A second-generation *Bacillus* cell factory for rare inositol production

Kosei Tanaka¹, Shinji Takanaka¹,², and Ken-ichi Yoshida¹,², *

¹Organization of Advanced Science and Technology; Kobe University; Kobe, Japan; ²Department of Agrobioscience; Graduate School of Agricultural Science; Kobe University; Kobe, Japan

Some rare inositol stereoisomers are known to exert specific health-promoting effects, including scylo-inositol (SI), which is a promising therapeutic agent for Alzheimer disease. We recently reported a *Bacillus subtilis* cell factory that performed the efficient production of SI from the cheapest and most abundant isomer myo-inositol (MI). In the cell factory all “useless” genes involved in MI and SI metabolism were deleted and overexpression of the key enzymes, IolG and IolW, was appended. It converted 10 g/L MI into the same amount of SI in 48 h of cultivation. In this addendum, we discuss further improvement in the cell factory and its possible applications.

**Introduction**

There are nine natural stereoisomers of inositol (1,2,3,4,5,6-cyclohexanehexol). One of the stereoisomers, scylo-inositol (SI) is rare but has been regarded as a promising therapeutic agent for Alzheimer disease.¹ In fact, SI has received a fast-track designation from the US Food and Drug Administration for the treatment of mild to moderate Alzheimer disease.²

*Bacillus subtilis* possesses two additional inositol dehydrogenases, IolX and IolW, which act specifically on SI with NAD⁺ and NADP⁺ reduction, respectively, to convert it to scylo-inosose.³ In vivo and in vitro analyses have revealed that IolX plays the major physiological role in SI catabolism, whereas IolW efficiently reduces scylo-inosose into SI with oxidation of NADPH.⁴ Scylo-inosose is metabolized sequentially in multiple steps involving the IolE, IolD, IolB, IolC, IolJ, and IolA enzymes to give common intermediates, dihydroxyacetone phosphate and acetyl-CoA, which enter glycolysis and the TCA cycle, respectively.⁵

We developed a *Bacillus subtilis* cell factory to produce SI, where MI was chosen as a starting material that is abundant in nature and cheaply available from rice bran. The first generation of the cell factory was realized in strain TM039, in which three genes including iolR, iolE, and iolI were disrupted and a missense mutation, iolE41, was introduced. Those modifications were introduced to enable the constitutive expression of the iolABCDEFGHJ operon, including iolG, elimination of SI dehydrogenase activity and inactivation of both isomerization and dehydration of the key intermediate scylo-inosose, and thus the accumulating scylo-inosose was expected to be converted readily to SI. In fact, nearly half of the initial MI was converted to SI after 72 h.
of cultivation, but the strain was found to consume and waste the other half of MI.2

**The Second-Generation *B. subtilis* Cell Factory**

The first-generation *B. subtilis* cell factory wasted half of the MI starting material. To solve this problem, *iolABCDEF_HIJ, iolX, and iolR* were deleted from the chromosome using a marker-free deletion technique6 to yield strain MYI04.7 As expected, MI was not wasted in MYI04, but the conversion rate of MI to SI was not improved. We accordingly attempted the overexpression of *iolG* and *iolW*, which encode the key enzymes for the conversion, under the control of strong and constitutively active promoters, even during stationary growth; the promoters were chosen from global *B. subtilis* transcriptome analysis data.8 Finally, strain KU106, in which *iolG* and *iolW* were simultaneously overexpressed under the control of the *rpsO* promoter, completely converted 10 g/L MI into the same amount of SI in 48 h of cultivation.7

**Aiming at Further Improvement of the Cell Factory**

In our preliminary attempt, the conversion was performed with 50 g/L MI; the resulting amount of SI produced in the medium was no more than 12 g/L. This amount of SI in the medium already exceeds its saturation point in water, but it does not have any cytotoxicity because KU106 was able to grow even in the presence of 20 g/L of SI. The results suggested that other unknown factors could limit conversion capacity. One such factor might be the transport of the substrate and product across the plasma membrane. IolT is responsible for uptake of MI9,10 and is constitutively expressed in KU106. To test the possible effect of enhanced MI import, we gave it a shot to overexpress *iolT* but found no obvious elevation in the conversion so far. However, we do not know how SI is secreted. Identification of the SI exporter is currently under investigation, and once such a gene is found, its overexpression will be attempted.

**Cofactors Required for Inositol Conversion**

The cofactors required for inositol dehydrogenases possibly play important roles in efficient conversion. In the SI-producing cell factory, MI is once converted to *scyllo*-inosose with NAD+ reduction, and then *scyllo*-inosose is reduced to SI with NADPH oxidation (Fig. 1). NAD+ is supposed to be easily regenerated by respiration, given that *B. subtilis* is a typical aerobic bacterium. However, given that *B. subtilis* is believed to have no transhydrogenase, NADPH must be regenerated by direct reduction of NADP+.11 In the previous study, we showed that the reduction of *scyllo*-inosose catalyzed by IolW requiring NADPH could be the most important step in SI conversion and also that the reduced amounts of the major nutrient Bacto soytone drastically reduced SI conversion.7 The results suggested that higher concentrations of Bacto soytone might stimulate NADPH regeneration. In addition, our preliminary observation suggested that inactivation of some of NADPH regenerating enzymes could result in decreased SI conversion even in the presence of higher concentrations of Bacto soytone. We are currently investigating the mechanism underlying this phenomenon. The regeneration of NADPH would be artificially and efficiently controlled, for instance, by enhancing the expression levels of NADPH regenerating enzymes. This might thus be applied not only to SI conversion but also to the production of...
other valuable chemicals, such as alcohols and fatty acids, which usually requires reaction steps driven by reducing power of NADPH and/or NADH.

### Possible Application to Producing Other Inositols

Not only SI but also some of other rare inositol stereoisomers are known to exert specific and useful biological activity. For example, DCI is claimed for its functional role in insulin action and its deficit in insulin resistance. As described above, B. subtilis IolG can act on MI and DCI but not on SI, whereas IolW and IolX act on SI, indicating the differential substrate specificities of inositol dehydrogenases. Within the B. subtilis genome are found additional iolG homologs, namely yfil, ndtC, yrbE, yteT, and yulF, and some of their gene products may exhibit inositol dehydrogenase activity. We also characterized three inositol dehydrogenases of Geobacillus kaustophilus, which exhibited mutually differing substrate specificities. This differential substrate specificity indicates that every inositol dehydrogenase has its own preference for dehydrogenation of the secondary alcohol at a specific position of an inositol isomer to generate a ketone group at the same position. Furthermore, there are inosose isomerases that can move the ketone group to the next position, similar to the IolI enzyme. We are currently collecting possible inositol dehydrogenases and inosose isomerases from databases and natural isolates.

Those enzyme genes are to be cloned in combination into the B. subtilis cell factory platform similarly to iolG and iolW, with the aim of identifying combinations that enable the conversion of MI to other rare inositol isomers.

### SI Production from Raw Materials

Phytic acid (MI-1,2,3,4,5,6-hexaphosphate) is the principal storage form of phosphorus in many plant tissues, in particular bran and seeds. Phytase is a special class of phosphatases that catalyze the sequential hydrolysis of phytic acid to liberate MI and phosphate. Because B. subtilis has high ability to secrete enzymes, the B. subtilis cell factory could be modified to secrete phytase, possibly enabling SI production from waste materials such as rice bran rich in phytic acid.

It is known that MI is synthesized from glucose-6-phosphate in two steps. Glucose-6-phosphate is the starting compound of glycolysis, appearing when glucose is incorporated into the cell via the phosphotransferase system. Glucose-6-phosphate is converted by inositol-3-phosphate synthase to MI 1-phosphate, which is then dephosphorylated by inositol monophosphatase to yield MI. We have already cloned a heterogenous inositol-3-phosphate synthase gene from Mycobacterium tuberculosis and have also identified ytkC encoding endogenous inositol monophosphatase in B. subtilis (details of these results will be reported elsewhere). If these two enzyme genes could be efficiently expressed in the future, it might be possible to develop a novel cell factory producing SI from glucose, sucrose, or starch.

### Conclusion

SI is a promising therapeutic agent for Alzheimer disease. Here we discussed that the B. subtilis cell factory converting MI into SI could have a potential to be improved further. Our previous study suggested that SI conversion might depend on NADPH-regeneration capacity in the cell, which seemed to be stimulated in the presence of higher concentration of Bacto soytone, and we are currently investigating the mechanism underlying this phenomenon. Applying the B. subtilis cell factory concept, we might be able to produce not only SI but also other rare inositol stereoisomers from raw materials in the future.

#### Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

#### Acknowledgments

This work was financially supported by the Ministry of Education, Culture, Sports, Science, and Technology, Japan, in part by Special Coordination Funds for Promoting Science and Technology, Creation of Innovative Centers for Advanced Interdisciplinary Research Areas; by the Advanced Low-Carbon Technology Research and Development Program; by KAKENHI; and by Grants-in-Aid from the NC-CARP project. The authors thank Enago (http://www.enago.jp) for English language review.
10. Morinaga T, Matsuse T, Ashida H, Yoshida K. Differential substrate specificity of two inositol transporters of Bacillus subtilis. Biosci Biotechnol Biochem 2010; 74:1312-4; PMID:20530884; http://dx.doi.org/10.1271/bbb.100125

11. Lerondel G, Doan T, Zamboni N, Sauer U, Aymerich S. YraJ has the major physiological role of the four paralogous malic enzyme isoforms in Bacillus subtilis. J Bacteriol 2006; 188:4727-36; PMID:16788182; http://dx.doi.org/10.1128/JB.00167-06

12. Larner J. D-chiro-inositol--its functional role in insulin action and its deficit in insulin resistance. Int J Exp Diabetes Res 2002; 3:47-60; PMID:11900279; http://dx.doi.org/10.1080/15604280212528

13. Shaltiel G, Dalton EC, Belmaker RH, Harwood AJ, Agam G. Specificity of mood stabilizer action on neuronal growth cones. Bipolar Disord 2007; 9:281-9; PMID:17430303; http://dx.doi.org/10.1111/j.1399-5618.2007.00400.x

14. Yoshida K, Sanbongi A, Murakami A, Suzuki H, Takenaka S, Takami H. Three inositol dehydrogenases involved in utilization and interconversion of inositol stereoisomers in a thermophile, Geobacillus kaustophilus HTA426. Microbiology 2012; 158:1942-52; PMID:22609753; http://dx.doi.org/10.1099/mic.0.059980-0

15. Oh BC, Choi WC, Park S, Kim YO, Oh TK. Biochemical properties and substrate specificities of alkaline and histidine acid phytases. Appl Microbiol Biotechnol 2004; 63:362-72; PMID:14586576; http://dx.doi.org/10.1007/s00253-003-1345-0

16. Ragon M, Aumelas A, Chemard M, Galvez S, Moulin G, Boze H. Complete hydrolysis of myo-inositol hexakisphosphate by a novel phytase from Debaryomyces castellii CBS 2923. Appl Microbiol Biotechnol 2008; 78:47-53; PMID:18046551; http://dx.doi.org/10.1007/s00253-007-1275-3

17. Escobin-Moqua L, Ohrahi M, Sekiguchi S, Sone T, Abe A, Tanaka M, Meervootisom V, Asano K. Purification and characterization of phytase from Klebsiella pneumoniae 9-5B. J Biosci Bioeng 2012; 113:562-7; PMID:22344916; http://dx.doi.org/10.1016/j.jbiosc.2011.12.010

18. Antelmann H, Tjalsma H, Voigt B, Ohlmeier S, von Dijl JM, Hecker M. A proteomic view on genome-based signal peptide predictions. Genome Res 2001; 11:1484-502; PMID:11544912; http://dx.doi.org/10.1101/gr.182801

19. van Dijl JM, Braun PG, Robinson C, Quax WJ, Antelmann H, Hecker M, Muller J, Tjalsma H, Bron S, Jongbloed JD. Functional genomic analysis of the Bacillus subtilis Tat pathway for protein secretion. J Biotechnol 2002; 98:243-54; PMID:12141990; http://dx.doi.org/10.1016/S0168-1656(02)00135-9

20. Reizer J, Saier MH Jr., Deutscher J, Grenier F, Thompson J, Hengstenberg W. The phosphoeneolpyruvate:sugar phosphotransferase system in gram-positive bacteria: properties, mechanism, and regulation. Crit Rev Microbiol 1988; 15:297-338; PMID:3060316; http://dx.doi.org/10.3109/10408418801904461

21. Movahedzadeh F, Smith DA, Norman RA, Dina-davala P, Murray-Rust J, Russell DG, Kendall SL, Rison SC, McAlister MS, Bancroft GJ, et al. The Mycobacterium tuberculosis ino1 gene is essential for growth and virulence. Mol Microbiol 2004; 51:1003-14; PMID:14763976; http://dx.doi.org/10.1046/j.1365-2958.2003.03900.x