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.

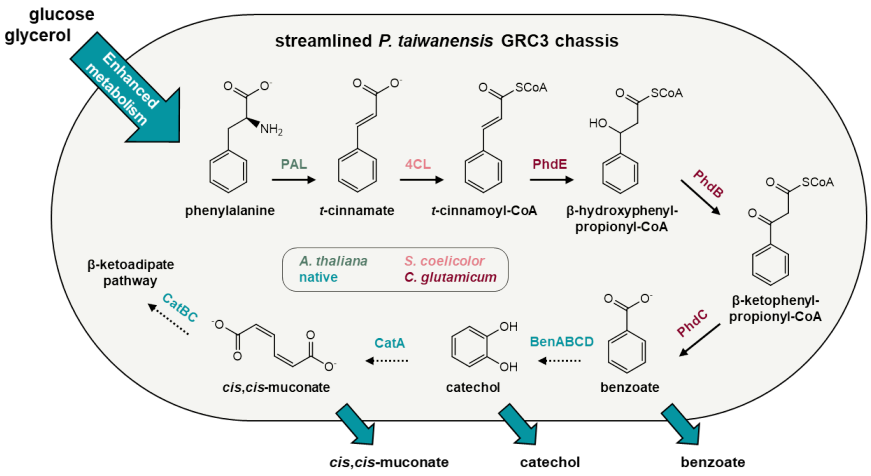

Figure 1 Biosynthetic pathway to convert l-phenylalanine into benzoate expressing heterologous enzymes and subsequent conversion of benzoate into catechol and *cis* ,*cis* -muconate by native enzymes.

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 conjugational 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 OD_{600} 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 (OD_{600}) 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 Δ8Δ*pykA -tap* Δ*benABCD* . Subsequently, the synthetic operon encoding the pathway from l-phenylalanine to benzoate was integrated at the*attTn7* -site under the control of the constitutive promoterP_{14f} [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}*-phdBCDE-4cl-pal* with Δ*benABCD* deletion for the production of benzoate (A,B) or Δ*catBCA* 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 Δ*catB* 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}-*phdBCDE-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 Δ8Δ*pykA -tap* Δ*catBCA attTn7* ::P_{14f}*-phdBCDE-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 Δ8Δ*pykA -tap* Δ*catBattTn7* ::P_{14f} *-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 Δ8Δ*pykA -tap* Δ*catBattTn7* ::P_{14f} *-phdBCDE-4cl-pal* was cultivated in dO_2-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 Δ8Δ*pykA -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.

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