A bacterial cell factory converting glucose into scyllo-inositol, a therapeutic agent for Alzheimer’s disease

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A rare stereoisomer of inositol, scyllo-inositol, is a therapeutic agent that has shown potential efficacy in preventing Alzheimer’s disease. *Mycobacterium tuberculosis* *ino1* encoding *myo*-inositol-1-phosphate (*MI1P*) synthase (*MI1PS*) was introduced into *Bacillus subtilis* to convert glucose-6-phosphate (*G6P*) into MI1P. We found that inactivation of *pbuE* elevated intracellular concentrations of NAD⁺-NADH as an essential cofactor of MI1PS and was required to activate MI1PS. MI1P thus produced was dephosphorylated into *myo*-inositol by an intrinsic inositol monophosphatase, YktC, which was subsequently isomerized into scyllo-inositol via a previously established artificial pathway involving two inositol dehydrogenases, IolG and IolW. In addition, both *glcP* and *glcK* were overexpressed to feed more G6P and accelerate scyllo-inositol production. Consequently, a *B. subtilis* cell factory was demonstrated to produce 2 g L⁻¹ scyllo-inositol from 20 g L⁻¹ glucose. This cell factory provides an inexpensive way to produce scyllo-inositol, which will help us to challenge the growing problem of Alzheimer’s disease in our aging society.
Dementia, most commonly caused by Alzheimer’s disease, reached a prevalence of 50 million people worldwide in 2018; this number is expected to increase to 152 million by 2050, with the biggest increase occurring in developing countries. Alzheimer’s disease involves the aggregation of amyloid β-proteins, engendering apoptosis of neurons and loss of cognitive function. Currently there is no cure for Alzheimer’s disease, but numerous attempts have been made to develop molecules capable of targeting these aggregations of amyloid β-proteins. Among these molecules, a rare stereoisomer of inositol, scyllo-inositol, has been shown to be promising. When taken orally, scyllo-inositol is able to reach the brain, and prevents amyloid β-proteins forming toxic amyloid fibrils and polymers. Scyllo-Inositol administered at 250 mg per day has been shown to have an acceptable level of safety. Patients given this dose had higher scyllo-inositol concentrations and fewer amyloid plaques in their cerebrospinal fluid. Slight but significant increases in brain ventricular volume were observed, but other markers of Alzheimer’s disease were unchanged. The small sample size of 250 mg per day did not provide evidence to support or refute any benefits associated with scyllo-inositol.

In natural biological systems, the biosynthesis of myo-inositol from glucose is widely conserved, especially in eukaryotes, due to the importance of myo-inositol as a moiety of the phospholipid phosphatidylinositol, which is found in the plasma membrane, and also because myo-inositol acts as a second messenger in the cell system. Myo-Inositol biosynthesis includes three steps: the phosphorylation of glucose into glucose–6-phosphate (G6P), commonly seen in many organisms; the conversion of glucose–6-phosphate (G6P) into myo-inositol 1-phosphate (MI1P) by MI1P synthase (MI1PS) (EC.3.1.3.25) encoded by ino1; and cleaving-off of a phosphate from MI1P to form myo-inositol by inositol monophosphatase (IMP).

In this study, we demonstrate a B. subtilis cell factory to produce 2 g L⁻¹ scyllo-inositol from 20 g L⁻¹ glucose. This cell factory provides an inexpensive way to produce scyllo-inositol.

**Results**

**Introduction of MI1PS into B. subtilis.** M. tuberculosis has efficient myo-inositol biosynthesis involving ino1 encoding MI1PS. In order to introduce functional M. tuberculosis ino1, its codon usage was optimized for expression in B. subtilis (Supplementary Fig. 1). The modified ino1 was cloned into the amyE locus on the chromosome of B. subtilis strain 168 to be expressed as a C-terminal His-tag fusion under the strong and constitutive rpsO promoter to give strain TK002 [amyE::(PrpsO-ino1Mt-His6 cat)] (Supplementary Table 1).

The ino1 transcript was accumulated during the culture process (Fig. 2a). In addition, MI1PS protein as a His-tag fusion was detected by Western blot analysis (Fig. 2b). These results suggested that M. tuberculosis ino1 was expressed, producing MI1PS as a protein in B. subtilis. However, MI1PS activity in TK002 could not be detected even in the presence of 10 mM NAD⁺ (Fig. 3a). In a previous study, the production of heterologous MI1PS was problematic due to its misfolding during the translation process. We therefore tried to express...
Inactivation of pbuE restored MI1PS activity in B. subtilis. It is known that the MI1PS enzyme requires NAD$^+$-NADH as an essential cofactor to become functional, and that NAD$^+$-NADH has to be properly located within the Rossmann-fold domain of the enzyme during its translation. We happened to discover that the intracellular levels of NAD$^+$ were significantly elevated in strain TK002 (Fig. 3b); the concentration was up to 20 mM, which was almost twice more than that in strain 168 cells. The MI1PS protein was detected at the expected size (41 kDa) in strain TK002 (lane 2) but not in strain 168 (lane 1) after culturing for 24 h. The arrowhead indicates the position of the band for the protein. Similar results were obtained for three independently repeated experiments, and a representative result is shown. (The original gel image is supplied as Supplementary Fig. 5).

conversion of glucose into myo-inositol, since its substrate G6P is naturally supplied from glucose and also because its product MI1P is dephosphorylated by the intrinsic and constitutive YktC to form myo-inositol. Accordingly, in order to enable the production of scylo-inositol from glucose, we next introduced a previously established artificial pathway to convert myo-inositol into scylo-inositol involving two inositol dehydrogenases, IolG and IolW. To achieve this, strain MC022 [amyE::(PrpsO-inolMt-His6 cat), column 2], TC003 [amyE::(PybK-inolMt-His6 cat), column 3], MC010 [pbuE::pMutin2(erm)] ΔiolABCDEFGHJ ΔiolX ΔiolR, column 4], MC001 [amyE::(PrpsO-inolMt-His6 cat) ΔiolABCDEFGHJ ΔiolX ΔiolR, column 5], and MC011 [amyE::(PrpsO-inolMt-His6 cat) pbuE::pMutin2(erm) ΔiolABCDEFGHJ ΔiolX ΔiolR, column 6] were cultured for 24 h as described in the Methods. The activity was expressed as the concentration of inorganic phosphate (Pi) present in the assay mixture. The values are means ± SEM from at least three independent experiments (Supplementary Data 1; the individual data-points are plotted as closed diamonds.) *Statistical significance was calculated using the Mann-Whitney U test with Prism (GraphPad software) based on the difference between MC001 and MC011 (p < 0.01). 2) NAD$^+$ - NADH levels in strains 168 (parental strain, column 1) and YDHld [pbuE::pMutin2(erm), column 2]. The total concentration of NAD$^+$ and NADH in cells after culturing for 24 h was measured as described in the Online Methods. The values are means ± SEM from at least three independent experiments (Supplementary Data 2; the individual data-points are plotted as closed diamonds.). *Statistical significance was calculated using the Mann-Whitney U test with Prism (GraphPad software) (p < 0.05).

SI production in B. subtilis. As described above, in B. subtilis MC011, which lacked both functional pbuE and myo-inositol catabolism, the artificially introduced M. tuberculosis ino1 was “restored” to produce an active enzyme. This could enable the transcription of ino1 in B. subtilis. The ino1 transcript was detected at the expected size (1.1 kb) by Northern blot analysis and accumulated as the cells grew after culturing for 10, 24, and 48 h (lanes 1, 2, and 3, respectively; each lane contained 30 μg of the RNA extract). The arrowhead indicates the position of bands for the transcript. Similar results were obtained for three independently repeated experiments, and a representative result is shown. (The original gel image is supplied as Supplementary Fig. 4).
conversion of myo-inositol into scylo-inositol (Supplementary Table 1). MC022 was grown in Soytone medium containing 20 g L\(^{-1}\) glucose, and scylo-inositol increasingly appeared in the culture medium as the growth time was extended. The amount of scylo-inositol produced was 0.11 ± 0.15 g L\(^{-1}\), 1.60 ± 0.33 g L\(^{-1}\), and 2.19 ± 0.92 g L\(^{-1}\) after being cultured for 24, 48, and 96 h, respectively (triangles, Fig. 4). These results demonstrated that we successfully created the first bacterial cell factory for the production of scylo-inositol from glucose.

In *B. subtilis*, it is known that the phosphoenolpyruvate-dependent phosphotransferase system (PTS) functions as the major mechanism for the uptake of glucose\(^{29,30}\). Therefore, we introduced an additional mechanism for the uptake of glucose involving the GlcP glucose transporter\(^{29}\) and GlcK glucose kinase\(^{30}\), anticipating that this could feed more G6P to be converted into myo-inositol. Thus, strain MC031 [\(\text{E. coli} \text{::} \Delta \text{iolHIJ} \text{::} \text{IO} \text{::} \text{iolG} \text{::} \text{iolW} \text{::} \text{iolT kan}\) \(\text{pbuE:} \text{PB} \text{::} \text{glcP-glcK ble}\)] was constructed, in which both \(\text{glcP}\) and \(\text{glcK}\) were overexpressed in a single artificial operon [\(\text{E. coli} \text{::} \Delta \text{iolHIJ} \text{::} \text{IO} \text{::} \text{iolG} \text{::} \text{iolW} \text{::} \text{iolT kan}\) \(\text{pbuE:} \text{PB} \text{::} \text{glcP-glcK ble}\)] on an MC022 background (Supplementary Table 1). The overexpression of \(\text{glcP}\) and \(\text{glcK}\) did not significantly affect the growth (Supplementary Fig. 2). MC031 was grown and produced 0.95 ± 0.12 g L\(^{-1}\) and 2.68 ± 0.45 g L\(^{-1}\) scylo-inositol after being cultured for 24 and 96 h, respectively (Fig. 4). These results indicated that in MC031 the simultaneous overexpression of \(\text{glcK}\) and \(\text{glcP}\) could accelerate the rate of scylo-inositol production by feeding more G6P to the earlier stage compared with MC022, although this failed to significantly increase the final concentration.

**Discussion**

In the present study, we demonstrated the production of scylo-inositol from glucose using a new *B. subtilis* cell factory, by combining two conversion steps: one step from glucose to myo-inositol and the second step from myo-inositol to scylo-inositol. To develop the former step and convert glucose into myo-inositol in *B. subtilis*, we introduced *M. tuberculosis* *iolI*, encoding MI1PS as the key enzyme. MI1PS has been reported to be a rate-limiting enzyme for the synthesis of myo-inositol in natural biological systems\(^{31}\). The MI1PS enzyme was first described in parsley leaves, rat testes, and *Saccharomyces cerevisiae*\(^{32–35}\). MI1PS requires NAD\(^{+}\)-NADH as an essential cofactor, since the reaction catalyzed by this enzyme involves the oxidation of G6P coupled with the reduction of NAD\(^{+}\) to form an intermediate, myo-2-inosose-1-phosphate, which is reduced to the product MI1P through being coupled with the oxidation of the previously formed NADH\(^{26}\). The cofactor NAD\(^{+}\)-NADH has been shown to be localized in the Rossmann-fold domain of MI1PS, a domain which is conserved throughout the evolutionary history of this family of enzymes\(^{37}\). It has been suggested that NAD\(^{+}\)-NADH is captured within the enzyme during its translation and passively involved in the folding process of the protein\(^{28}\). In this study, we decide to employ *iolI* of *M. tuberculosis*, since this gene is known to function efficiently in *M. tuberculosis*, and the enzyme it encodes has been well characterized, both biochemically and structurally\(^{23}\). Therefore, once the enzyme had been produced in *B. subtilis*, it was intended to function as MI1PS. However, despite the detection of specific MI1PS mRNA and protein in strain MC001 (Fig. 2), no specific activity was detected (Fig. 3a). However, when *pbuE* was inactivated in *B. subtilis*, MI1PS became active as a functional enzyme (Fig. 3a). *B. subtilis* *pbuE* encodes a purine base/nucleoside efflux pump, which is induced when intracellular concentrations of purine bases increase\(^{38}\) and thus helps to maintain the homeostasis of purine bases/nucleosides in cells, although it remains unclear as to why intracellular levels of purine bases/nucleosides are kept low under normal conditions. The inactivation of *pbuE* might increase the intracellular pool of purine bases/nucleosides, which could then be used for the biosynthesis of NAD\(^{+}\)-NADH (Fig. 3b). Previously, the apparent K\(_{D}\) value for NAD\(^{+}\) binding to *M. tuberculosis* MI1PS was reported to be 36 ± 4 µM, which is much weaker than values reported for other, similar enzymes characterized from *Archaeoglobus fulgidus* (1 µM) and *Arabidopsis thaliana* (≈0.2 µM)\(^{25}\). In addition, *B. subtilis* cell extracts, in which *M. tuberculosis* MI1PS encoded by *iol1Mt* was expressed with active *pbuE*, exhibited no specific activity in *vitro* even in the presence of 10 mM NAD\(^{+}\) (Fig. 3a). It is likely that under natural conditions in *B. subtilis* the level of NAD\(^{+}\)-NADH is too low to make *M. tuberculosis* MI1PS functional or to stabilize it as an active enzyme.

It has previously been reported that the MI1PS reaction could be the rate-limiting step in natural myo-inositol biosynthesis\(^{31}\). In addition, we found that MI1PS became active in *B. subtilis* only when the intracellular level of NAD\(^{+}\)-NADH was elevated. Therefore, one of the key elements for improved scylo-inositol production is the performance of MI1PS. It was only recently that an uncharacterized *iolI* was found in the genome of a strain of *Bacillus thuringiensis*\(^{25}\). Although nothing is known about its inositol biosynthesis, this bacterium is one of the closest relatives of *B. subtilis*, and this enzyme might therefore be more suitable in *B. subtilis* than the one from *M. tuberculosis*. On the other hand, it was reported that Mck1, a homolog of glycogen synthase kinase 3, could act as a novel positive regulator of *de novo* myo-inositol synthesis in *S. cerevisiae*\(^{35}\), suggesting that there might be other such additional factors required to make the enzyme function properly.

In the version of a *B. subtilis* cell factory for the production of scylo-inositol from glucose reported here, scylo-inositol corresponding to 10% of the initial glucose was produced during the later period of an extended culturing process (Fig. 4). These results suggested that 90% of the glucose was used for energy and/or as a carbon source for bacterial growth. In *B. subtilis*, the phosphoenolpyruvate-dependent phosphotransferase system is the major mechanism for the uptake of glucose, importing glucose as G6P, while another mechanism, involving the glucose transporter GlcP and glucose kinase GlcK, is thought to play a minor role\(^{29,30}\). Therefore, we tried to enhance the latter mechanism to feed more G6P by overexpressing both *glcP* and *glcK* simultaneously and constitutively (Fig. 1). Although the results indicated accelerated scylo-inositol production, the final
concentration of scyllo-inositol did not substantially increase (Fig. 4). It is likely that the major metabolic pathways, such as glycolysis and the pentose phosphate pathway, are so efficient in G6P consumption that only a limited amount of G6P is available for conversion into scyllo-inositol. In order to redirect more G6P to scyllo-inositol production, we plan to manipulate the two key enzymes that metabolize G6P: Pgi, which converts G6P into fructose-6-phosphate in glycolysis, and Zwf, which transforms G6P into D-glucono-1,5-lactone-6P in the pentose phosphate pathway. However, since the manipulation of these two genes could possibly disturb the holistic metabolism of the cell system, we will need to first devise a proper strategy to avoid such problems.

To conclude, in this study we have demonstrated the first example of a bacterial cell factory for the production of scyllo-inositol from glucose, by coupling the process by which glucose is converted into myo-inositol with the previously established process of converting myo-inositol into scyllo-inositol. To enable the former process, pbuE had to be inactivated to elevate the intracellular concentration of NAD+·NADH and activate MIP5 functional. At the same time, glucose is sold at the price about USD 0.45-0.60 per kg, while myo-inositol is at USD 10.00-30.00 per kg. The bacterial cell factory created in this study gave a production efficiency about 2 g L−1 of scyllo-inositol from 20 g L−1 of glucose, and the produced scyllo-inositol would not be cheaper than USD 4.50-6.00 per kg considering the material cost. On the other hand, one of the most efficient cell factories previously created was capable of producing 27.6 g L−1 of scyllo-inositol from 50 g L−1 of myo-inositol13. In this case, the produced scyllo-inositol would be more expensive than USD 18.12-54.35 per kg. Therefore, the new cell factory possibly makes the scyllo-inositol production at least 3-times cheaper than the previous ones, which could be improved in our future studies involving the strategies mentioned above. scyllo-inositol is an important compound that will help us to challenge the growing problem of Alzheimer’s disease, and our bacterial cell factory will ensure an inexpensive way to produce this rare compound.

Methods

Bacterial strains, culture conditions, and primers. Bacterial strains and oligonucleotide primers used in this study are listed in Supplementary Tables 1 and 2, respectively. Bacterial strains were maintained in lysogeny broth (LB) medium38, USA, 0.5% (w/v) Bacto yeast extract (Becton, Dickinson and Co.), and 20 g L−1 glucose.

Construction of bacterial strains. B. subtilis 168 was our standard strain. Strains MY104, TM3031, and KU302 were constructed as previously described15,18,39. Strain YDHLd [pbuE::mutuin2 erm] was obtained from the National Bioresource Project, National Institute of genetics, Mishima, Japan. Strain KS001 was constructed from MY104 using the marker-free deletion technique, as follows39 (Supplementary Fig. 3). Four PCR fragments, including fragments A, B, C, and the mazF cassette were prepared. Fragment A corresponds to upstream of the sigG region as the target of deletion. Fragment B downstream of the deletion target. Fragment C inside the deletion, and the mazF cassette contained mazF for a suicidal toxin under the control of an IPTG-inducible promoter (Ppac) regulated by lacI and a spectinomycin-resistance gene. Fragments A, B, and C were amplified from genomic DNA of strain 168 by PCR using the primer pairs DioGAF/DioGAR, DioGFB/DioGCR, and DioGCF/DioGCC (Supplementary Table 2), respectively, and the mazF cassette was amplified from DNA of TM3031 with MazF/MazFr39-41. These four fragments, A, B, the mazF cassette, and C were ligated in this order by PCR using the primer pair DioGAF/DioGCC. The ligated PCR product was used to transform strain MY104 into a spectinomycin-resistant pop-in mutant with integration of the PCR product through homologous recombination within the regions corresponding to fragments A and C. The spectinomycin-resistant transformants obtained in this way were grown in the absence of spectinomycin and then screened on IPTG-containing plates for spectinomycin-sensitive mutants, which could appear following intrachromosomal recombination between the two direct-repeat regions. To fragment B corresponding to the mazF cassette as the target of deletion. One of the spectinomycin-sensitive mutants was selected to have the desired marker-free deletion of sigG, to yield strain K5001. Strain TK001 was constructed as follows. Three PCR fragments, D, E, and F were prepared. Fragment D, containing the C-terminal region of the amyE locus, a chloramphenicol resistance gene (erm) and the promoter of the rpsO gene (rpsO promoter), was amplified from strain 168 DNA by PCR using the primer pair AmyAF/AmyBR. Fragment E, containing the N-terminal region of the amyE locus, was amplified from DNA of strain 168 by PCR using the primer pair AmyBF/AmyBB. Fragment F, containing the 5′-end of M. tuberculosis (ino1Mt) codon-optimized scyllo-inositol gene (https://mymicrobrowser.epfl.ch/genes/Rv00046c), was amplified from custom-made synthetic DNA (Eurofins Scientific, Brussels, Belgium) by PCR using the primer pair inol1F/inol1B to generate the ribosome-binding site of rpsO and the tufA terminator of B. subtilis, flanking the head and tail, respectively (Supplementary Fig. 1). The three fragments D, E, and F were ligated in this order by PCR using the primer pair AmyAF/AmyBB. The ligated PCR product was used to transform strain 168, making it resistant to chloramphenicol, and resulting in strain TK001, whose correct construction was confirmed by DNA sequencing.

Strain TK002 was constructed as follows. Two PCR fragments, G and H, were prepared. Fragment G, containing the C-terminal region of the amyE locus, the chloramphenicol resistance gene, andrpsO, was codon-optimized inol1 gene fused with a His-tag (His6) in the C-termius (ino1Mt-His6), was amplified from TK001 by PCR using the primer pair AmyAF/inol1hi. Fragment H, containing the His-tag and the the tufA terminator followed by the N-terminal region of amyE, was amplified using the primer pair liasthen/AmyBB from TK002 DNA. The two fragments, G and H, were ligated by PCR using the ligated PCR product and the primers AmyAF/AmyBB. The ligated PCR product was also used to transform strain 168, making it resistant to chloramphenicol, and resulting in strain TK002, whose correct construction was confirmed by DNA sequencing.

Strain TK003 was constructed as follows. Three PCR fragments, I, J, and K were prepared. Fragment I, containing the C-terminal part of amyE and the chloramphenicol resistance gene, was amplified from KU302 DNA using the primer pair AmyAF/AmyBR. Fragment J, containing the ribosome-binding site of rpsO, the inol1Mt-His6 gene, the tufA terminator, and the N-terminal part of AmyE was amplified using the primer pair inol1F/AmyBB from TK002 DNA. Fragment K, which contains the promoter of pbuE and the terminator of B. subtilis, was amplified from DNA of strain 168 using the primer pair PbuyF/PbuyKBB. The three fragments I, J, and K were ligated in this order by PCR using the primer pair AmyAF/AmyBB. The ligated PCR product was used to transform strain 168, making it resistant to chloramphenicol, and resulting in strain TK003, whose correct construction was confirmed by DNA sequencing.

Strain MC021 was constructed as follows. Three PCR fragments, L, M, and N were prepared. Fragment L, containing the C-terminal region of the pbuE locus, was amplified from DNA of strain 168 by PCR using the primer pair pbuEAF/pbuEBF. Fragment M, which corresponds to the N-terminal region of the pbuE locus, was amplified from DNA of strain 168 using the primer pair pbuEF/pbuEBB. Fragment N, containing the chloramphenicol resistance gene and the inol1Mt-His6 gene, was amplified from TK002 using the primer pair cmino1F/cmino1B. The three fragments L, M, and N were ligated in this order by PCR using the primer pair pbuEA/pbuEBB. The ligated PCR product was used to transform strain 168, making it resistant to chloramphenicol and resulting in strain MC021, whose correct construction was confirmed by DNA sequencing.

Strain MC030 was constructed as follows. Six PCR fragments, O, P, Q, R, S, and T were prepared. Fragment O, containing the C-terminal region of the epr locus, was amplified from DNA of strain 168 by PCR using the primer pair EpraF/EpraB. Fragment P, containing PpraO, was amplified from TK002 using the primer pair PpraOA/PpraOB. Fragment Q, containing gplK, was amplified from DNA of strain 168 using the primer pair GlcKF/GlckB. Fragment R, containing gplK, was amplified from DNA of strain 168 using the primer pair PpraOA/PpraOB. Fragment S, containing the bluelocus, was amplified from DNA of strain 168 using the primer pair pbuEAF/pbuEBF. Fragment T, containing the N-terminal part of epr, was amplified from DNA of strain 168 using the primer pair EpraF/EpraB. The six fragments O, P, Q, R, S, and T were ligated by PCR using the primer pair EpraF/EpraB and used to transform strain 168, making it resistant to chloramphenicol and resulting in strain MC030, whose correct construction was confirmed by DNA sequencing.

The genetic elements constructed in the above mutant strains were combined in various combinations, as follows. Strain MC001 [ΔiolABCΔCDGFIHJ ΔiolX Δiolr amyE::(Pprep-o1molMt-His6 cat)] was made from K5001 transformed to be resistant to chloramphenicol by using the primer pair AmyAF/AmyBR. Strain K5001 [ΔiolABCΔCDGFIHJ ΔiolX Δiolr amyE::(Pprep-o1molMt-His6 cat)] was made from K5001 transformed to be resistant to chloramphenicol, erythromycin by using YDHLd DNA. Strain MC011 [ΔiolABCΔCDGFIHJ ΔiolX Δiolr amyE::(Pprep-o1molMt-His6 cat)] was made from MC001 transformed to be resistant to chloramphenicol, erythromycin by using YDHLd DNA. Strain MC020 [ΔiolABCΔCFDΔHIJΔiolX Δiolr amyE::(Pprep-o1olMt-wt-Iol cat)] was made from MC010 transformed to be resistant to chloramphenicol, erythromycin by using YDHLd DNA. Strain MC022 [ΔiolABCΔCFDΔHIJΔiolX Δiolr amyE::(Pprep-o1olMt-wt-Iol cat)] was made from MC020 transformed to be resistant to chloramphenicol by using MC021 DNA. Strain MC031 [ΔiolABCΔCFD].
Western blot analysis. B. subtilis strains were grown in Sotyone medium at 37 °C with shaking. Bacterial cells were harvested when the cultures reached 50 units at OD600 and washed three times with cold lysis buffer containing 20 mM Tris/HCl (pH 8), 10 mM NaCl, 10 mM EGTA, 5 mM EDTA, and 50 mM 2-mercaptoethanol, then stored at −80 °C. The cells were suspended in 10 mL lysis buffer containing 100 μL Halt Protease Inhibitor Single-Use Cocktail (Thermo Fisher Scientific, Waltham, MA, USA) and 35 μL 2-mercaptoethanol, then disrupted by three passages at 120 bar in Avestin Emulsiflex B15 cell disruptor (ATC Scientific, New South Wales, Australia). Following centrifugation, supernatants were mixed with 1% (w/v) proteamine sulfate to precipitate nucleic acids. After further centrifugation, supernatants were subjected to 12% PAGE and the proteins separated in the gel were transferred to an iBlot PVDF membrane (Thermo Fisher Scientific, Waltham, MA, USA), where they were subsequently reacted with 2,000-times diluted HRP substrate (Takara Bio, Shiga, Japan) and visualized with ChemiDoc (Bio-Rad, Hercules, CA, USA).

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Author contributions

K.Y. conceived the study and wrote the final manuscript. C.M. designed and performed the experiments. C.K., S.K., and K.T. contributed to the construction of the bacterial strains. All authors were involved in drafting the manuscript, and S.I. contributed to the critical reading and revising of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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