Benzoate synthesis from glucose or glycerol using engineered *Pseudomonas taiwanensis*

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Abstract

Benzoic acid is one of the most commonly used food preservatives, but currently exclusively produced in petrochemical processes. In this study, we describe a bio-based production pathway using an engineered strain of *Pseudomonas taiwanensis*. In a phenylalanine-overproducing strain, we heterologously expressed bacterial, yeast, and plant genes to achieve production of benzoate via a β-oxidation pathway. Strategic disruption of the native *Pseudomonas* benzoate degradation pathway further allowed the production of catechol and cis,cis-muconate. Taken together, this work demonstrates new routes for the microbial production of these industrially relevant chemicals from renewable resources.

1. Introduction

Benzoic acid and its salts are widely used in food, pharmaceuticals, and cosmetics as preservative, as they inhibit growth of several yeasts and bacteria. Commercially, benzoate is produced by partial oxidation of toluene with oxygen, catalyzed by cobalt or manganese naphthenates [1]. This conversion can also be performed by microbes, e.g., through the upper pathway encoded on the TOL plasmid pWW0 from *Pseudomonas putida* mt-2 [2]. Besides environmental issues that arise from its petrochemical production process, microbially produced benzoate is considered to be “natural”, which is a major benefit for applications in food and cosmetics. This, however, requires the production to start from a bio-based substrate.

Despite its industrial relevance and simple structure, only minor efforts have been made to develop a microbial host for the bioproduction of benzoate with only one study demonstrating its de novo production [3]. About 460 mg L⁻¹ (3.8 mM) of benzoate was produced with *Streptomyces maritimus* in a fermentation process using a complex medium (5% tryptone, 3% cornstarch). So far this is the only prokaryotic organism that has been described to natively synthesize benzoate from l-phenylalanine via β-oxidation of trans -cinnamoyl-CoA as part of the enterocin biosynthesis pathway [4].

*Pseudomonas taiwanensis* is a promising microbial cell factory, especially regarding the production of aromatics. This has been recently demonstrated by our group in multiple studies for de novo synthesis of phenol, 4-hydroxybenzoate, and trans -cinnamate [5-8]. In this study, a previously generated *Pseudomonas taiwanensis* trans -cinnamate overproducer was further engineered to enable benzoate production from renewable resources in a mineral medium without supplementation of complex substances or antibiotics. To our knowledge, this is the first de novo synthesis of benzoate in a recombinant microbial cell factory. The intrinsic benzoate catabolic pathway of *P. taiwanensis* was exploited to produce other industrially relevant chemicals, namely catechol and cis,cis -muconate, thereby establishing a novel biosynthesis pathway for these molecules.
Figure 1 Biosynthetic pathway to convert l-phenylalanine into benzoate expressing heterologous enzymes and subsequent conversion of benzoate into catechol and cis,cis-muconate by native enzymes.

2. Experimental Section

2.1 Media and culture conditions

Plasmids and strains used in this study can be found in Table S1 in the supplementary information. For cloning purposes, *Escherichia coli* and *P. taiwanensis* cells were cultivated at 37 or 30 °C, respectively, either in liquid LB medium containing 5 g L⁻¹ sodium chloride or on solid LB agar plates (with 1.5 % (w/v) agar). After conjugal mating procedures, Pseudomonads were isolated on cetrimide agar (Sigma Aldrich) plates supplemented with 10 mL L⁻¹ glycerol. Kanamycin (50 mg L⁻¹) or gentamicin (20 mg L⁻¹) was added to cultures or plates when necessary. Growth and production experiments were performed in mineral salts medium (MSM) [9] with 20 mM glucose or 40 mM glycerol as sole carbon source.

In production experiments, liquid cultures of *P. taiwanensis* were performed in MSM with 20 mM glucose or 40 mM glycerol without the addition of antibiotics. Main cultures were inoculated at an OD₆₀₀ of ~0.2, from seed cultures grown in MSM containing glucose. Batch production experiments were performed in 500 mL Erlenmeyer flasks with a culture volume of 50 mL, cultivated in a rotary shaker with a frequency of 200 rpm and a throw of 50 mm. Fed batch fermentations were performed in DASbox(r) mini-bioreactors (Eppendorf) according to the setup and procedure described in Otto et al. [8].

2.2 Plasmid construction and genomic modification

Deletion and expression plasmids were cloned as described in detail in the supplementary information. Plasmids derived from pEMG and pSEVA412S were transformed into *E. coli* DH5α λpir cells, pBG-based plasmids into *E. coli* PIR2. Integration at the attTn7-site was achieved by four-parental patch mating as described by Wynands et al. [5]. Genomic deletions were realized using the I-SceI-based method by Martinez-Garcia and de Lorenzo [10] using a streamlined protocol adapted from Wynands et al. [5]. Genomic modifications were verified by colony PCR.

2.3 Analytical methods

Optical densities (OD₆₀₀) were measured using an Ultrospec 10 Cell Density Meter (GE Healthcare).

Culture supernatants were analyzed in a 1260 Infinity II HPLC equipped with a 1260 DAD WR (Agilent Technologies) and an ISAspher 100-5 C18 BDS reversed-phase 202 column (ISERA) at 30 °C and a flow rate of 0.8 mL min⁻¹. Elution took place with a binary mixture of 0.1% (v/v) aqueous trifluoroacetic acid and acetonitrile according to the following program: 0-2 min: 10% acetonitrile; 2-6 min: linear increase to
100% acetonitrile; 6-8 min: 100% acetonitrile; 8-10 min: linear decrease to 10% acetonitrile; 10-14 min: 10% acetonitrile. trans-Cinnamate and benzoate were detected at 245 nm, muconate at 260 nm, and catechol at 280 nm.

3. Results and Discussion

To prevent the degradation of benzoate by the engineered Pseudomonas strain, the benABCD operon (PVLB_12215-12230) responsible for the conversion of benzoate to catechol (Figure 1) was deleted in a previously described phenylalanine-producing chassis [8] to yield P. taiwanensis GRC3 Δ8ΔpykA -tap ΔbenABCD. Subsequently, the synthetic operon encoding the pathway from l-phenylalanine to benzoate was integrated at the attTn7 -site under the control of the constitutive promoter P14f [11]. The phenylalanine ammonia-lyase (PAL) deaminates l-phenylalanine to trans-cinnamate that is subsequently CoA-activated by the 4-coumarate CoA-ligase (4CL) [10]. The resulting trans-cinnamoyl-CoA is converted into benzoate by the enzymes encoded by the phd cluster [12]. The Phd pathway from C. glutamicum was a key enabling factor for benzoate production because it accepts non-hydroxylated cinnamoyl-CoA as a substrate, unlike the ferulic acid pathway from P. putida, which only converts hydroxylated phenylpropanoids [5]. Shake flask cultivations were performed to characterize benzoate production from glucose and glycerol (Figure 2A,B).

The strain reached a final OD_{600} of ~3 while producing 1.9 ± 0.0 or 3.0 ± 0.0 mM benzoate from 20 mM glucose or 40 mM glycerol, respectively. Assuming complete carbon utilization, this corresponds to yields of 10.8 ± 0.1 on glucose and 17.3 ± 0.1 % (Cmol Cmol⁻¹) on glycerol. In the course of the cultivation, no accumulation of trans-cinnamate was observed, confirming the efficient operation of the Phd pathway. To the best of our knowledge, this is the first approach demonstrating de novo benzoate biosynthesis applying a synthetic pathway. Moreover, high titers and yields were achieved for a microbial benzoate production process with a minimal medium, thereby producing benzoate solely from glucose or glycerol.

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Figure 2 Biosynthesis of benzoate and derivatives by strains of P. taiwanensis GRC3 [?]8[?]pykA -tapattTn7 ::P_{14f}-phdBBCDE-4cl-pal with ΔbenABCD deletion for the production of benzoate (A,B) or ΔcatBCA deletion for the production of catechol (C,D) in shake flask in 50 ml MSM with 20 mM glucose (A,C) or 40 mM glycerol (B,D). Fed batch fermentations of the ΔcatB strain controlled at pH 7 for the production of cis,cis-muconate from glucose (E). Error bars represent the standard error of the mean. Shake flask cultivations were performed in triplicates, fed batch fermentations in duplicates.

P. taiwanensis is natively able to assimilate benzoate via the intermediates catechol and cis,cis-muconate. Targeted disruption of this pathway thus allows the synthesis of these derivatives (Figure 1). Due to their potential as bio-based building blocks, the microbial production of these molecules is intensively studied [13-17]. The pathway via l-phenylalanine described in this study is a novel strategy that adds a new aspect to this highly active field of research. To allow catechol or cis,cis-muconate accumulation, the genes catBCA(PVLB_12240-50) or catB (PVLB_12240) (Figure 1) were deleted in the l-phenylalanine-overproducing Pseudomonas chassis [8], and the benzoate biosynthesis module (P_{14f}-phdBBCDE-4cl-pal) was integrated.

On both carbon sources, this strain grew to an OD_{600} of ~3.1 (Figure 2C,D). By the end of cultivation, 0.43 ± 0.01 mM catechol were produced from glucose, and 0.67 ± 0.01 mM from glycerol. This corresponds to yields of 2.2 ± 0.04 and 3.3 ± 0.04% (Cmol Cmol⁻¹), respectively. These catechol titers and yields are relatively low compared to those of trans-cinnamate [8] and benzoate (Figure 2A,B). One possible explanation might be the greater toxicity of catechol related to the formation of reactive oxygen species and protein damage [18]. However, Pseudomonads were reported to tolerate higher amounts of catechol than produced during these experiments [19]. The concentrations produced by P. taiwanensis GRC3 Δ8ΔpykA -tap ΔcatBCA attTn7 ::P_{14f}-phdBBCDE-4cl-pal should thus not yet lead to a high impairment of cellular fitness, especially considering that this strain is more solvent-tolerant [7]. Alternatively, the low titers of catechol may be due
to its instability in the presence of oxygen and water [18, 20].

The quantification of cis,cis-muconate produced by strain _P. taiwanensis_ GRC3 Δ8ΔpykA -tap ΔcatBattTn7 ::P14f -phdBCDE-4cl-pal in shake flasks was complicated by its isomerization. During HPLC analysis of supernatants, peaks appeared broadened and split, thereby impairing reliable quantification. This effect is due to the isomerization of cis,cis-muconate into cis,trans-muconate and subsequent lactonization, which occurs readily under acidic conditions [21]. Already a slight pH shift occurring during cultivation from initially pH 7.0 to 6.8 leads to isomerization. A five-fold increase of the medium’s buffer capacity did not reduce this effect. To avoid this, _P. taiwanensis_ GRC3 Δ8ΔpykA-tap ΔcatBattTn7 ::P14f-phdBCDE-4cl-pal was cultivated in dO2-stat fed batch fermentations with strict pH control (≥7; Figure 2E). Indeed, no isomerization was observed in the course of the fermentation and a titer of 7.2 ± 0.4 mM of cis,cis-muconate was achieved after 170 h. However, around 0.2 mM of catechol started to accumulate after 52h, followed by strong accumulation of trans-cinnamate to up to 2.9 ± 0.0 mM by the end of cultivation, indicating inhibition of the downstream pathway. The _catBCA_ cluster is subject to Crc regulation [22] possibly creating a bottleneck of the CatA-catalyzed reaction once a certain cis,cis-muconate concentration is reached. Furthermore, _P. taiwanensis_ harbors one copy of the _catA_ gene, while _P. putida_ KT2440 holds a second chromosomal copy (catA2), offering a “safety valve” in the presence of high catechol concentrations [19, 23]. An overexpression of a modified CatA could thus not only counteract Crc regulation, but also enhance catechol conversion, thereby limiting the accumulation of this toxic intermediate.

4. Concluding remarks

This study describes the adaptation of the previously engineered phenylalanine-overproducing chassis _P. taiwanensis_ GRC3 Δ8ΔpykA -tap to enable microbial production of bio-benzoic acid. The applied heterologous pathway converts phenylalanine via trans-cinnamate to benzoate. Further, the catabolic versatility of _Pseudomonas_ was exploited to establish novel pathways for the production of catechol and cis,cis-muconate. The catechol yields were relatively low compared to previously achieved production parameters for other aromatics, likely at least partially related to the high toxicity of catechol. However, the yields achieved for benzoate and cis,cis-muconate are very promising and future efforts should be made to increase the titers in a fed-batch fermentation. Benzoate has significant relevance as food preservative and as a starting point for the production of many other platform chemicals and secondary metabolites, thus expanding the product spectrum of _P. taiwanensis_ as robust biotechnological workhorse.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

References

1. Dakka, J., Z. Amikam, and Y. Sasson, _Process for the manufacture of benzoic acid and salts thereof_. US patent US4965406A, 1989.
2. Silva-Rocha, R. and V. de Lorenzo, _The pWW0 plasmid imposes a stochastic expression regime to the chromosomal ortho pathway for benzoate metabolism in Pseudomonas putida_. FEMS Microbiol Lett, 2014.356 (2): p. 176-83.
3. Noda, S., et al., _Βενζοις αςιδ φερμεντατιον φρομ σταρςη ανδ ςελλυλοσε vια α πλαν-λικε β-οξιδατιον πατηωαψ ω Στρεπτομψςς μαριτιμυς_. Microb Cell Fact, 2012. 11 : p. 49-49.
4. Hertweck, C. and B.S. Moore, *A plant-like biosynthesis of benzoyl-CoA in the marine bacterium ‘Streptomyces maritimus’*. Tetrahedron, 2000. 56 (46): p. 9115-9120.

5. Wynands, B., et al., *Metabolic engineering of Pseudomonas taiwanensis VLB120 with minimal genomic modifications for high-yield phenol production*. Metab Eng, 2018. 47 : p. 121-133.

6. Lenzén, C., et al., *High-yield production of 4-hydroxybenzoate from glucose or glycerol by an engineered Pseudomonas taiwanensis VLB120*. Front Bioeng Biotechnol, 2019. 7 : p. 130.

7. Wynands, B., et al., *Streamlined Pseudomonas taiwanensis VLB120 chassis strains with improved bioprocess features*. ACS Synth Biol, 2019. 8 (9): p. 2036-2050.

8. Otto, M., et al., *Rational engineering of phenylalanine accumulation in Pseudomonas taiwanensis to enable high-yield production of trans-cinnamate*. Front Bioeng Biotechnol, 2019. 7 : p. 312.

9. Hartmans, S., et al., *Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading Xanthobacter strain 124X*. Appl Environ Microbiol, 2011. 77 (10): p. 2702-16.

10. Martinez-Garcia, E. and V. de Lorenzo, *Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of Pseudomonas putida KT2440*. Environ Microbiol, 2011. 13 (10): p. 2850-5.

11. Zobel, S., et al., *Tn7-based device for calibrated heterologous gene expression in Pseudomonas putida*. ACS Synth Biol, 2015. 4 (12): p. 1341-51.

12. Kallscheuer, N., et al., *Identification of the phd gene cluster responsible for phenylpropanoid utilization in Corynebacterium glutamicum*. Appl Microbiol Biotechnol, 2016. 100 (4): p. 1871-1881.

13. Balderas-Hernandez, V.E., et al., *Catechol biosynthesis from glucose in Escherichia coli anthranilate-overproducer strains by heterologous expression of anthranilate 1,2-dioxygenase from Pseudomonas aeruginosa PAO1*. Microb Cell Fact, 2014. 13 : p. 136.

14. Sun, X., et al., *A novel muconic acid biosynthesis approach by shunting tryptophan biosynthesis via anthranilate*. Appl Environ Microbiol, 2013. 79 (13): p. 4024-30.

15. Johnson, C.W., et al., *Enhancing muconic acid production from glucose and lignin-derived aromatic compounds via increased protocatechuate decarboxylase activity*. Metab Eng Commun, 2016. 3 : p. 111-119.

16. Jha, R.K., et al., *Sensor-enabled alleviation of product inhibition in chorismate pyruvate-lyase*. ACS Synth Biol, 2019. 8 (4): p. 775-786.

17. Thompson, B., et al., *Muconic acid production via alternative pathways and a synthetic "metabolic funnel"*. ACS Synth Biol, 2018. 7 (2): p. 565-575.

18. Schweigert, N., A.J. Zehnder, and R.I. Eggen, *Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals*. Environ Microbiol, 2001. 3 (2): p. 81-91.

19. Kohlstedt, M., et al., *From lignin to nylon: Cascaded chemical and biochemical conversion using metabolically engineered Pseudomonas putida*. Metab Eng, 2018. 47 : p. 279-293.

20. Baláž, J., T. Kiss, and R.F. Jameson, *Copper(II)-catalyzed oxidation of catechol by molecular oxygen in aqueous solution*. Inorg Chem, 1992. 31 (1): p. 58-62.

21. Carrara, J.M., et al., *cis,cis-Muconic acid isomerization and catalytic conversion to biobased cyclic-C_6_1,4-diacid monomers*. Green Chem, 2017. 19 (13): p. 3042-3050.

22. Johnson, C.W., et al., *Eliminating a global regulator of carbon catabolite repression enhances the conversion of aromatic lignin monomers to muconate in Pseudomonas putida KT2440*. Metab Eng Commun, 2017. 5 : p. 19-25.
23. Jiménez, J.I., et al., *Genomic analysis of the aromatic catabolic pathways from Pseudomonas putida KT2440*. Environ Microbiol, 2002. 4 (12): p. 824-41.