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Rescuing Auxotrophic Microorganisms with Non-Enzymatic Chemistry

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Chemical manipulations of small molecule metabolites take place in all living systems and are essential for sustaining life through cellular metabolism.[1] The vast majority of these transformations are carried out by macromolecular, enzymatic catalysts. In contrast to the natural world, most chemical reactions employed in laboratory syntheses utilize non-biological reagents and catalysts.[2] Although non-biological catalysts and reagents have been shown to function in the presence of cells,[3] the question of whether their reactivity can interface with cellular metabolism and influence biological function remains underexplored. We are interested in further developing this aspect of reaction methodology, which we term biocompatible chemistry: non-enzymatic chemical transformations that can be used to manipulate the structures of small molecules in the presence of living organisms. Successful integration of biocompatible chemistry with cellular metabolism would have implications for biotechnology as it could expand the capabilities of biotransformation in ways that are not possible using enzymes. Establishing a link between non-enzymatic catalysis and metabolism could also have potential implications for prebiotic chemistry, as it would suggest that primitive cells could have relied upon non-enzymatic transformations occurring in the surrounding environment to generate essential metabolites.[4] Here we describe biocompatible chemical reactions that interact with microbial metabolism and rescue auxotrophy through the production of essential metabolites. This experimental approach enables us to directly connect the growth of a living organism to the success of a non-biological chemical transformation.

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Auxotrophs are organisms that lack the ability to synthesize key nutrients required for growth and survival.[5] The necessary compounds must instead be obtained from external sources, typically the diet or metabolic activities of other organisms living in close proximity. The numerous examples of auxtotrophs in Nature include humans, who are unable to synthesize many essential vitamins and metabolites. It is possible to rescue auxotrophy in bacteria by transformation with genes encoding enzymes that can replace or complement the missing metabolic activity. Restoration of growth in this way constitutes an extremely powerful strategy for selection of evolved biomolecules in the context of directed evolution.[6] Recently, Hecht and co-workers demonstrated that it is possible to rescue the growth of *E. coli* auxotrophs using de novo protein scaffolds not found in natural systems.[7] Significantly, this result implies that catalysts other than native enzymes can replace key metabolic functions in living cells.

Our interest in merging non-enzymatic chemistry with metabolism led us to explore whether reactions carried out by non-biological catalysts could support the growth of auxotrophic microorganisms (Figure 1A). Rescue of auxotrophy in this manner requires linking a transformation of interest to production of an essential cellular metabolite. We decided to initially apply a ruthenium-catalyzed Alloc deprotection reaction developed by Streu and Meggers that had been previously used in human cell culture.[8] We envisioned connecting this reaction to release of *p*-aminobenzoic acid (PABA), a biosynthetic precursor to folic acid (Figure 1B). Organisms that cannot biosynthesize PABA are unable to grow because of defects in nucleotide metabolism. By using the Alloc deprotection to generate PABA, the success of this non-enzymatic reaction would enable growth of PABA auxotrophs.

We accessed putative rescue substrate Alloc-PABA (**1**) in a single step as reported previously.[9] We then evaluated whether this transformation could take place in growth media. Using 20 μM [Cp*Ru(cod)Cl] (**2**) as a catalyst in PABA-free M9 glycerol minimal medium at 37 °C, we were able to detect formation of PABA from 100 μM **1** (Table 1, Entry 1). As was observed previously, inclusion of a thiol additive boosted conversion (Entries 2, 3). Production of PABA was dependent on the presence of catalyst **2** (Entry 4) and was also observed at lower substrate concentrations (Entry 5). Overall, these experiments verified that deprotection could proceed in media, albeit with low efficiency.

Testing the ability of this reaction to rescue PABA auxotrophy required a microorganism that was unable to synthesize this metabolite. We obtained three different *E. coli* mutants from the Keio collection that are PABA auxotrophs (Figure 1C).[10] Two of these strains (Δ*pabA* and Δ*pabB*) have mutations in the genes encoding the two subunits of aminodeoxychorismate synthase, which catalyzes the penultimate step in PABA biosynthesis.[11] The third mutant (Δ*aroC*) is missing the gene encoding key shikimate pathway enzyme chorismate synthase, and is unable to synthesize not only PABA but also the aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan, as well as the aromatic metabolites *para*-hydroxybenzoic acid (PHBA) and 2,3-hydroxybenzoic acid.[12] We confirmed that all three strains were unable to grow in PABA-free M9 glycerol minimal media and established each strain's inability to utilize substrate **1** in place of PABA. All media used for the ΔaroC mutant were supplemented with the additional required nutrients. As strains from the Keio collection contain a kanamycin resistance cassette, we routinely

cultivated the auxotrophs in media containing this antibiotic to prevent contamination with wild type strains.

We evaluated whether Ru-catalyzed deprotection of **1** could restore the growth of these strains by serially diluting overnight cultures of Δ*pabA*, Δ*pabB*, and Δ*aroC* mutants into PABA-free M9 glycerol minimal media, inoculating each strain into PABA-free M9 glycerol minimal media containing 10 μM **1**, adding catalyst **2** to the cultures at a final concentration of 2 μM, and then incubating the cultures at 37 °C. At the same time, we also inoculated the auxotrophs into M9 glycerol minimal media containing 10 μM PABA (positive control), M9 glycerol minimal media lacking both PABA and **1**, and PABA-free M9 glycerol minimal media containing 10 μM **1** (negative controls). Measurement of the optical density of the cultures (OD_{600}) after 48 h revealed considerable growth of each of the mutant strains in the presence of substrate **1** and catalyst **2** (Figure 2A,B). The final optical densities were comparable to the cultures grown in the presence of PABA. As expected, no growth was observed in the absence of PABA. We evaluated the time course of growth under each of these conditions by recording growth curves over 72 h. Growth curves for mutants rescued by **1** and **2** and mutants grown in media containing 10 μM PABA were similar, with only the rescued Δ*pabA* mutant reaching a lower optical density (Figure 2C). We delineated the requirements for successful auxotroph rescue with a series of control reactions, varying the components of the defined minimal media and measuring final OD_{600} after 48 h at 37 °C (Table S3). We found that the presence of substrate **1** and catalyst **2** are both essential. Thiol addition had a minimal impact on growth, perhaps indicating that the improved conversion observed with these additives does not affect the efficiency of rescue. This result could also suggest that thiols generated by *E. coli* are involved in the deprotection in vivo.

An alternate explanation for auxotroph rescue could be the accumulation of mutations during the course of the experiment that either alleviate PABA auxotrophy or enable the strains to generate PABA from substrate **1**. We investigated these possibilities by re-inoculating rescued cultures back into minimal media lacking PABA (Figure S3, Table S4). None of the rescued cultures grew under these conditions, confirming that they were still PABA auxotrophs. The rescued strains also remained unable to grow in PABA-free media containing 10 μM substrate **1**, indicating that they had not acquired the ability to deprotect Alloc-PABA. All of the rescued cultures retained the ability to grow in media containing PABA and could be rescued a second time in PABA-free medium containing substrate **1** and catalyst **2**. Finally, the fact that three auxotrophic strains, each containing a distinct mutation, were rescued using this deprotection reaction adds to the weight of evidence against genetic changes leading to bacterial growth in these experiments.

A final piece of evidence supporting the link between Alloc deprotection and growth was the detection of PABA in rescued cultures. We determined the concentration of PABA necessary to achieve rescue by inoculating Δ*pabA* and Δ*aroC* mutants into M9 glycerol minimal media containing varying amounts of PABA (10 μM – 10 pM). We found that at least 10 nM of PABA was required to rescue both mutant strains (Table S5). We then used liquid chromatography-mass spectroscopy (LC-MS) to quantify the PABA produced during a rescue experiment (Table S6). Analysis of culture medium after rescue of a Δ*pabA* mutant

revealed 60 nM PABA; therefore, the concentration of PABA generated from the Ru-catalyzed deprotection of **1** is sufficient to allow growth of these auxotrophs. Although we detected PABA in the medium, we do not know to what extent deprotection occurs intracellularly vs. extracellularly; gaining a deeper understanding of where biocompatible reactions take place is a future area of interest.

This initial demonstration of auxotroph rescue relied upon a reaction that had previously been applied in living cells. We decided to explore the generality of this phenomenon by rescuing a different auxotrophy using iron(II)-catalyzed hydroxylation, a chemically challenging transformation that had not previously been utilized in the presence of living organisms. Hydroxylation of aromatic substrates in water using low valent transition metals and molecular oxygen has been extensively investigated, as these systems mimic the reactivity of monooxygenase enzymes.[13] In particular, oxygenation using iron(II) complexes, molecular oxygen, and reducing agents (ascorbic and citric acids) has been previously carried out in aqueous solvent at ambient temperatures, conditions that could potentially be compatible with living organisms.[14] These hydroxylations are thought to proceed via formation of reactive oxygen species, including hydroxyl radicals, making it unclear whether this type of transformation could be biocompatible.[15] However, this reaction has biological relevance, as it resembles chemistry that may have occurred during the oxygenation of the Earth's atmosphere as molecular oxygen reacted with iron present in the environment.[16]

We envisioned rescuing the Δ*aroC* mutant, a *p*-hydroxybenzoic acid (PHBA) auxotroph, using iron-catalyzed *para* hydroxylation of benzoic acid (**3**) (Figure 3A). We first confirmed that the Δ*aroC* mutant could not grow in PHBA-free M9 glycerol minimal media and that media containing 100 μM benzoic acid did not rescue growth (Figure 3B). In analogy to the earlier rescue of PABA auxotrophy, growth media contained all nutrients required for growth except PHBA. We then inoculated the mutant into PHBA-free minimal media containing **3** (10, 100 or 200 μM), $FeSO_4$ (10, 100 or 200 μM), and citric acid (0.2, 2 or 4 mM).[14e] Both of the larger scale cultures were rescued. Control experiments revealed that all reaction components were required for growth (Table S7). Comparison of growth curves for cultures grown in the presence of PHBA and rescued cultures revealed a more pronounced delay of entry into exponential growth phase during rescue than was observed for the PABA auxotrophs (Figure 3C). We hypothesize that this difference may be due to cell death arising from reactive oxygen species generated during the hydroxylation reaction, which would reduce the inoculum. Similarly to the rescued PABA auxotrophs, rescued PHBA auxotrophs remained unable to grow in the presence of **3**, indicating a key role for catalysis in restoration of growth (Table S8, Figure S4). Culturing the Δ*aroC* mutant in media containing varying amounts of PHBA revealed that 10–100 nM PHBA is sufficient for growth (Table S9). We confirmed that PHBA was produced in rescued cultures using LC-MS, detecting 70 nM of PHBA in media post-rescue (Table S10).

Unlike most enzymatic transformations that functionalize aromatic scaffolds, the iron-catalyzed hydroxylation used to generate PHBA is not expected to be regioselective. To establish the selectivity of this reaction, we performed the hydroxylation in culture media using 1 mM **3**. At this higher substrate concentration we could separate and quantify all

three monohydroxylation products using HPLC (Figure S5 and Table S11). As anticipated, the reaction provided a mixture of *para*, *meta*, and *ortho* hydroxylation products (Figure 4D). This result indicates that a non-enzymatic transformation does not have to proceed with the same degree of selectivity as an enzymatic reaction to support growth, an observation that may have significance for prebiotic chemistry and the evolution of new enzymatic function. It also raises the question of whether there is residual promiscuity in native metabolic reactions and pathways that may provide insights into their origins.

In summary, we have demonstrated for the first time that biocompatible, non-enzymatic transformations can rescue the growth of auxotrophic microorganisms by generating essential metabolites. More broadly, this approach enables us to link growth of a living organism to the success of a non-biological reaction. We anticipate that this phenomenon will constitute a powerful strategy for screening and discovering new biocompatible chemistry. Important future challenges include establishing methods to discover more efficient transformations, as well as identifying reactions that can directly replace missing cellular enzymes for use within metabolic engineering and medicine. Overall, this work represents an important step toward interfacing biocompatible transformations with metabolism and constitutes a new way to apply synthetic chemistry in the context of biological systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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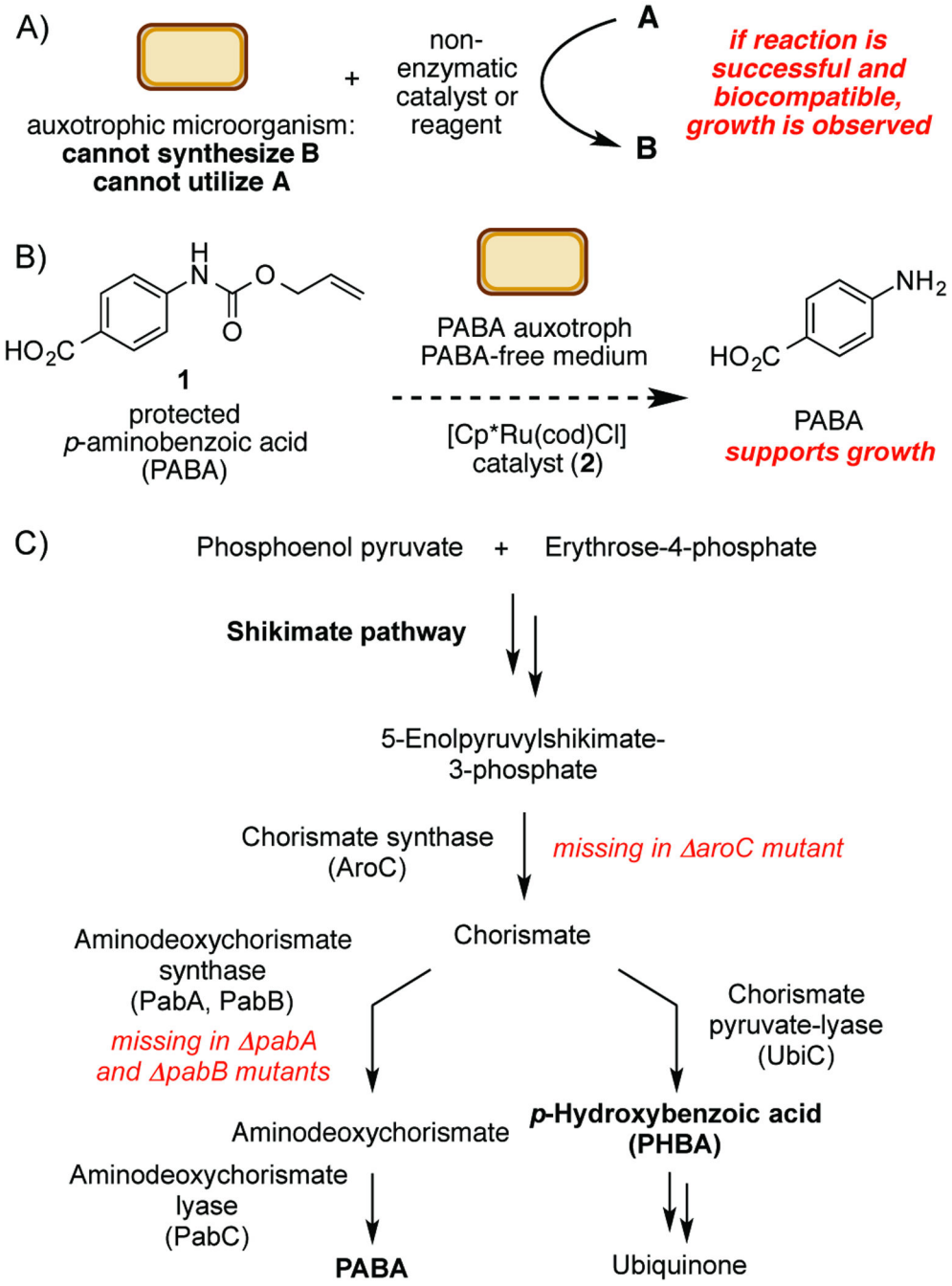


Figure 1.

Rescuing auxotrophy with biocompatible chemistry. A) Overall strategy for auxotroph rescue. B) Plan for connecting biocompatible Alloc deprotection reaction to generation of the essential metabolite *p*-aminobenzoic acid (PABA). C) Biosynthesis of aromatic metabolites in *E. coli* and mutations leading to PABA and *p*-hydroxybenzoic acid (PHBA) auxotrophies.

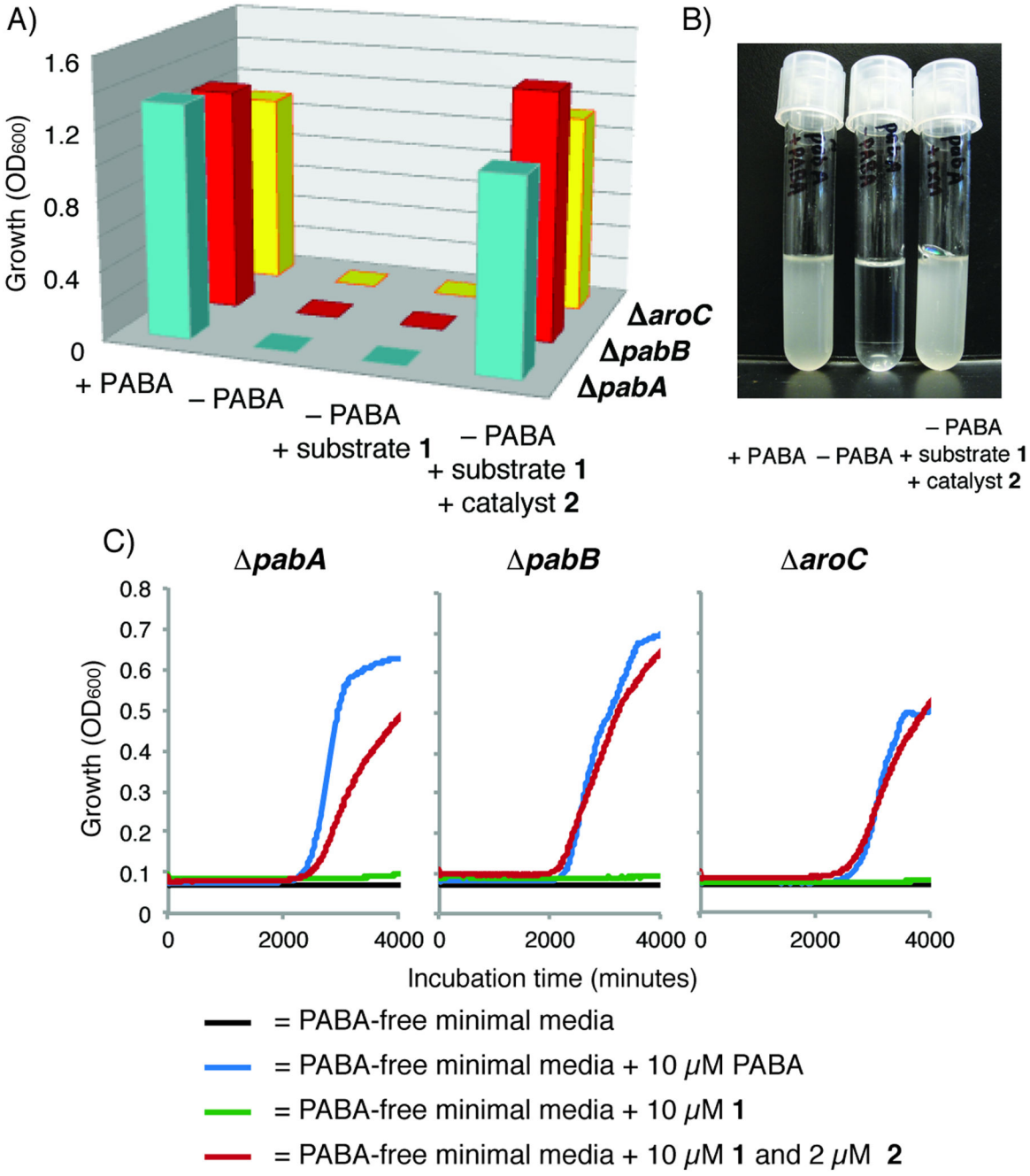


Figure 2.

Biocompatible Alloc deprotection rescues growth of *E. coli* PABA auxotrophs. A) Final optical densities of *E. coli* mutants grown in 5 mL PABA-free M9 glycerol minimal media for 48 h at 37 °C. B) Image of Δ*pabA* mutant cultures after 48 h of growth. C) Growth curves of *E. coli* mutants grown in 150 μL of PABA-free M9 glycerol minimal media at 37 °C. Growth curves shown are the average of three replicate cultures.

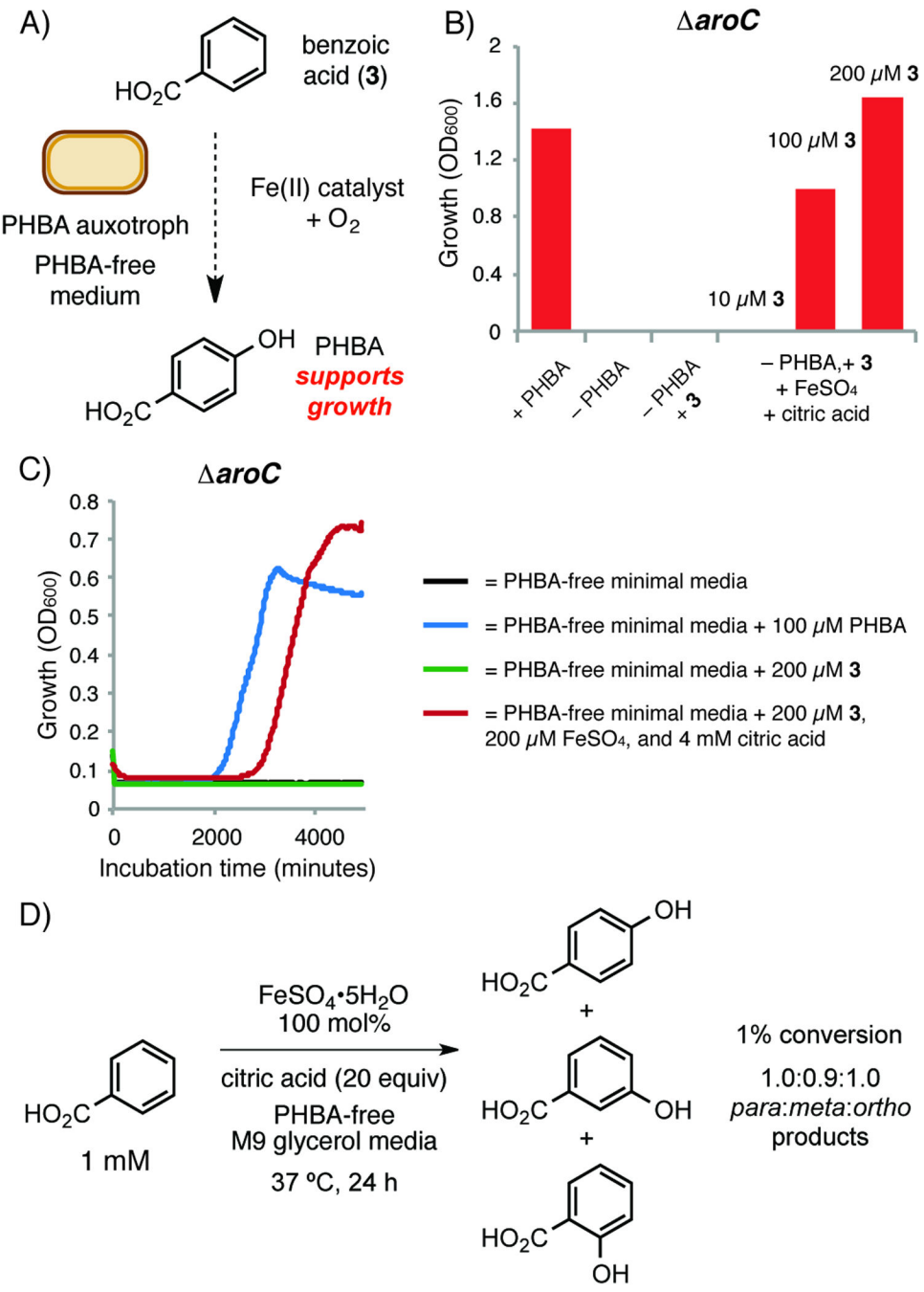


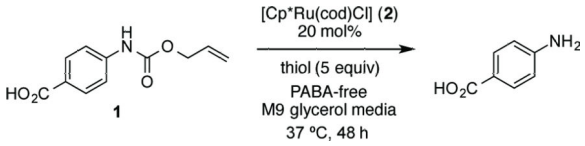
Figure 3.

Rescue of *p*-hydroxybenzoic acid (PHBA) auxotrophs using iron-catalyzed hydroxylation. A) Plan for generating PHBA through hydroxylation of benzoic acid. B) Final optical densities of *E. coli* Δ*aroC* mutant grown in 5 mL PHBA-free M9 glycerol minimal media for 48 h at 37 °C. C) Growth curves of Δ*aroC* mutant grown in 150 μL of PHBA-free M9 glycerol minimal media at 37 °C. Growth curves shown are the average of three replicate

cultures. D) Selectivity of the $FeSO_4$-catalyzed hydroxylation of benzoic acid. Product ratio determined by HPLC analysis.

Table 1

Deprotection of **1** in the absence of cells.



Entry[a]	1 (μM)	2 (μM)	Thiol	Conversion[%][b]
1	100	20	none	8
2	100	20	PhSH	9
3	100	20	GSH	14
4	100	0	PhSH	0
5	10	2	GSH	2

[a] Reactions were run on 5 mL scale for 48 h.

[b] Conversions were determined by HPLC analysis of reaction mixtures.

Abbreviations: PhSH = thiophenol, GSH = glutathione.