Biotechnology Journal
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Benzoate Synthesis from Glucose or Glycerol Using Engineered *Pseudomonas taiwanensis*

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Benzoic acid is one of the most commonly used food preservatives, but currently exclusively produced in petrochemical processes. In this study, a bio-based production pathway using an engineered strain of *Pseudomonas taiwanensis* is described. In a phenylalanine-overproducing strain, bacterial and plant genes are heterologously expressed to achieve production of benzoate via a β-oxidation pathway. Strategic disruption of the native *Pseudomonas* benzoate degradation pathway further allows 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 could also be performed by microbes, for example, through the upper pathway encoded on the TOL plasmid pWW0 from *Pseudomonas putida* mt-2, but this has not been studied so far in a biotechnological context.[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.

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DOI: 10.1002/biot.202000211

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] Previously, a route for the de novo production of benzaldehyde via the intermediates (*S*)-mandelate and phenylglyoxylate was established.[5] In principle, this route gives access to the production of benzoate when a benzylaldehyde dehydrogenase is expressed simultaneously. However, this pathway involves two decarboxylation reactions and is therefore less carbon-efficient. The β-oxidation pathway applied in this study releases acetyl-CoA fueling the TCA cycle and enabling regeneration of reducing equivalents.

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.[6–9] Benzoate as a product was thus far elusive, mainly because the ferulic acid pathway from *P. putida* only converts hydroxylated phenylpropanoids.[7] In this study, a previously generated *P. 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. 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.

2. 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[9] 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

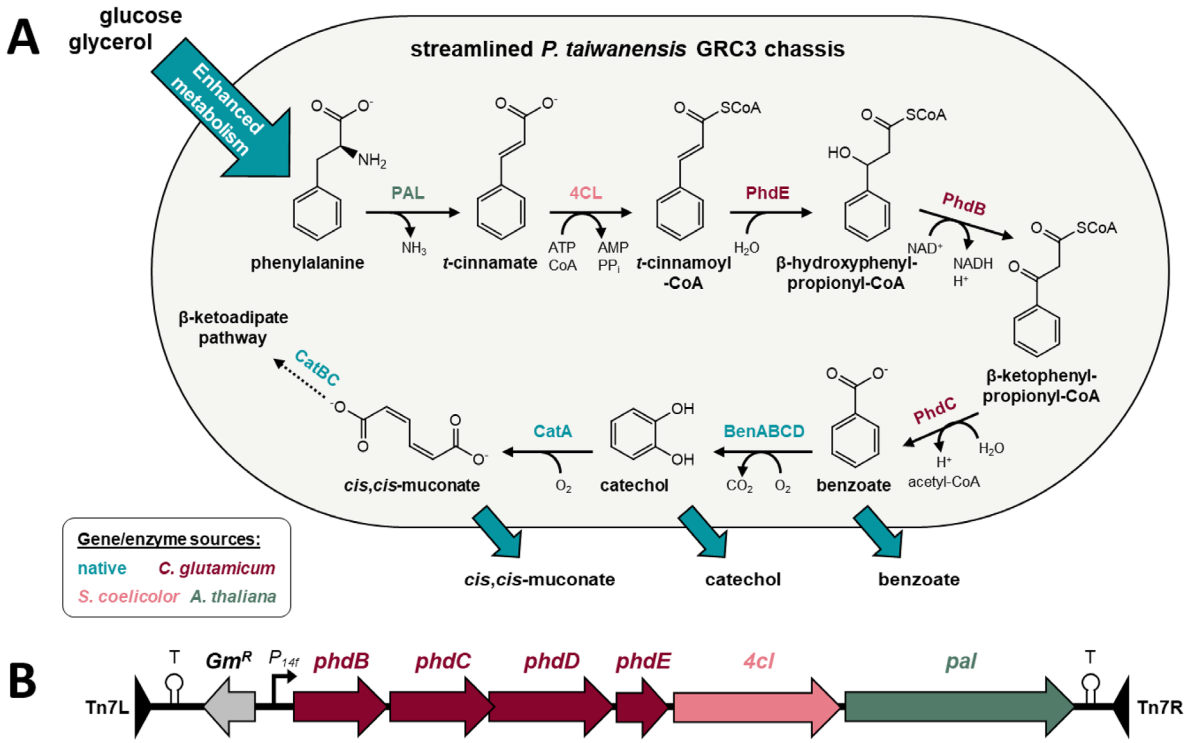

Figure 1. A) Biosynthetic pathway to convert L-phenylalanine into benzoate expressing heterologous enzymes and subsequent conversion of benzoate into catechol and *cis,cis*-muconate by native enzymes. B) Sketch of the synthetic operon integrated into the *attTn7*-site of *P. taiwanensis*. The full annotated sequence is available in the Supporting Information.

under the control of the constitutive promoter P_{14f}.[10] The phenylalanine ammonia-lyase (PAL) deaminates L-phenylalanine to *trans*-cinnamate that is subsequently CoA-activated by the 4-coumarate CoA-ligase (4CL).[11] 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*.[7] Shake flask cultivations were performed to characterize benzoate production from glucose and glycerol (**Figure 2**A). Alternatively, glycerol was used as sole carbon source (Figure 2B). This major by-product of biodiesel was shown to enable higher product yields in other aromatics producers.[6,7,9]

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, which is reasonable considering comparable cultivations with *P. taiwanensis* strains producing similar aromatics,[6,7,9] 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. These results confirm the *de novo* benzoate biosynthesis applying a synthetic pathway, achieving high titers and yields solely from glucose or glycerol.

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,[9] 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[9] 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] which may have caused incomplete substrate utilization. A limitation in the conversion from benzoate to catechol is unlikely, given the lack of production of *trans*-cinnamate or benzoate. Fed-batch fermentations of a recombinant *Escherichia coli*, led to the production of 4.5 g L^{-1} catechol from glucose,[13] even though this microbe has a lower catechol tolerance than *Pseudomonas* species.[19] Pseudomonads were reported to tolerate higher amounts of catechol than produced during the current experiments,[20] and this is also the case for *P. taiwanensis* GRC3, which can still grow in the presence of 5 mM catechol and in fact is more tolerant than the wildtype[7] (Figure S2, Supporting Information). Given that *P. taiwanensis* GRC3 can tolerate much higher externally added catechol concentrations,

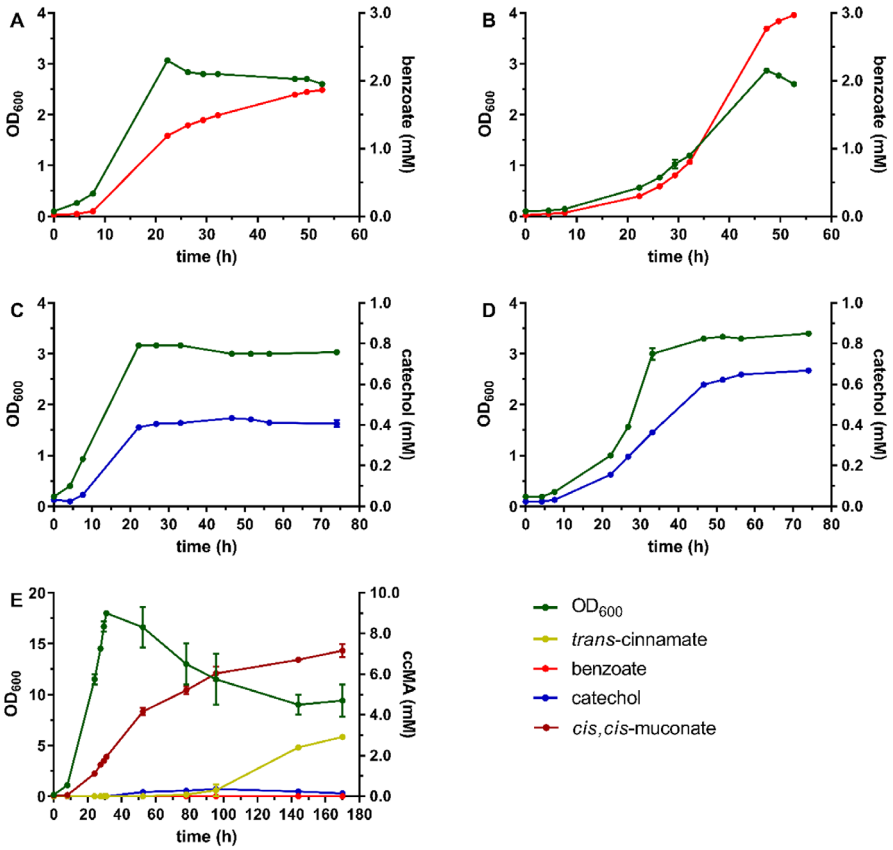

Figure 2. Biosynthesis of benzoate and derivatives by strains of *P. taiwanensis* GRC3 Δ8Δ*pykA-tap attTn7*::P_{14f}-*phdBCDE-4cl-pal* with Δ*benABCD* deletion for the production of benzoate (A,B) or Δ*catBCA* deletion for the production of catechol (C,D) in shake flask in 50 mL MSM with 20 mM glucose (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.

perhaps there is a limitation in catechol export leading to cytoplasmic accumulation and polymerization. Alternatively, the low titers of catechol may be due to its instability in the presence of oxygen and water.[18,21] Clearly, more research is needed here to fully understand and exploit the solvent-tolerance of this strain with respect to this product.

Production of *cis*,*cis*-muconate with *P. taiwanensis* GRC3 Δ8Δ*pykA-tap*Δ*catB attTn7*::P_{14f}-*phdBCDE-4cl-pal* was assessed in dO_2-stat fed batch fermentations with strict pH control (>7) (Figure 2E) to prevent isomerization into *cis*,*trans*-muconate and subsequent lactonization, which occurs readily under acidic conditions.[22] A titer of 7.2 ± 0.4 mM was achieved after 170 h. This proves the feasibility of this novel pathway for *cis*,*cis*-muconate, although the titer is lower than that achieved in other studies of engineered *Pseudomonas* sp., where up to 155 mM was produced.[23] Thus, significant increases can be expected from further strain and process optimization. Around 0.2 mM of catechol started to accumulate after 52 h, 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[24] 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.[20,25] 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.

3. 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.

4. Experimental Section

Media and Culture Conditions: Plasmids and strains used in this study can be found in Table S1, Supporting Information. For cloning purposes, *E. 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)[26] 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 mini-bioreactors (Eppendorf) according to the setup and procedure described in Otto et al.[9]

Plasmid Construction and Genomic Modification: Deletion and expression plasmids were constructed as described in detail in the Supporting 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.[6] Genomic deletions were performed using the I-SceI-based method by Martinez-Garcia and de Lorenzo[27] using a streamlined protocol adapted from Wynands et al.[6] Genomic modifications were verified by colony PCR.

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.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors acknowledge funding by the German Research Foundation (DFG) through the Emmy Noether program (WI 4255/1-1) and the German Federal Ministry of Education and Research (BMBF) through the NO-STRESS project (FKZ 031B0852A). The laboratory of Lars M. Blank is partially funded by the DFG under Germany's Excellence Strategy within the Cluster of Excellence FSC 2186 "The Fuel Science Center." The authors would like to thank Gregg T. Beckham for technical advice regarding the production of *cis*,*cis*-muconate.

Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

benzoate, catechol, *cis*,*cis*-muconate, microbial catalysis, *Pseudomonas*

Received: May 14, 2020
Revised: June 25, 2020
Published online: August 20, 2020

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