Design and Application of a Novel High-throughput Screening Technique for 1-Deoxynojirimycin

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High-throughput screening techniques for small molecules can find intensive applications in the studies of biosynthesis of these molecules. A sensitive, rapid and cost-effective technique that allows high-throughput screening of endogenous production of the natural iminosugar 1-deoxynojirimycin (1-DNJ), an α-glucosidase inhibitor relevant to the pharmaceutical industry, was developed in this study, based on the inhibitory effects of 1-DNJ on the activity of the β-glycosidase LacS from Sulfolobus solfataricus. This technique has been demonstrated effective in engineering both the key enzyme and the expression levels of enzymes in the 1-DNJ biosynthetic pathway from Bacillus atrophaeus cloned in E. coli. Higher biosynthetic efficiency was achieved using directed evolution strategies.

Directed evolution is a powerful tool frequently used in engineering the strains or biosynthetic pathways to improve the production of high-value small molecules1,2. The production of small molecules from the biosynthetic pathways can be impacted by the activities of key enzymes, the balance of the expression levels of pathway enzymes, and even the interactions of the host strain’s native genetic network with the biosynthetic pathways3–6. Directed evolution of the biosynthetic pathways or the host genomes, is an efficient way to develop hyper-producing strains by optimization of the potential bottlenecks of the pathways to direct the carbon flux toward the desired products7,8. Elegant high-throughput screening techniques for small molecules can greatly aid the engineering processes5. The majority of small molecules are not ready to be easily detected with colorimetric or fluorescent screening techniques. Lack of proper screening techniques, especially in vivo screening techniques, has become the bottleneck in optimization of the biosynthetic pathways for higher yields of small-molecule products.

Iminosugars constitutes a group of sugar mimic alkaloids produced by plants or microorganisms as the secondary metabolites. As the eversible and competitive inhibitors of glycosidases, they are relevant to the pharmaceutical industry for the treatment of various diseases especially in the treatment of non-insulin-dependent (type II) diabetes9–10. 1-DNJ is one of the iminosugars. Known as α-glucosidase inhibitors, 1-DNJ and its derivatives have shown potential therapeutic effects on diabetes, HIV infection as well as Gaucher’s disease11–14. 1-DNJ is mainly found in plants15, however, low amounts are found in some microorganisms16–20. The preparation of 1-DNJ include extraction from plants, microbial fermentation as well as chemical synthesis21. A combined biotechnological-chemical synthesis method is used for industrial production of 1-DNJ due to its short and economical process. The involved biotransformation step is the regioselective oxidation of 1-amino-1-deoxy-D-sorbitol to 6-amino-6-deoxy-1-sorbose, which is flanked by four chemical reactions22. Although the biosynthesis of 1-DNJ has attracted great interests, the studies are still preliminary. Genetic engineering and process optimization are still in great demand for improved 1-DNJ yield in microbial stains.

It has been demonstrated that the TYB gene cluster is responsible for catalysis of the first three steps of the 1-DNJ biosynthetic pathway in two Bacillus species. This cluster contains the gabT1, yktc1 and gutB1 genes, which encode a putative transaminase (GabT1), a phosphatase (Yktc1), and an oxidoreductase (GutB1), respectively23. The expression of the TYB gene cluster in E. coli led to 1-DNJ production. However, the genes encoding the epimerase and the reductase responsible for catalysis of the last two steps involving the conversion of mannojirimycin (MJ) to 1-DNJ remain unknown (Figure 1)18. Despite the report that MJ and 1-DNJ could be specifically

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assayed with α-mannosidase and trehalase inhibition reactions, respectively, these methods are laborious and time-consuming\(^{16}\). In order to engineer the biosynthesis of 1-DNJ for higher efficiency, a high-throughput screening technique for 1-DNJ is necessary. Here a solid phase-based sensitive high-throughput screening method for 1-DNJ was developed. The effectiveness of this method has been demonstrated through its applications in optimizing the TYB gene cluster for higher production of 1-DNJ.

**Results**

The development of 1-DNJ high-throughput screening technique. \(\beta\)-glycosidase from the archae Sulfolobus solfataricus (LacS) has broad substrate specificity and catalyzes the hydrolysis of aryl \(\beta\)-gluco, \(\beta\)-xylo and \(\beta\)-galactosides\(^{24}\). 1-DNJ was found able to inhibit LacS on its \(\alpha\)-nitrophenyl-\(\beta\)-D-galactopyranoside (\(\alpha\)NPG) hydrolysis activity (Figure 2a). The inhibitory effect of 1-DNJ on the LacS activity toward 5-bromo-4-chloro-3-indolyl \(\beta\)-D-galactopyranoside (X-GAL) was also demonstrated (Figure 2b). Five constructs expressing the TYB gene cluster (pDNJ1~5) were constructed. *E. coli* BWLacS was used as the expression host for all except pDNJ4 which was expressed in BL21(DE3) (Figure S1). Significant LacS inhibitory activity was detected in the cultures of BWLacS harboring pDNJ5, compared with the strain harboring a control plasmid without the gene cluster. This indicated that the inhibition was due to the products of the TYB gene cluster (Figure S2a).

The intermediate 2-amino-2-deoxy-D-mannitol (ADM) was found to not inhibit LacS (Figure S3). The effect of the unstable product of the TYB gene cluster, MJ (due to the unstable aminal functionality), on LacS activity was assayed with the reaction mixture of purified GutB1 with ADM and NAD\(^+\) as substrates\(^{25}\). MJ was also found to inhibit the activity of LacS (Figure S3). The production of MJ and 1-DNJ in strain BWLacS harboring pDNJ5 was also confirmed by the MJ-specific \(\alpha\)-mannosidase assay and the 1-DNJ-specific trehalase assay (Figure S2b). The production of 1-DNJ was also confirmed by HPLC (Figure S4), NMR (Table S3) and ESI-MS.

Based on the inhibitory effects of 1-DNJ, MJ and probably nojirimycin (Figure 1) on the activity of LacS, a solid-phase high-throughput screening method was developed. When cells coexpressing the TYB gene cluster and lacS were plated onto LB agar plates containing X-GAL, the cells producing higher titers of MJ and 1-DNJ...
were expected to show lighter blue (or whiter) color compared with lower producers. This high-throughput screening method was used to optimize the TYB gene cluster for higher production of 1-DNJ.

**Engineering of the key enzyme GutB1 in the TYB gene cluster.** To explore the catalytic efficiency of the enzymes in the TYB gene cluster, random mutagenesis libraries of gabT1, yktc1 and gutB1 were constructed and transformed into strain BWLacS. The strains harboring the mutant libraries were grown for ~20 h on LB agar supplemented with 10 mM glucose as the precursor for 1-DNJ biosynthesis and X-GAL as substrate for LacS. Compared with BWLacS which harbored the wild-type TYB gene cluster and was grown under the same conditions, a higher ratio of light blue (or white) colonies was observed for GutB1 random mutagenesis library than for GabT1 or Yktc1 library, indicating that GutB1 plays a critical role in the biosynthetic efficiency of the pathway. Thus, we proceeded to use the GutB1 library for high-throughput screening. Eight whiter colonies of BWLacS harboring the GutB1 randomly mutated variants were selected (from a total of 10^5 colonies screened), and the inhibitory effects of their LB liquid cultures on LacS activity were measured. Five mutant strains exhibited higher inhibitory effects on LacS activity toward oNPG, compared with the strain harboring the wild-type TYB gene cluster (Figure S5a). Plasmids from these clones were purified and retransformed into BWLacS, and the enhanced inhibitory activities on LacS were confirmed. Sequencing of the gutB1 genes in the five variants revealed three mutants (Gu2, Gu5 and Gu18) with one base-pair silent mutation (no change to amino acid sequence, the codon was changed to more frequently used codon in *E. coli*), mutant Gu1 and Gu30 with a D163G and an I236V substitutions, respectively (Table S4).

The results of the trehalase inhibition assay showed that, at 14 h, the 1-DNJ production of BWLacS harboring the I236V mutant was 54% higher than that of strain expressing wild-type GutB1 (Figure 3). A time course profile of MJ/1-DNJ production is presented in Figure S5b. It showed the inhibition rate of the culture on LacS activity upon oNPG and the cell growth (OD600) of BWLacS harboring either the I236V mutant or wild-type GutB1. The results showed that MJ/1-DNJ was produced in both the exponential and stationary phases. The supplemented glucose was almost exhausted in 3 h and led to a maximum inhibition rate of ~55% for the strain harboring wild-type GutB1 and 65% for strain harboring the I236V mutant.

**Characterization of the wild-type GutB1 and its I235V mutant.** Wild-type GutB1 and the I236V mutant were expressed in *E. coli* BL21(DE3) as N-terminal His-tagged fusion proteins and purified for an activity assay. D-sorbitol, D-mannitol and ADM were used as the substrates. Among the three substrates, wild-type GutB1 and the I236V mutant all showed the highest activities upon ADM,

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**Figure 3** | The 1-DNJ production of strain BWLacS harboring various plasmids cultured for 14 h.

**Figure 4** | The specific activity of wild type (□) and mutant (■) GutB1 on various substrates.
indicating that the amino group substitution at C2 of the hexitol chain of the substrate is preferred. The two enzymes also accepted \(D\)-sorbitol and \(D\)-mannitol as substrates albeit at reduced rates. For all three substrates, the I236V mutant showed \(\sim 2\)-fold higher specific activity compared with wild-type GutB1 (Figure 4). The results of kinetic studies revealed that the I236V mutant exhibited higher affinity for the substrate NAD\(^+\) than the wild-type enzyme. For both substrates, \(k_{cat}\) of the I236V mutant was higher than that of the wild-type enzyme. Therefore, the catalytic efficiency of I236V mutant was higher than that of the wild-type enzyme for both substrates (Table 1).

**Table 1 | Kinetic parameters of the wild-type GutB1 and its I236V mutant for substrates ADM and NAD\(^+\)**

| Enzymes   | \(K_m\) (mM) | \(k_{cat}\) (min\(^{-1}\)) | \(k_{cat}/K_m\) (mM\(^{-1}\) min\(^{-1}\)) |
|-----------|-------------|----------------|-------------------|
| Wildtype (ADM) | 0.063 ± 0.005 | 0.25 ± 0.005 | 4.0 |
| I236V (ADM)    | 0.064 ± 0.007 | 0.48 ± 0.02 | 7.5 |
| Wildtype (NAD\(^+\)) | 0.10 ± 0.008 | 0.41 ± 0.02 | 4.1 |
| I236V (NAD\(^+\)) | 0.054 ± 0.002 | 0.50 ± 0.01 | 9.3 |

LacS, a \(\beta\)-glucosidase with broad substrate specificity. Therefore, a blue-white screening technique developed based on the inhibition of 1-DNJ on the hydrolysis activity of LacS on X-GAL was designed and successfully applied for the high-throughput screening of endogenous 1-DNJ production on agar plates.

It has been demonstrated that the production of 1-DNJ could be improved by either elevating the activity of the rate-limiting enzyme GutB1 or balancing the relative expression levels of the pathway enzymes. The results from these two studies all revealed that higher activity of GutB1 relative to Yktc1 was helpful for 1-DNJ production improvement, indicating again that GutB1 catalyzes the rate-limiting step in the wild-type biosynthetic pathway. After the optimization of the biosynthetic pathway, the rate-limiting step may change and further additional improvements of 1-DNJ is foreseeable by identification and engineering of the rate-limiting enzymes, optimization of the genetic networks of the host strain for better supply of the biosynthetic precursors and cofactors, as well as optimization of process fermentation.

From Figure 2, the inhibitory effect of 1-DNJ on LacS showed high sensitivity at low concentrations of 1-DNJ. Therefore, the current screening system may not be suitable for screening when the productivity of 1-DNJ is relatively high because the colonies may all appear white on the agar plates. Then a LacS mutant which is less sensitive to the 1-DNJ inhibition needs to be developed. The problem may also be partly solved by engineering the rate-limiting enzyme in a lower-producing strain and then put the evolved mutant enzyme back into the higher-producing strain.

Due to the broad substrate specificity of LacS, it was also found to be inhibited by MJ, and probably by other iminosugars of similar structures as 1-DNJ, which will further broaden the application of this high-throughput screening technique. This technique also has potential applications in the screening of inhibitors of glycosidases.

**Methods**

**General.** Restriction enzymes were purchased from New England Biolabs (Beijing, China) and DNA polymerases were purchased from Takara Bio Inc. (Dalian, China).

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**Characterization of the wild-type and RBS-mutated TYB gene clusters.** To measure the expression levels of Yktc1 and GutB1 from BWLacS harboring pDNJ6 or pM13, sfGFP was fused to the C-terminal of GutB1 or Yktc1 (Figure S8). The sfGFP fluorescence, representing the expression levels of Yktc1 or GutB1 in both plasmids, was measured (Figure 5). The expression level of GutB1 in pM13 was slightly higher than that in pDNJ6, while the expression level of Yktc1 in pM13 was \(\sim 1/3\) of that in pDNJ6. This suggests that a lower ratio of Yktc1 to GutB1 expression led to a higher production of MJ/1-DNJ. These results demonstrate that an appropriate ratio of pathway enzyme expression levels, rather than the high expression of all of them, leads to greater biosynthetic efficiency.

**Discussion**

We have demonstrated the design and application of a novel high-throughput screening technique for endogenous 1-DNJ production in this study. 1-DNJ was mainly found as an inhibitor of \(\alpha\)-glucosidases, rarely as a \(\beta\)-glucosidase or galactosidase inhibitor\(^{20}\). For example, it does not have inhibitory effect on the \(\beta\)-galactosidase from *E. coli* (LacZ). In this study, 1-DNJ was found able to inhibit LacS, a \(\beta\)-glucosidase with broad substrate specificity. Therefore, a blue-white screening technique developed based on the inhibition of 1-DNJ on the hydrolysis activity of LacS on X-GAL was designed and successfully applied for the high-throughput screening of endogenous 1-DNJ production on agar plates.

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**Figure 5 | The sfGFP fluorescence measured from strain BWLacS expressing sfGFP-fused Yktc1 or GutB1 in both wild-type (pDNJ6, □) and mutant (pM13, ■) TYB gene clusters.**
T4 DNA ligase was purchased from Life Technologies (Shanghai, China). Oligonucleotides and the promoter P_araC were synthesized by Life Technologies (Shanghai, China). 1-Deoxynojirimycin (1-DNJ) was purchased from Carbosynth (Basingstoke, England). α-Mannosidase and α-mannosidase from C. rugosa (MegaBiotech, Seoul, South Korea) were purchased from Sigma-Aldrich (St. Louis, USA). The strain E. coli HK022 (from narrative: Escherichia coli) was used as megaprimer to perform MEGAWHOP PCR using pDNJ6 as template. The CRIM plasmid pH15756 was used for transformation and molecular cloning experiments. The CRIM plasmid pH15756 was used for transforming the Bacillus subtilis genome. The CRIM plasmid pH15756 was used for transforming the Bacillus subtilis genome. All colonies from the agar plates were used for plasmid isolation to prepare the plasmid library.

Preparation of samples for 1-DNJ/MJ inhibition assays. A single colony of strain BWLacS carrying plasmid pBADLacS was picked for quantifying DNJ production. All reported data in Figures 2a, 2a, 3a, 3b, 3c, 4a and 4b represent the mean of three independent data points. The error bars represent standard deviations.

Library Construction

The β-glycosidase gene laeC (Genbank accession No. AF133096) was amplified with the genomic DNA of S. solfataricus (kindly provided by Prof. Li Huang from Institute of Microbiology, Chinese Academy of Sciences) as template using primers LacS-for/NdeI and LacS-rev/XhoI. This fragment was then ligated into plasmid pBADLacS. The DNA fragment containing arac and lacS under control of promoter P_araC was PCR-amplified with plasmid pBADLacS as template using primers Arac-LacS-XbaI-for and LacS-rev-XhoI. This fragment was then ligated into the PCR product amplified using primers P_H15756-NheI and P_araC-XhoI with the CRIM plasmid pH15756 as template after digestion with XbaI and XhoI, resulting in a construct in which lacS is under control of P_DNj promoter, adjacent to the ggtC gene. The ggtC gene and flanked by regions homologous to the E. coli HK022 integration site. The construct was integrated into the E. coli BW25113 chromosome using helper plasmid pH15756 as described, resulting in strain BWLacS. The integration was verified by PCR.

Kinetic analysis of wild-type and mutant GutB1. 0.2 ~ 5 mM of ADM (0.5 mM NAD) or 0.05 ~ 1 mM of NAD (0.5 mM ADM) were used in determining the average kinetic parameters of GutB1 wild-type and 1236V mutant enzymes with the method described above. All assays were performed in three replicates and the kinetic parameters in Table 1 were obtained using Lineweaver-Burk plots. All reported data in Figures 1a and 1b represent the mean of three independent data points. The error bars represent standard deviations.

Fluorescence assay. A colony of BWLacS cells harboring plasmid pDNJ6-yktc-sfgfp, pM13-yktc-sfgfp, pDNJ6-gutB-sfgfp or pM13-gutB-sfgfp was grown overnight at 37 °C in LB medium containing ampicillin, then diluted to OD_560 = 0.8 in the same medium containing 0.5 mM IPTG, and allowed to grow at 30 °C for 10 h. A total of 200 μL of culture was centrifuged, and the cells were washed with 10 mM potassium phosphate buffer (pH 7.4) and resuspended in 200 μL of the same buffer. The cell suspension optical density (OD_560) and fluorescence emission were measured with a SynergyMx Multi-Mode Microplate Reader (BioTek, Vermont, USA) (485 nm excitation filter, 520/20 nm emission filter). The background fluorescence due to buffer served as the blank in all measurements.

All reported data in Figures 4 and 5 represent the mean of three independent data points. The error bars represent standard deviations.
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**Author contributions**

P.J., S.M., H.L., Y.L. and C.F., performed the experiments and analyzed the data. S.-Y.T. and J.-M.J., planned the experiments and wrote the manuscript. All authors reviewed the manuscript.

**Additional information**

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