MethodsX 7 (2020) 101143

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Method Article

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

Julian L. Wissner, Julian Ludwig, Wendy Escobedo-Hinojosa, Bernhard Hauer*

Institute of Technical Biochemistry, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany

A B S T R A C T

cis-Dihydrodiendiols are valuable compounds, finding multiple application as chiral synthons in organic chemistry. The biotechnological route for the generation of *cis*-dihydrodiendiols 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*-dihydrodiendiols 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*-dihydrodiendiols. Here, we provide a fast and easy strategy for the identification of promiscuous alcohol dehydrogenases in *E. coli* BW25113, catalyzing the degradation of *cis*-dihydrodiendiols 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*-dihydrodiendiols was validated for *cis*-1,2-dihydrocatechol as substrate
- Corresponding knock-outs can be used for recombinant production of *cis*-dihydrodiendiols
- Simple analysis based on liquid chromatography with diode array detector (HPLC-DAD)

A R T I C L E I N F O

Method name: Strategy for identification of *cis*-dihydrodiendiol-degrading dehydrogenases in *E. coli* BW25113
Keywords: KEIO collection, Screening, HPLC-DAD, Rieske non-heme iron dioxygenase, 96 deepwell plate, **c**is-1,2-dihydrocatechol
Article history: Received 20 September 2020; Accepted 7 November 2020; Available online 13 November 2020

DOI of original article: 10.1016/j.jbiotec.2020.09.012
* Corresponding author.
E-mail address: bernhard.hauer@itb.uni-stuttgart.de (B. Hauer).

https://doi.org/10.1016/j.mex.2020.101143

Specifications table

Subject Area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	Biodegradation
Method name:	Strategy for identification of *cis*-dihydrodiendiol-degrading dehydrogenases in *E. coli* BW25113
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 ***HPLC-DAD method for cis-dihydrodiendiols and catechols:*** • J.L. Wissner, W. Escobedo-Hinojosa, P.M. Heinemann, A. Hunold, B. Hauer, Methods for the detection and analysis of dioxygenase catalyzed dihydroxylation in mutant derived libraries, in: Enzym. Eng. Evol. Incl. Dir. Evol., 1st ed., Elsevier Inc., 2020: pp. 1–31. https://doi.org/10.1016/bs.mie.2020.04.022 • S.-P. Ouyang, S.-Y. Sun, Q. Liu, J. Chen, G.-Q. Chen, Microbial transformation of benzene to cis-3,5-cyclohexadien-1,2-diols by recombinant bacteria harboring toluene dioxygenase gene tod, Appl. Microbiol. Biotechnol. 74 (2007) 43–49. https://doi.org/10.1007/s00253-006-0637-6
Resource availability:	N/A

Method details

Background

cis-Dihydrodiendiols are valuable chemical compounds used as synthons 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 multicomponent TDO system in *E. coli* to produce *cis*-dihydrodiendiols in large quantities [4,8–10]. Nevertheless, none of these publications has explored the *E. coli* catalyzed degradation of *cis*-dihydrodiendiols. In most cases, *cis*-dihydrodiols 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-((5*S*,6*R*)-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*-dihydrodiendiol, 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

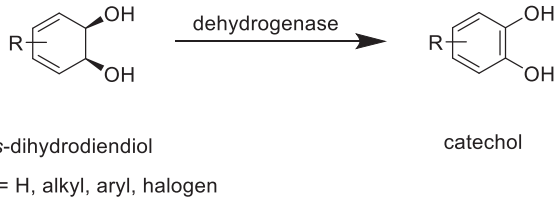

Fig. 1. Dehydrogenase catalyzed degradation of *cis*-dihydrodiol to corresponding catechol.

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, Δ*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-dihydrocatchol is not commercially available, it was biosynthesized [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. Lois, US). The KEIO collection was purchased from GE Healthcare Dharmacon (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; Δ*entA*, Δ*hcaB,* and Δ*gldA* (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*-dihydrodiendiol 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 mg_{cww} per well

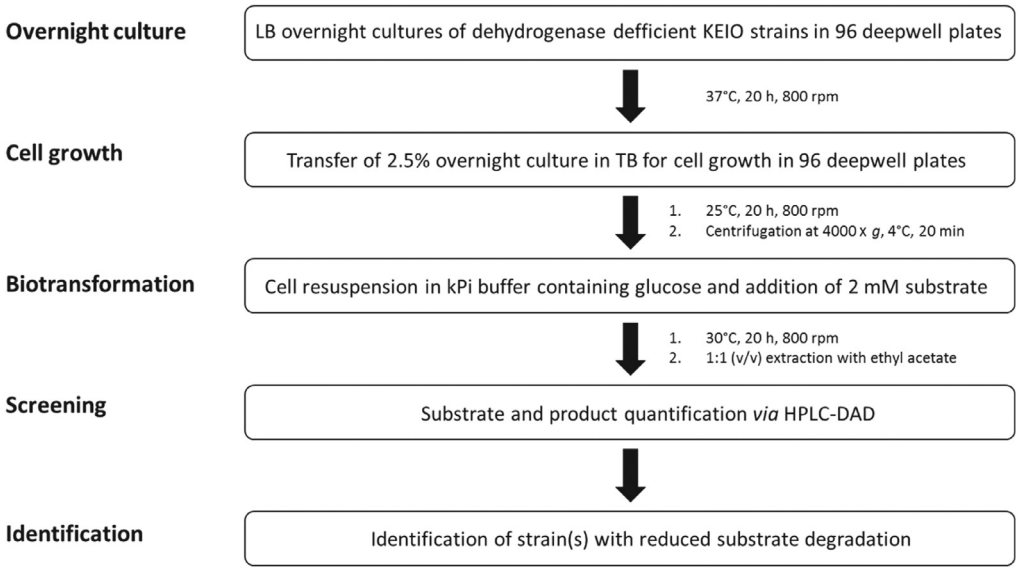

Fig. 2. KEIO collection based screening approach to determine *E. coli* BW25113 knock-out strains, incapable of degrading *cis*-dihydrodiendiols.

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*-dihydrodiendiol and the corresponding catechol.

A result showing a knock-out variant unable to degrade the supplied *cis*-dihydrodiendiol 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, *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, *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 *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 *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*-dihydrodiendiols in recombinant *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.

Acknowledgements

This research was supported by the BMBF in the course of the project PowerCart 031B0369A. W. E.-H. thanks the Science and Technology Council of Mexico (Consejo Nacional de Ciencia y Tecnología, CONACYT) for financial support granted to work in this project.

Supplementary materials

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

References

[1] 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), doi:10.1038/msb4100050.

[2] 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, doi:10.1128/JB.01982-07.

[3] J.L. Wissner, W. Escobedo-Hinojosa, P.M. Heinemann, A. Hunold, B. Hauer, Methods for the detection and analysis of dioxygenase catalyzed dihydroxylation in mutant derived libraries, in: Enzym. Eng. Evol. Incl. Dir. Evol., 1st ed., Elsevier Inc, 2020, pp. 1–31, doi:10.1016/bs.mie.2020.04.022.

[4] S.-P. Ouyang, S.-Y. Sun, Q. Liu, J. Chen, G.-Q. Chen, Microbial transformation of benzene to cis-3,5-cyclohexadien-1,2-diols by recombinant bacteria harboring toluene dioxygenase gene tod, Appl. Microbiol. Biotechnol. 74 (2007) 43–49, doi:10.1007/s00253-006-0637-6.

[5] J.J. Shie, J.M. Fang, C.H. Wong, A concise and flexible synthesis of the potent anti-influenza agents tamiflu and tamiphosphor, Angew. Chemie - Int. Ed. 47 (2008) 5788–5791, doi:10.1002/anie.200801959.

[6] P. Mukherjee, S.J.S. Roy, T.K. Sarkar, A diversity-oriented synthesis of bicyclic cis -dihydroarenediols, cis -4-hydroxyscytalones, and bicyclic conduritol analogues, Org. Lett. 12 (2010) 2472–2475, doi:10.1021/ol100557f.

[7] R.E. Parales, S.M. Resnick, Biocatalysis in the Pharmaceutical and Biotechnology Industries, CRC Press, 2006, doi:10.1201/9781420019377.

[8] M.G. Quintana, H. Dalton, Biotransformation of aromatic compounds by immobilized bacterial strains in barium alginate beads, Enzyme Microb. Technol. 24 (1999) 232–236, doi:10.1016/S0141-0229(98)00116-1.

[9] M.A. Endoma, V.P. Bui, J. Hansen, T. Hudlicky, Medium-scale preparation of useful metabolites of aromatic compounds via whole-cell fermentation with recombinant organisms, Org. Process Res. Dev. 6 (2002) 525–532, doi:10.1021/op020013s.

[10] M.A. Vila, M. Brovetto, D. Gamenara, P. Bracco, G. Zinola, G. Seoane, S. Rodríguez, I. Carrera, Production of cis-1,2-dihydrocatechols of high synthetic value by whole-cell fermentation using Escherichia coli JM109 (pDTG601): a detailed study, J. Mol. Catal. B Enzym. 96 (2013) 14–20, doi:10.1016/j.molcatb.2013.06.003.

[11] M. Sakaitani, F. Rusnak, N.R. Quinn, C. Tu, T.B. Frigo, G.A. Berchtold, C.T. Walsh, Mechanistic Studies on trans-2,3-Dihydro-2,3-dihydroxybenzoate Dehydrogenase (Ent A) in the Biosynthesis of the Iron Chelator Enterobactin, Biochemistry 29 (1990) 6789–6798, doi:10.1021/bi00481a006.

[12] E. Díaz, A. Ferrández, J.L. García, Characterization of the HCA cluster encoding the dioxygenolytic pathway for initial catabolism of 3-phenylpropionic acid in Escherichia coli K-12, J. Bacteriol 180 (1998) 2915–2923.

[13] J.L. Wissner, J. Ludwig, W. Escobedo-Hinojosa, B. Hauer, An enhanced toluene dioxygenase platform for the production of cis-1,2-dihydrocatechol in Escherichia coli BW25113 lacking glycerol dehydrogenase activity, J. Biotechnol. Under revision, JBIOTEC-D-20-00805

[14] V.G. Debabov, Microbial Metabolic Engineering, Springer New York, New York, NY, 2019, doi:10.1007/978-1-4939-9142-6.

[15] E.C.A. Goodall, A. Robinson, I.G. Johnston, S. Jabbari, K.A. Turner, A.F. Cunningham, P.A. Lund, J.A. Cole, I.R. Henderson, The essential genome of Escherichia coli K-12, MBio 9 (2018) 1–18, doi:10.1128/mBio.02096-17.