Method Article

Strategy for identification of cis-dihydrodiendiol-degrading dehydrogenases in E. coli BW25113

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A B S T R A C T

cis-Dihydrodiendiol are valuable compounds, finding multiple application as chiral synths in organic chemistry. The biotechnological route for the generation of cis-dihydrodiendiol involves the dihydroxylation of aromatic compounds, catalyzed by Rieske non-heme iron dioxygenases. To date, numerous examples of recombinant E. coli, harboring such dioxygenases, can be found in the literature. Nevertheless, there is only a minor number of publications, addressing the E. coli catalyzed degradation of cis-dihydrodiendiol into catechols via dehydrogenases. Identification and elimination of such dehydrogenase catalyzed degradation is key for the establishment of enhanced recombinant E. coli platforms pursuing the production of cis-dihydrodiendiol. Here, we provide a fast and easy strategy for the identification of promiscuous alcohol dehydrogenases in E. coli BW25113, catalyzing the degradation of cis-dihydrodiendiol into catechols. This approach is based on the screening of dehydrogenase deficient KEIO strains, regarding their incapability of degrading a cis-dihydrodiendiol of choice.

- Novel screening strategy for E. coli BW25113 dehydrogenase knock-outs, incapable of degrading cis-dihydrodiendiol was validated for cis-1,2-dihydrocatechol as substrate
- Corresponding knock-outs can be used for recombinant production of cis-dihydrodiendiol
- Simple analysis based on liquid chromatography with diode array detector (HPLC-DAD)

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Specifications table

| Subject Area: | Biochemistry, Genetics and Molecular Biology |
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| Name and reference of original method: | Cultivation of KEIO collection in 96 well plates: |
| | • C. Tamae, A. Liu, K. Kim, D. Sitz, J. Hong, E. Becket, A. Bui, P. Solaimani, K.P. Tran, H. Yang, J.H. Miller, Determination of antibiotic hypersensitivity among 4000 single-gene-knockout mutants of Escherichia coli, J. Bacteriol. 190 (2008) 5981–5988. https://doi.org/10.1128/JB.01982-07. |
| | • T. Baba, T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K.A. Datsenko, M. Tomita, B.L. Wanner, H. Mori, Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: The Keio collection, Mol. Syst. Biol. 2 (2006). https://doi.org/10.1038/msb4100050 |
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Method details

Background

cis-Dihydrodiiodiols are valuable chemical compounds used as synths for various organic syntheses. They are generated via the dearomatizing dihydroxylation of aromatic compounds catalyzed by Rieske non-heme dioxygenases (ROs). Interestingly, the direct cis-dihydroxylation of aromatic double bonds by chemical catalysts has so far not been reported [5]. The chemical synthesis of such compounds is rather challenging and consists, if even possible, of multiple reaction steps [6]. One of the best-characterized ROs is the toluene dioxygenase (TDO) from P. putida F1, which comprises a broad substrate scope with over 100 compounds [7]. There are numerous publications reporting the recombinant expression of the multi-component TDO system in E. coli to produce cis-dihydrodiiodiols in large quantities [4,8–10]. Nevertheless, none of these publications has explored the E. coli catalyzed degradation of cis-dihydrodiiodiols. In most cases, cis-dihydrodiiodiols seem to be not degraded by E. coli under the tested conditions, since catechol formation is not mentioned. However, there are known dehydrogenases in E. coli, which can dehydrogenate some of these valuable compounds to the corresponding catechols (Fig. 1). For instance, the trans-2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase EntA [11], which is able to convert cis-1,2-dihydrocatechol into catechol, and the 3-phenylpropionate dioxygenase HcaB [12], which converts 3-((55,6R)-5,6-dihydroxycyclohexa-1,3-dien-1-yl)propanoic acid into 3-(2,3-dihydroxyphenyl)propanoic acid. Furthermore, recently we showed that the glycerol dehydrogenase GldA seems to have a major influence in the degradation of cis-1,2-dihydrocatechol into catechol, and a minor activity for the degradation of cis-1,2-dihydro-3-methylcatechol into 3-methylcatechol [13]. In this study, we provide a fast and easy screening system for dehydrogenases in E. coli, for the conversion of a targeted cis-dihydrodiiodiol, using the single-gene knock-out strains from the KEIO collection [1]. E. coli BW25113, a descendant of K-12 strain, is the genetic background of the KEIO collection and includes in-frame deletions in every
single gene of all 3985 non-essential genes. These E. coli mutants were created by replacing the open reading frame coding regions with a kanamycin resistance cassette. KEIO strains provide a resource for the analysis of gene-protein associated functions. Among various applications, the most extensive use of the KEIO collection has been in the assessment of how loss of specific gene function influences phenotypes [2, 14]. Our described screening approach was evaluated with cis-1,2-dihydrocatechol and enlightened the glycerol dehydrogenase GldA, as the main responsible for the degradation of this compound. Furthermore, we could show that by using the identified knock-out strain of the KEIO collection, ∆gldA, the degradation of cis-1,2-dihydrocatechol could be dramatically reduced.

**Method**

**Reagents and standards**

Chemicals and solvents used in this work were obtained at the highest purity degree available from Sigma-Aldrich (St. Louis, US) and Carl Roth (Karlsruhe, DE). Since cis-1,2-dihydrocatechol is not commercially available, it was biosynthetized [13]. Polypropylene 96 deep well plates (Riplate SW 2 mL 96 Wells) were purchased from Ritter (Schwabmünchen, DE), Breathe-EASIER sealing membranes from Sigma-Aldrich (St. Louis, US). The KEIO collection was purchased from GE Healthcare Dharmaco (Lafayette, US).

**Candidate enzyme pool**

In total 156 annotated and putative dehydrogenases and their corresponding genes were identified by using the online database EcoCyc (ecocyc.org) for E. coli K-12, using as query the term “dehydrogenase”. E. coli K-12 is the direct parent strain of E. coli BW25113. The corresponding knock-outs of the KEIO collection can be found in table S1. We recommend to examine first strains BW25113; ΔentA, ΔhcaB, and ΔgldA (table S1), before proceeding with other dehydrogenases.

**Cultivation of the KEIO strains**

The strains of the KEIO collection are stored in the form of glycerol stocks in 96-well microtiter plates at −80 °C. For cell cultivation, the microtiter plates were placed on ice and 2 μL of the glycerol stocks were transferred (using sterile pipette tips) into 96 deep well plates, containing 0.5 mL of LB media supplemented with 50 μg mL⁻¹ of kanamycin. The plates were sealed with Breathe-EASIER sealing membranes for multiwell plates (Sigma, St. Louis, US) and incubated for 20 h at 37 °C and 800 rpm in a microplate shaker (VWR International, Radnor, US). For consistent cell quantities, 2.5% (v/v) of precultures were transferred into new 96 deep well plates, containing 1.0 mL TB media supplemented with 50 μg mL⁻¹ of kanamycin and incubated at 37 °C and 800 rpm for 20 h.

**Screening of KEIO strains for the inability of cis-dihydrodiol consumption**

Cells were harvested by centrifugation at 4000 x g and 4 °C for 20 min, and the supernatant was discarded. At this point uniform cell growth was checked. Approximately 72 mgsw per well
were obtained. Cells were resuspended in 490 μL of 0.1 M potassium phosphate buffer (pH 7.4), supplemented with 20 mM glucose for cofactor regeneration, yielding a resting cell suspension. A volume of 10 μL of the 0.1 M substrate solution (in ethanol or DMSO, final concentration 2 mM), was added to each resting cell suspension. It is important to keep in mind that ethanol may have an influence at inducing dehydrogenase reactions. Therefore, we recommend for most screenings DMSO as substrate solvent. Biotransformation plates were incubated at 30 °C and 800 rpm for 20 h in a microplate shaker. The reaction temperature as well as the reaction time and the extraction solvent is dependent on the substrate or product of interest, and should be established accordingly. Therefore, we suggest to work under conditions where *E. coli* wild type strain displays the highest substrate conversion. Biotransformations were stopped by addition of 1:1 (v/v) ethyl acetate (alternatively MTBE) and vigorous shaking, using a mat (WebSeal Mat, 96 square, 8 mm, Thermo Fisher Scientific, Waltham, US). The deep well plates were centrifuged for 5 min at 4000 x g and room temperature. Afterwards, the ethyl acetate layer was separated, transferred into 2 mL autosampler vials (Wicom, Heppenheim, DE) and analyzed by HPLC-DAD to quantify cis-1,2-dihydrocatechol consumption and catechol formation.

Substrate and product quantification via HPLC-DAD

cis-Dihydodiendiols and their corresponding catechols can be analyzed via HPLC-DAD due to their conjugated π-system at 262 nm. Extracted samples were analyzed by HPLC-DAD [3]. An Agilent 1260 Infinity II system (Santa Clara, US), equipped with a C18-column (Agilent Eclipse XDB-C18, 5 μm, 4.6 × 150 mm, Santa Clara, US) and a diode array detector (Agilent 1260 Infinity II DAD HS, Santa Clara, US) was operated isothermally at 30 °C. Measurements were run at a flow rate of 1.0 mL min⁻¹. For most cis-dihydodiendiols and catechols a 30 min long isocratic method with 40/60 water/acetonitrile can be used. For quantification, the wavelengths 210 nm and 262 nm were employed. Peak areas were measured by the integrator and transformed into concentration using the correspondent standard curves of cis-dihydodiendiol and the corresponding catechol.

A result showing a knock-out variant unable to degrade the supplied cis-dihydodiendiol suggests, that the knocked out dehydrogenase is responsible for its degradation (Fig. 2). If the consumption
is only partially reduced, it is likely that other dehydrogenase(s) might be involved in the substrate degradation. To fully corroborate the role of the identified proteins in substrate consumption, \textit{E. coli} BW25113 strains harboring and lacking the encoding gene should be analyzed. Mutational polar effects or that the responsible gene is not within the 156 knock-outs of table S1 have to be considered in cases when not a single of the 156 knock-outs of table S1 displays a major or partial decrease in the cis-dihydrodiendiol degradation. An option would be to screen the whole KEIO collection, or to search for more knock-out strains encoding dehydrogenase-type enzymes.

**Method validation**

The method described above was evaluated with the substrate cis-1,2-dihydrocatechol. The substrate was dissolved in ethanol (0.1 M stock solution). Biotransformations were performed at 30 °C for 20 h at 800 rpm. Ethyl acetate was used as extraction solvent. For substrate and product quantification a shorter HPLC-DAD method was used. Measurements were run at a flow rate of 0.6 mL min$^{-1}$, using as mobile phase water/acetonitrile with a linear gradient of; $t = 0$ min, 35/65 (v/v); $t = 5.5$ min, 10/90 (v/v); $t = 6.0$ min, 35/65 (v/v); $t = 10.0$ min, 35/65 (v/v) was used. Peak areas were measured by manual integration and transformed into concentration using the corresponding standard curves (Fig. S4 and S5) of catechol and cis-1,2-dihydrocatechol. The employed wavelength to detect cis-1,2-dihydrocatechol and catechol were 262 and 210 nm, respectively (Fig S2 and S3). Under the mentioned conditions, \textit{E. coli} BW25113 wild type degraded the total amount of the supplied cis-1,2-dihydrocatechol into catechol.

The biotransformations of the 156 knock-outs were successively evaluated in biological triplicates, until a knock-out was found, which converted only 5% of substrate (knock-out 88; Fig. S1). Therefore, the remaining 68 knock-out strains were not analyzed. Knock-out 88 was identified as the glycerol dehydrogenase GldA. The reduced substrate conversion in knock-out number 8, the lipoamide dehydrogenase Lpd was mostly due to its reduced growth, compared with the \textit{E. coli} wild type, and was not further investigated [15]. All 156 dehydrogenase deficient strains can be easily screened within 2 weeks in biological triplicates (half of the library in biological triplicates, resulting in 3 plates per week). We recommend including, in each single plate, the wild type strain as well as the substrate in buffer in biological triplicates, for comparison reasons.

**Conclusion**

A new screening system for the identification of cis-dihydrodiendiol-degrading dehydrogenases in \textit{E. coli} BW25113 was developed and validated. By employing the identified knock-out of the KEIO collection lacking the undesired degradation activity, the cis-dihydrodiendiol consumption can be drastically decreased. This approach enables the production of valuable cis-dihydrodiendiol in recombinant \textit{E. coli}, harboring a Rieske-non heme iron dioxygenase, in higher concentrations [13].

**Declaration of Competing Interest**

The Authors confirm that there are no conflicts of interest.

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**Supplementary materials**

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2020.101143.
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