A cell factory of *Bacillus subtilis* engineered for the simple bioconversion of *myo*-inositol to *scyllo*-inositol, a potential therapeutic agent for Alzheimer’s disease

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Abstract

**Background:** A stereoisomer of inositol, *scyllo*-inositol, is known as a promising therapeutic agent for Alzheimer’s disease, since it prevents the accumulation of beta-amyloid deposits, a hallmark of the disease. However, this compound is relatively rare in nature, whereas another stereoisomer of inositol, *myo*-inositol, is abundantly available.

**Results:** *Bacillus subtilis* possesses a unique inositol metabolism involving both stereoisomers. We manipulated the inositol metabolism in *B. subtilis* to permit the possible bioconversion from *myo*-inositol to *scyllo*-inositol. Within 48 h of cultivation, the engineered strain was able to convert almost half of 10 g/L *myo*-inositol to *scyllo*-inositol that accumulated in the culture medium.

**Conclusions:** The engineered *B. subtilis* serves as a prototype of cell factory enabling a novel and inexpensive supply of *scyllo*-inositol.

**Background**

Alzheimer’s disease is one of the most common and problematic forms of dementia [1]. In 2006, 26.6 million people suffered from Alzheimer’s disease worldwide, and by 2050, the disease is predicted to increase, affecting 1 in every 85 individuals [2]. Hundreds of clinical trials have been conducted to identify possible treatment strategies for the disease, but despite these efforts no promising strategy has been established. At present, most of the treatment strategies currently used offer only a small symptomatic benefit, and in fact no treatment is available to stop the progression of the disease.

Inositol (1,2,3,4,5,6-cyclohexanehexol) has nine possible stereoisomers due to epimerization of the six hydroxyl groups. The most abundant stereoisomer in nature is *myo*-inositol (compound 1, Figure 1), which is the structural basis for a number of secondary messengers in eukaryotic cells [3]. In plants, *myo*-inositol hexakisphosphate or phytic acid often occurs in high bran cereals, serving as the principal storage form of phosphate. This is the main industrial source of *myo*-inositol [4]. The other inositol stereoisomers are relatively rare in nature but some exert specific health-promoting activities. D-*chiro*-inositol (compound 2) is beneficial for patients with hyperglycemia and polycystic ovary syndrome since it restores insulin sensitivity [5] and recovers normal ovulation [6], respectively. Accumulating evidence indicates that another isomer, *scyllo*-inositol (compound 3), has exceptional potential as a therapeutic agent for Alzheimer’s disease [7].

*scyllo*-Inositol is sometimes found in plants and also occurs naturally in human brain as a minor substance that readily crosses the blood-brain barrier [8]. Aggregation of the beta-amyloid peptide in brain cells is a key pathological feature of Alzheimer’s disease [9]. *scyllo*-Inositol directly interacts with the beta-amyloid peptide and blocks the development of fibers, alleviating memory deficits and other symptoms associated with beta-amyloid accumulation [10,11]. Furthermore, mice fed with *scyllo*-inositol have been demonstrated to have
myo-inositol to scyllo-inositol and its inactivation inhibits cell growth in the presence of the metabolic pathway described above [18]. Phase 2 clinical trials of scyllo-inositol treatment have already been conducted with positive results, and plans for Phase 3 trials have been announced [13].

*Bacillus subtilis* is one of the best studied Gram-positive bacteria, and its unique inositol metabolism involves at least three of the stereoisomers, *myo*-inositol, *scyllo*-inositol, and *D*-chiro-inositol (Figure 1) [14]. The *B. subtilis* *iolABCDEFGH*I operon encodes enzymes involved in multiple steps of inositol metabolism [15]. In the first step, *myo*-inositol is converted to *scyllo*-inosose (compound 4, Figure 1) by *myo*-inositol dehydrogenase, *iolG*, with NAD⁺ reduction. *iolG* also dehydrogenates *D*-chiro-inositol to 1-keto-*D*-chiro-inositol (compound 5), which is subsequently isomerized by *iolI* to *scyllo*-inosose [16]. In the second step, *scyllo*-inosose is dehydrated by *iolE* to 3D-(3,5/4)-trihydroxycyclohexane-1,2-dione (compound 6) [17]. In the third step, *iolD* catalyzes the hydrolysis of 3D-(3,5/4)-trihydroxycyclohexane-1,2-dione to yield 5-deoxy-*D*-glucuronic acid (compound 7). In the next step, *iolB* catalyzes the isomerization of 5-deoxy-*D*-glucuronic acid to 2-deoxy-5-keto-*D*-gluconic acid (compound 8), which is subsequently phosphorylated by *iolC* kinase to 2-deoxy-5-keto-*D*-gluconic acid 6-phosphate (compound 9) [18]. 2-Deoxy-5-keto-*D*-gluconic acid 6-phosphate is an intermediate that acts as an inducer by antagonizing DNA binding of IolR, a repressor controlling transcription of the *iol* operon [19]. Finally, *iolI* aldolase catalyzes the conversion of 2-deoxy-5-keto-*D*-gluconic acid 6-phosphate to dihydroxyacetone phosphate (compound 10) and malonic semialdehyde (compound 11). The former is a glycolytic intermediate and the latter is converted to acetyl-CoA (compound 12), which enters the tricarboxylic acid cycle, and CO₂ by the IolA reaction.

*B. subtilis* possesses two additional and distinct inositol dehydrogenases, *iolX* and *iolW*, which specifically act on *scyllo*-inositol with NAD⁺ and NADP⁺ reduction, respectively [14]. Each of these enzymes convert *scyllo*-inositol to *scyllo*-inosose, which is the same compound produced from *myo*-inositol by *iolG* and is readily degraded further via the metabolic pathway described above [18]. *iolX* transcription is induced after the addition of *scyllo*-inositol to the growth medium of cells, and its inactivation inhibits cell growth in the presence of the metabolic pathway described above [18].

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**Figure 1** Inositol metabolism in *B. subtilis*. *B. subtilis* *iol* genes encoding enzymes for reactions in the inositol catabolic pathway and the respective intermediate compounds are shown. The intermediate compounds are *myo*-inositol (compound 1), *D*-chiro-inositol (compound 2), *scyllo*-inositol (compound 3), *scyllo*-inosose (compound 4), 1-keto-*D*-chiro-inositol (compound 5), 3D-(3,5/4)-trihydroxycyclohexane-1,2-dione (compound 6), 5-deoxy-*D*-glucuronic acid (compound 7), 2-deoxy-5-keto-*D*-glucanic acid 6-phosphate (compound 8), dihydroxyacetone phosphate (compound 9), malonic semialdehyde (compound 11), and acetyl-CoA (compound 12).
of scyllo-inositol as a carbon source, indicating that IolX could function physiologically as a catabolic enzyme [14]. In contrast, iolW transcription is constitutive and its inactivation does not alter the cell growth in the presence of scyllo-inositol. Our previous study suggested that IolW may generate scyllo-inositol from scyllo-inosose with NADPH oxidation, although its physiological role remains unidentified [14].

In this study, we manipulated the inositol metabolism in B. subtilis to control the interconversion among inositol stereoisomers secreted into the culture medium, permitting a simple and inexpensive bioconversion of myo-inositol to scyllo-inositol. The engineered B. subtilis materialized our cell factory concept for scyllo-inositol production.

Results and Discussion

Analyzing mutations for efficient bioconversion of myo-inositol to scyllo-inositol

Previously we demonstrated that B. subtilis strain YF256 (Table 1) was capable of bioconverting myo-inositol to D-chiro-inositol [16]. Because of iolR inactivation [19], strain YF256 constitutively expresses iolG, iolE41, and iolI. The missense mutation allele iolE41 abolishes iolE dehydratase activity, resulting in the intracellular accumulation of scyllo-inosose from myo-inositol due to the IolG reaction [16]. scyllo- Inosose is isomerized by IolI to 1-keto-D-chiro-inositol and subsequently reduced by IolG to D-chiro-inositol; thus, myo-inositol is converted to D-chiro-inositol, which then appears in the inositol bioconversion medium [16].

In this study, we re-examined the medium in which strain YF256 was cultured for 24 h by an improved GC-TOFMS system [20]. We were surprised to detect not only D-chiro-inositol but also scyllo-inositol (Figure 2A), thus revealing that our previous high-performance liquid chromatography analysis failed to detect scyllo-inositol [16]. Furthermore, this result suggested that some of the accumulated scyllo-inosose could be converted to scyllo-inositol, probably involving IolX and/or IolW.

When iolI was inactivated (strain TM038, Figure 2B), conversion of scyllo-inosose to D-chiro-inositol decreased to a negligible level, whereas that of scyllo-inositol increased; however only slightly. These results indicated that we could eliminate the metabolic pathway for D-chiro-inositol by inactivating iolI, although this was not sufficient to increase the conversion to scyllo-inositol. Therefore, iolX and/or IolW were inactivated to examine their involvement in scyllo-inositol production. When iolW was functional, iolX inactivation alone drastically elevated the concentration of scyllo-inositol present in the medium (strain TM039, Figure 2C), suggesting that IolX may degrade scyllo-inositol. In contrast, IolW was essential for efficient scyllo-inositol production (strains TM040 and TM041, Figure 2D and 2E, respectively). These results clearly indicate that IolW is crucial for the conversion of scyllo-inosose to scyllo-inositol.

Properties of the scyllo-inositol producing B. subtilis cell factory

After 48-h culture of strain TM039 in the inositol bioconversion medium (Figure 3A), almost half of the myo-inositol was converted to scyllo-inositol. This simple bioconversion was so successful that it realized our concept of cell factory to produce scyllo-inositol with higher translational potential in the yield and purity of product than the other methods previously described [21,22]. Further culture resulted in the consumption of not only myo-inositol but also some of the scyllo-inositol, and particularly myo-inositol was consumed almost completely after 96 h of culture whereas scyllo-inositol remained relatively intact. The results implied that the mutated IolE41 enzyme, which did not support cell growth using myo-inositol as the carbon source [17], might possibly retain a certain lower level of scyllo-inosose degrading activity, which resulted in the decrease in the two inositol isomers; because myo-inositol is preferentially converted to scyllo-inosose by IolG reaction, it decreased more obviously. Although we do not have

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Table 1 Bacterial strains used in this study.

| Strain   | Relevant genotype | Source or reference |
|----------|-------------------|---------------------|
| 60015    | Wild-type         | Laboratory stock    |
| BFS3018  | iolX::pMutin2(erm) | National Institute of Genetics [14] |
| FU341    | ansH::spc         | [24]                |
| TM038    | iolE41 iolI::spc iolR::cat | This work |
| TM039    | iolE41 iolI::spc iolR::cat iolX::pMutin4(erm) | This work |
| TM040    | iolE41 iolI::spc iolR::cat iolW::pMutin2(erm::tet) | This work |
| TM041    | iolE41 iolI::spc iolR::cat iolW::pMutin2(erm::tet) iolX::pMutin4(erm) | This work |
| TM043    | iolW::pMutin2(erm::tet) | [14] |
| YF256    | iolE41 iolR::cat   | [17]                |

The integration vector pMutin2 originally possesses the erm marker [26], which was replaced with the tet marker in TM043 as described previously [23].
experimental evidences to prove the possibility mentioned above, nevertheless the selective consumption of myo-inositol could be even practical for a cell factory making the scylo-inositol product free from significant myo-inositol contamination. However, to avoid wastage of both the material and the product, possible strategies to stop the inositol degradation will be considered, including in-frame deletion of iolE.

In principal, we could assume that IolG reaction is due to a lowering of the intracellular NAD\(^+\)/NADH ratio, whereas the IolW reaction elevates the NADP\(^+\)/NADPH ratio (Figure 1). If this is actually the case, the inevitable problem of cofactor imbalance must have been induced in the cells. Although considerably efficient bioconversion has already been achieved (Figure
3A), it may be improved by correcting the possible cofactor imbalance under the given conditions. Interestingly, when the major nutrient (Bacto soytone) contained in the conversion medium was reduced by half, no severe effect on the cell growth was observed, nevertheless only scyllo-inositol production was impaired significantly while myo-inositol was consumed almost completely (Figure 3B). The results imply that there might be some nutritional and/or metabolic factors limiting the bioconversion, which should be identified and then controlled in the next generation of realistic cell factory with optimized efficiency in scyllo-inositol production.

It was demonstrated that *B. subtilis* possesses two distinct inositol transporters, IolT and IolF, which are different in substrate specificity to myo-inositol and D-chiro-inositol [23]. Our preliminary observations suggested that scyllo-inositol transport into the cells could mainly depend on IolT, which is similar to myo-inositol (data not shown), and overexpression of *iolT* may not be effective for improving the conversion efficiency. However, it has not yet been explained how the inositol stereoisomers are secreted into the medium after conversion; hence, we speculate the involvement of an unknown efflux pump, which is also currently being investigated as one of the possible targets to be engineered.

**Conclusions**

*B. subtilis* is one of the best studied gram-positive bacteria. Its unique inositol metabolism has been completely elucidated to involve at least three inositol stereoisomers, including scyllo-inositol and myo-inositol. Our previous knowledge and findings suggested possible interconversion among the stereoisomers. Therefore, we manipulated the metabolic pathway to realize a cell factory concept enabling the simple and inexpensive bioconversion of myo-inositol to scyllo-inositol, which is a promising therapeutic agent for Alzheimer’s disease.

**Methods**

**Bacterial strains and their constructions**

Bacterial strains used in this study are listed in Table 1. The *ioll* gene of strain YF256 was inactivated by inserting a spectinomycin resistance gene (*spc*) cassette as follows. A DNA fragment carrying the *spc* cassette was amplified from strain FU341 DNA [24] by PCR using *IolI3F* and *IolI4R* primer pair (Table 2). Further, two DNA fragments corresponding to the upstream and downstream regions of *ioll* were amplified from strain 60015 DNA by PCR using *IolI1F/IolI2R* and *IolI5F/IolI6R* primer pairs, respectively (Table 2). The 5’ end sequences of *IolI3F* and *IolI4R* were complementary to *IolI2R* and *IolI5F*, respectively. The three PCR fragments were mixed and ligated using PCR performed with the outside primer pair *IolI1F/IolI6R*. The resulting PCR fragment was used to transform strain YF256 to obtain strain TM038 with spectinomycin resistance (*iolI*: *spc*; Table 1).

Strain TM038 was transformed with strain BFS3018 DNA [14] to obtain strain TM039 with erythromycin resistance (*iolIX*: *p*Mutin4(*erm*); Table 1) and with strain TM043 DNA [14] to obtain strain TM040 with tetracycline resistance (*iolIW*: *p*Mutin2(*erm*:*tet*); Table 1). Strain TM040 was further transformed with strain BFS3018 DNA to obtain strain TM041 with erythromycin resistance (*iolIW*: *p*Mutin2(*erm*:*tet*) *iolIX*: *p*Mutin4(*erm*); Table 1).

**Culture conditions**

Bacterial strains were maintained in Luria-Bertani medium [25]. For inositol bioconversion, a 500-ml Sakaguchi flask with a breathable foam stopper, containing 30 ml of inositol bioconversion medium, containing 2% or 1% (w/v) Bacto soytone (Becton, Dickinson and Co., Sparks, MD), 0.5% Bacto yeast extract (Becton, Dickinson and Co.), 0.5% NaCl, and 1% myo-inositol, was inoculated with one of the *B. subtilis* strains at an optical density of 0.05 at 600 nm, and incubated at 37°C with shaking at 150 rpm.

**Measurement of inositol stereoisomers in the medium**

After bioconversion, aliquots of the culture medium were subjected to gas chromatography-time-of-flight mass spectrometry (GC-TOFMS) analysis as described [20] but with some modifications as follows. After removing bacterial cells by centrifugation, an aliquot of the culture medium was evaporated in a test tube under vacuum. The dried pellet was dissolved in the extraction mixture of water, chloroform, and methanol (2:2:5), and incubated at 150°C for 30 min with vigorous shaking. After centrifugation at 40°C for 3 min at 16,000 g, the supernatant was transferred to another tube and diluted appropriately with pure water. After another centrifugation, part of the supernatant was dried completely in another tube. The dried pellet was dissolved, and the substances present in it were derivatized with methoxyamine hydrochloride in pyridine at 30°C for 90 min with

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**Table 2 Oligonucleotide primers used in this study**

| Primer | Sequence |
|--------|----------|
| *IolI1F* | 5’-TGGGGTTGAACCTGAAGTGG-3’ |
| *IolI2R* | 5’-TCTTCTGCTGTCAACAGC-3’ |
| *IolI3F* | 5’-GCTTGTGACAGCAGGAAACATGCTATGGGAG-3’ |
| *IolI4R* | 5’-GAACCATGCTGAAAGTTATGCTCTTGC-3’ |
| *IolI5F* | 5’-CAGTCGAGCAGTGGTC-3’ |
| *IolI6R* | 5’-ATATTGATCTCCGCTGGCC-3’ |
vigorously shaken. After the addition of N-methyl-N-(tri-
methyisilsilyl) trifluoroacetamide (GL Science, Tokyo, 
Japan), the derivatized substances were further incu-
bated at 37°C for 30 min, and then injected via an Agi-
lent 7683B autosampler into an Agilent 7890A GC 
(Agilent Technologies, Palo Alto, CA) coupled to a 
Pegasus HT TOFMS (Leco Corp., St Joseph, MI) with 
the settings as described [20]. Peak deconvolution iden-
tification and quantification were performed using the 
Pegasus ChromaTOF software package ver. 4.21 (Leco 
Corp.). Commercially available authentic inositols were 
derivatized and analyzed in parallel with the experimen-
tal samples. The mass spectra and retention time 
time were used to identify the stereoisomers, and 
ion intensities were used to calculate concentration of 
the stereoisomers.

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contributed to experiments. ST was involved in data analysis and 
interpretation. KY designed and conducted this research project as the 
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Competing interests
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