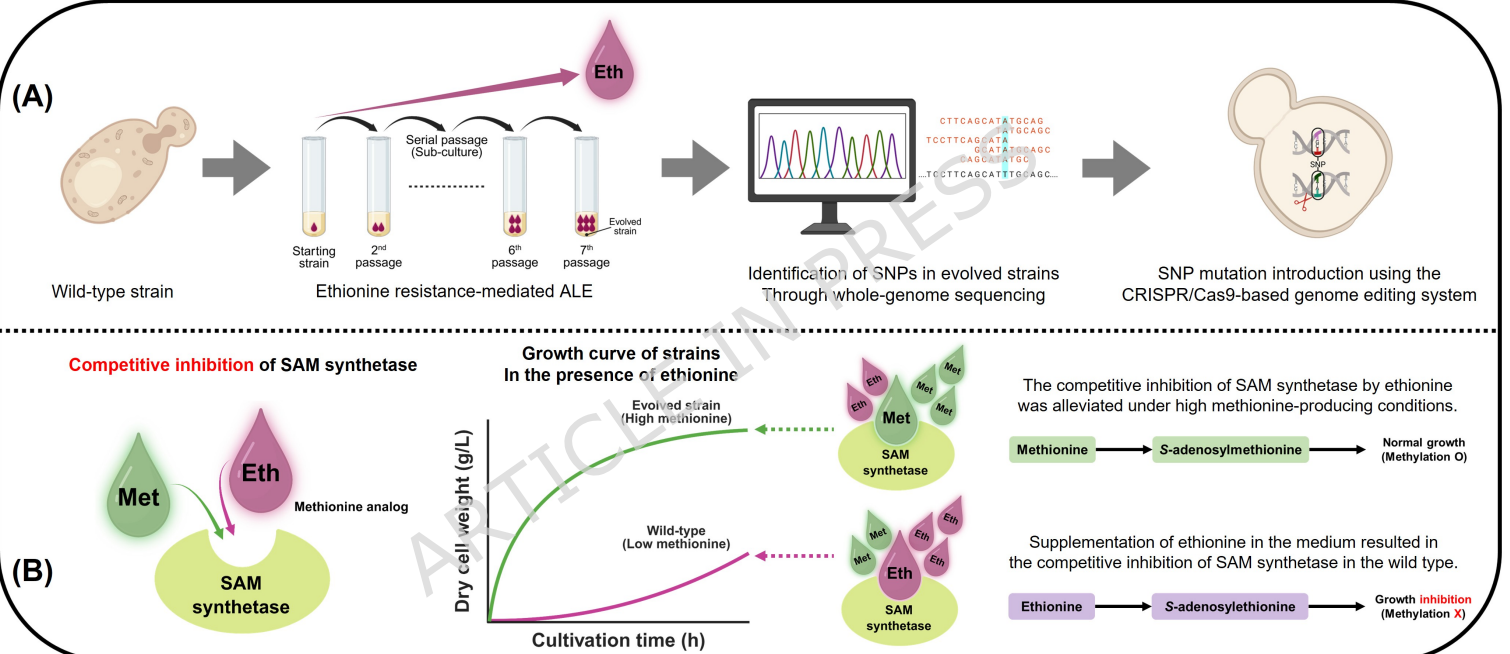
Fig. 3. Comparison of free methionine content (A) and relative protein content (B) of parental and evolved strains. Free methionine content was measured in the parental D452-2 strain and evolved strains obtained from the third passage to the seventh passage of ethionine-mediated ALE. Relative protein content was determined using the Bradford assay. Results are the mean of $n \geq 2$ experiments, and error bars indicate the standard deviation. Different letters represent significantly different means (Tukey's honestly significant difference tests, $p < 0.05$).

Fig. 4. Comparison of individual amino acid content (A) and total amino acid content (B) of parental and evolved strains. Amino acid profiling was performed for the parental D452-2 strain and evolved strains obtained after the fourth and fifth passages of ethionine-mediated ALE. Results are the mean of two experiments, and error bars indicate the standard deviation. Different letters represent significantly different means (Tukey's honestly significant difference tests, $p < 0.05$).

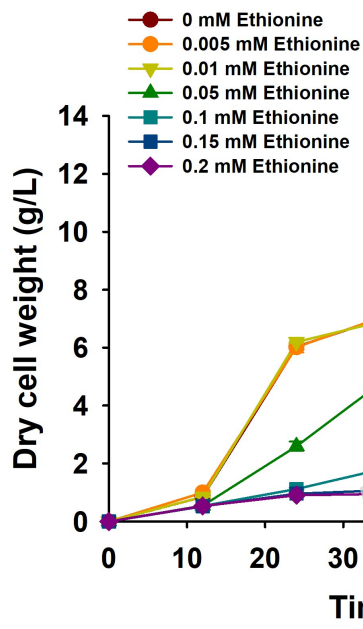
Fig. 5. Comparison of free methionine content (A) and relative protein content (B) of the parental strain and SNP-introduced strains. Free methionine and protein concentrations were measured in the parental D452-2 strain and recombinant strains carrying SNPs in specific candidate genes. The specific amino acid substitutions corresponding to each SNP-introduced strain are summarized in Table 1. Results are the mean of two experiments, and error bars indicate the standard deviation. Asterisks (*) indicate statistically significant

differences compared with the parental strain (Student's t-test, $p < 0.05$).

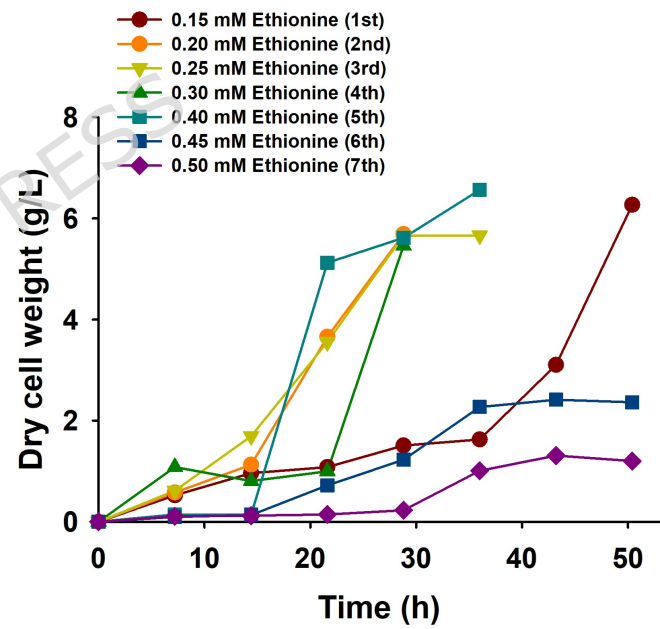
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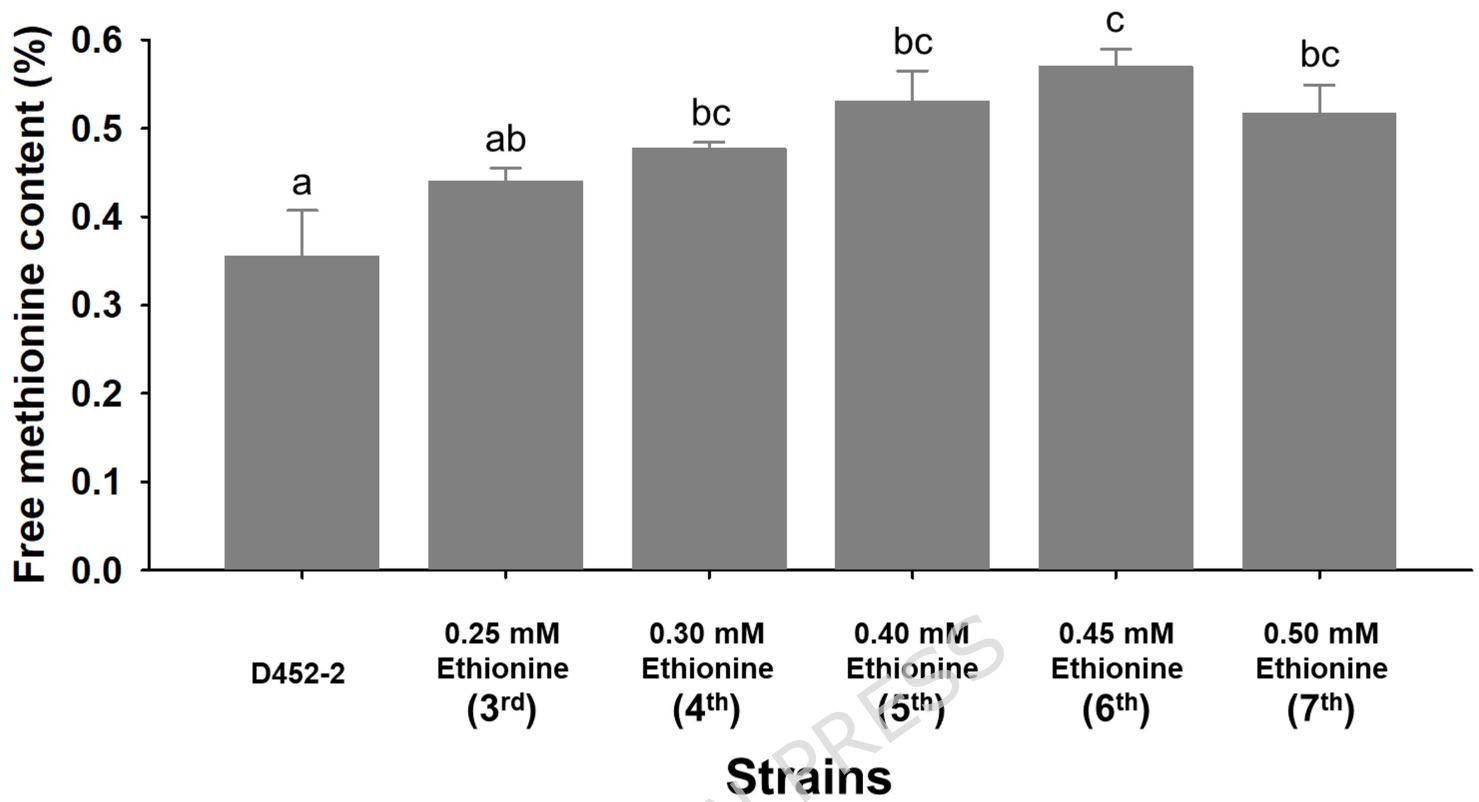
(A)



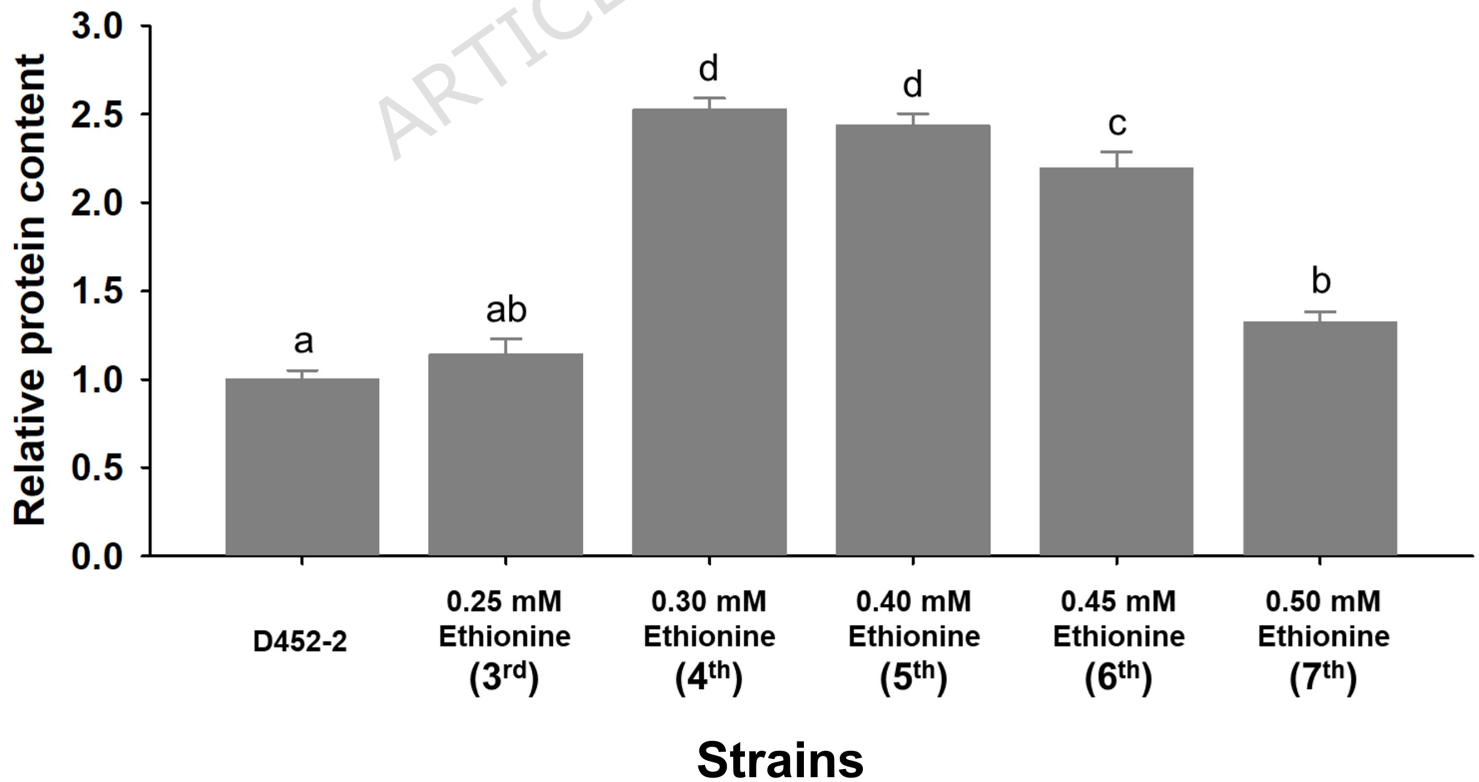
(B)

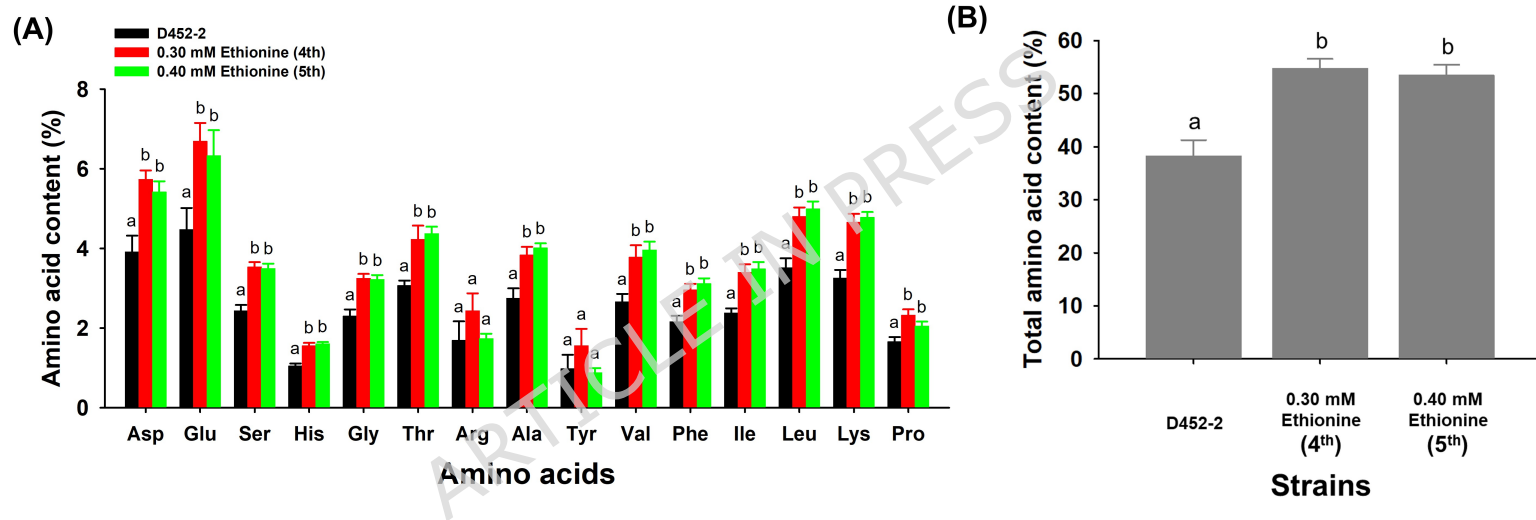


(A)

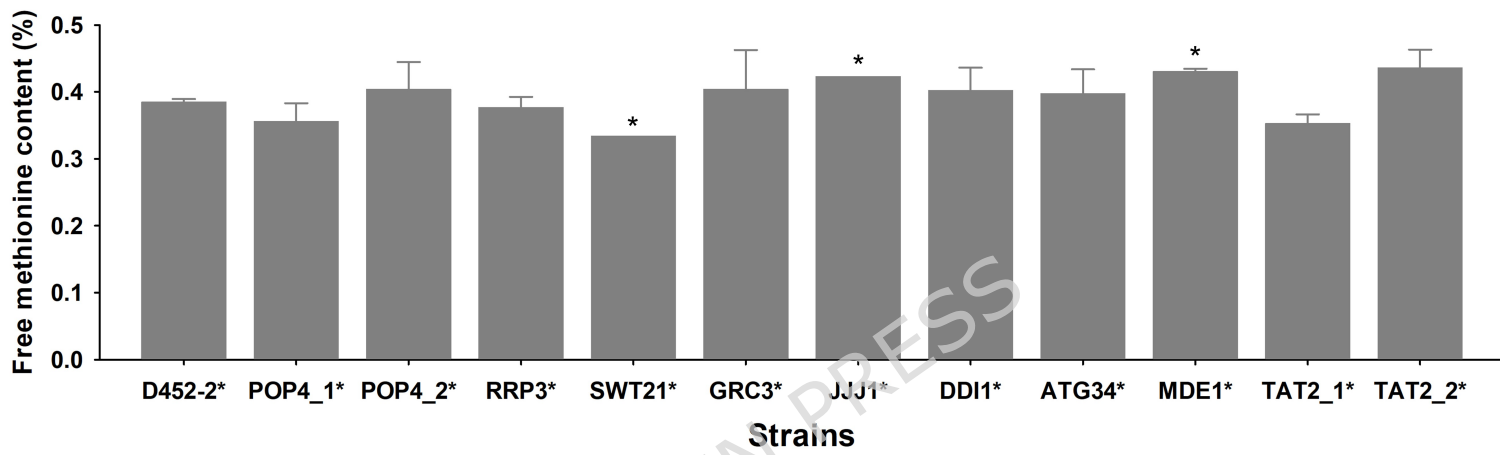


(B)





(A)



(B)

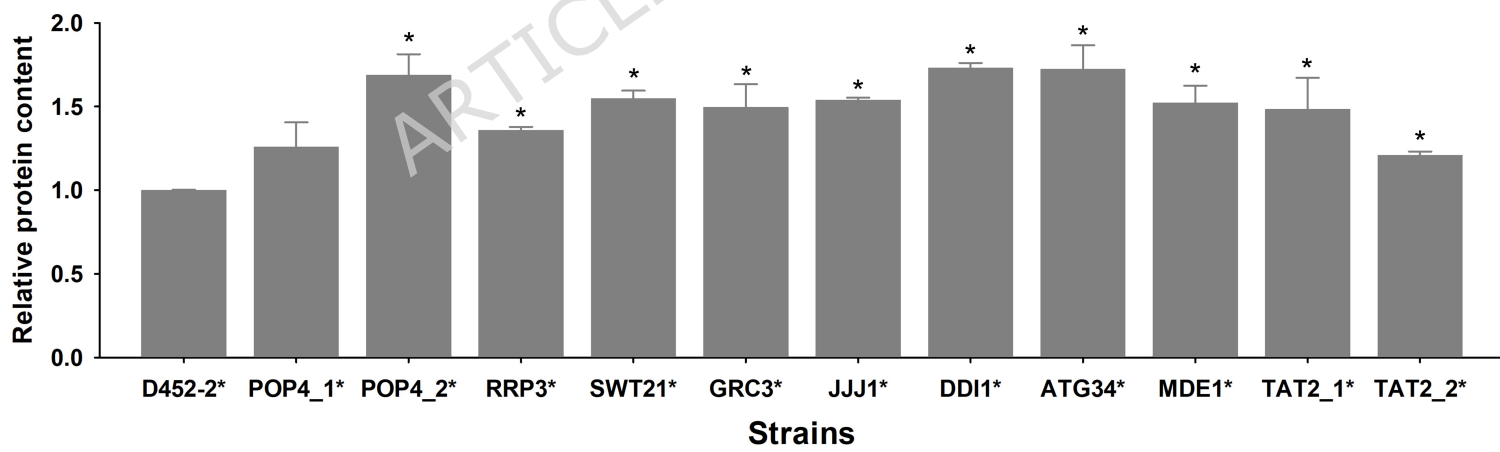


Table 1. Key candidate gene mutations associated with increased protein content in the fifth-passage strain

Gene	Nucleotide change	Amino acid change	Function
<i>POP4</i> (<i>YBR257W</i>)	A ⁴¹ → C	Lys ¹⁴ → Thr	Ribonuclease complex subunit required for tRNA maturation and rRNA processing
<i>RRP3</i> (<i>YHR065C</i>)	G ⁶⁰⁷ → A	Val ²⁰³ → Ile	DEAD-box RNA helicase involved in pre-rRNA processing and ribosome biogenesis
<i>SWT21</i> (<i>YNL187W</i>)	G ⁵⁸⁰ → A	Val ¹⁹⁴ → Met	Spliceosome regulator involved in mRNA splicing
<i>GRC3</i> (<i>YLL035W</i>)	G ⁵¹¹ → A	Asp ¹⁷¹ → Asn	Polynucleotide 5'-hydroxyl-kinase involved in rRNA processing and termination of RNA polymerase I transcription
<i>JJJ1</i> (<i>YNL227C</i>)	C ¹⁶²¹ → T	Pro ⁵⁴¹ → Ser	J-domain co-chaperone (Hsp40 family) involved in rRNA processing and ribosomal large subunit biogenesis and nuclear export
<i>DDI1</i> (<i>YER143W</i>)	G ¹⁸² → A	Arg ⁶¹ → Lys	Ubiquitin-like protease involved in protein degradation
<i>ATG34</i> (<i>YOL083W</i>)	G ¹¹⁵³ → A	Val ³⁸⁵ → Met	Autophagy receptor involved in selective transport of endocytic vesicle cargo to vacuoles

<i>MDE1</i>	G ³⁰¹ → A	Asp ¹⁰¹ → Asn	Methionine salvage pathway enzyme involved in methionine recycling
<i>(YJR024C)</i>	G ³⁵⁵ → C	Val ¹¹⁹ → Leu	
<i>TAT2</i>	C ¹⁴⁸ → A	His ⁵⁰ → Asn	High-affinity tryptophan permease mediating uptake of aromatic amino acids
<i>(YOL020W)</i>	A ¹²⁵⁶ → A	Asp ⁵⁰⁹ → Gly	
