Addicting *Escherichia coli* to New-to-Nature Reactions

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**ABSTRACT:** Biocontainment is an essential feature when deploying genetically modified organisms (GMOs) in open system applications, as variants escaping their intended operating environments could negatively impact ecosystems and human health. To avoid breaches resulting from metabolic cross-feeding, horizontal gene transfer, and/or genetic mutations, synthetic auxotrophs have been engineered to become dependent on exogenously supplied xenobiotics, such as noncanonical amino acids (ncAAs). The incorporation of these abiological building blocks into essential proteins constitutes a first step toward constructing xenobiological barriers between GMOs and their environments. To transition synthetic auxotrophs further away from familiar biology, we demonstrate how bacterial growth can be confined by transition-metal complexes that catalyze the formation of an essential ncAA through new-to-nature reactions. Specifically, using a homogeneous ruthenium complex enabled us to localize bacterial growth on solid media, while heterogeneous palladium nanoparticles could be recycled and deployed up to five consecutive times to ensure the survival of synthetic auxotrophs in liquid cultures.

**Synthetic biology aims to take advantage of designer organisms for diverse applications including the sustainable synthesis of fine and bulk chemicals, the cleanup of environmental pollutants, or their use in biomedicine.** However, deploying genetically modified organisms (GMOs) in such real-world applications bears the risk that variants escaping their intended operating environments could harm both ecosystems and human health. As a result, any GMO with the potential of being (deliberately or unintentionally) released into the environment must feature effective biocontainment safeguards that restrict its proliferation in space and time (= trophic containment).

Exploiting the inability of an organism to synthesize a compound that is essential for growth (= auxotrophy) is a straightforward means to achieve biocontainment. For example, rendering essential genes in key metabolic pathways inoperable in addicted organisms, whose survival becomes strictly dependent on the exogenous supply of this metabolite (= metabolic auxotrophy). In practice, this type of biocontainment is often ineffective, as auxotrophic organisms can either regain functional genes via horizontal gene transfer and/or obtain the essential metabolite by metabolic cross-feeding (e.g., the lysine contingency in *Jurassic Park*). To avoid the possibility of genetic and metabolic cross-talk (= semantic containment), an amended version of this approach has recently been introduced, in which survival is made dependent on compounds that are not naturally occurring (= xenobiotics).

Both noncanonical amino acids (ncAAs) and unnatural nucleobases have been successfully employed for restricting bacterial growth to artificial environments in which these xenobiotics are supplied (= synthetic auxotrophy, Figure 1B). For example, the suppression of in-frame stop codons by orthogonal translation systems (OTS, Figure 1C) enables the site-specific incorporation of ncAAs into essential proteins. In the absence of the non-natural building block, premature termination of translation is triggered, resulting in a truncated and inactive protein fragment. As a result, this functional connection renders ncAAs essential metabolites and also can prevent metabolic cross-feeding, if the ncAA is not readily available in natural habitats. Moreover, this strategy also provides a path toward halting genetic cross-talk between designer microbes and organisms in the environment, since the genetic code used by the former cannot be readily understood by the latter. An extension of this strategy is the creation of designer organisms that have undergone full-genome recoding and make use of an unambiguous, alternative genetic code.

In addition to providing a proof-of-concept for synthetic biocontainment strategies, these studies also facilitate further xenobiological sophistications that could transition contain-
Figure 1. Employing auxotrophy in (synthetic) biocontainment strategies. (A) In metabolic auxotrophy, an essential metabolite is provided to an organism that lacks the ability for its biosynthesis. (B) Synthetic auxotrophy takes advantage of recoded organisms that have been made dependent on the supply of xenobiotics, such as ncAAs or unnatural nucleobases. (C) Schematic representation of genetic code expansion by amber stop-codon suppression: an ncAA is charged onto an orthogonal suppressor tRNA through the action of an engineered aminoacyl-tRNA-synthetase (aaRS). Once charged, the acylated tRNA is recruited to the ribosome, where it suppresses an in-frame stop codon located on an mRNA, resulting in the site-selective incorporation of the ncAA into a protein of interest. (D) Synthetic biocontainment strategies lend themselves to further xenobiological sophistication. For example, we employ biocompatible catalysts that are able to convert appropriate precursors into essential xenobiotics to add new-to-nature reactions.

Figure 2. Establishing a functional link between E. coli survival and the supply of ncAAs. (A) The plasmid pAddict contains an OTS selective for 3iY/3nY and a β-lactamase variant that only functions when either of these ncAAs is incorporated. As a result, survival of pAddict-harboring E. coli in the presence of ampicillin is dependent on the exogenous supply of 3iY or 3nY. (B) From left to right: pAddict-harboring E. coli cannot proliferate in the presence of ampicillin (5 μg mL⁻¹) but grow when 3nY or 3iY (500 μM) are supplied or in absence of ampicillin. Images were taken after incubating plates at 30 °C for 48 h (see Figure S1 for additional time points and higher ampicillin concentrations). (C) Schematic representation of cell densities measured after 30 h across ampicillin and ncAA gradients. Average OD₆₀₀ values obtained from two biological replicates are represented in blue shades matching the legend provided on the left. Values obtained for individual experiments as well as a time course of the addiction experiment can be found in Figure S2 and Videos S1 and S2.

To verify addiction to either of these ncAAs, bacterial survival was first evaluated on permissive and nonpermissive solid media by monitoring colony-forming units (c.f.u.) over time. As anticipated, when spreading ~500 bacterial cells on LB agar containing ampicillin (5–50 μg mL⁻¹), colonies were only observed when 3nY or 3iY was supplied (500 μM, Figure 2B and Supporting Figure S1). Similarly, when following cell density (OD₆₀₀) over 30 h at 30 °C in liquid cultures, increasing ncAA concentrations enabled bacterial growth at higher antibiotic concentrations (Figure 2C, Supporting Videos S1 and S2 and Figure S2). Consistent with a strict ncAA-dependency, in the absence of either 3nY or 3iY, pAddict-harboring E. coli could not proliferate in a nonpermissive solid or liquid media (Figure 2B,C). As 3nY consistently outperformed 3iY and enabled growth at higher ampicillin concentrations (Figures S1, S2), we selected the former for all further experiments.

The ability to alter cellular environments by promoting transformations in the presence of live cells makes biocompatible catalysts a promising tool for the envisioned xenobiological sophistictions of synthetic biocontainment strategies. For example, transition-metal catalysts have previously been shown that homogeneous catalysts can be used to localize ncAAs or unnatural nucleobases. Biocompatibility properties and ncAA gradients. Average OD₆₀₀ values obtained from two biological replicates are represented in blue shades matching the legend provided on the left. Values obtained for individual experiments as well as a time course of the addiction experiment can be found in Figure S2 and Videos S1 and S2.

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employed for promoting abiological transformations in the presence of or inside cells and allowed for replacing inoperative enzymes in metabolic auxotrophs. To confine bacterial growth by employing ncAAs and new-to-nature transformations, we selected the transition-metal-catalyzed deprotection of allyloxy carbonyl-α-substituted amines as a model new-to-nature transformation and prepared alloc-3nY as a suitable ncAA precursor (Figure 3A). From the biocompatible catalysts known to promote the target reaction and function in presence of or inside cells, we made use of a ruthenium-based complex, Ru1 (Figure 3B), as this catalyst is able to perform up to 60 turnovers in the presence of E. coli, while not being deactivated and displaying minimal toxicity.

To evaluate the ability of Ru1 to rescue bacterial growth when alloc-3nY was provided (Figure 3C), OD600 values were monitored for 30 h in 96-well plates across Ru1 and ampicillin gradients in the presence of a fixed concentration of alloc-3nY (500 μM, Figure 3D, Supporting Video S3 and Figure S3). While alloc-3nY proved metabolically stable and did not promote bacterial growth under nonpermissive conditions (≥5 μg mL\(^{-1}\) ampicillin), Ru1 was indeed able to rescue 3nY-addicted bacteria (Figure 3D). Specifically, increasing Ru1 concentrations promoted growth under more stringent conditions, and for catalyst loadings > 2.5%, cell proliferation was comparable to directly supplementing 500 μM 3nY in the absence of the catalyst. Notably though, the combination of Ru1 and alloc-3nY resulted in growth under more stringent conditions, when compared to adding Ru1 to 3nY (Figure 3D). We ascribe this result to both the catalyst and the ncAA displaying some toxicity (vide infra), while the alloc- precursor does not. As such, in the presence of Ru1, the continuous production of 3nY by the catalyst over an extended time period is advantageous when compared to the direct supplementation of the ncAA itself.

With Ru1 being able to rescue synthetic auxotrophs in liquid media, we next explored whether the catalyst’s ability to continuously generate 3nY in its proximity could also allow us to confine bacterial growth on solid media. Toward this end, we first spread ~500 addicted E. coli cells on the surface of permissive and nonpermissive LB agar plates containing alloc-3nY (500 μM). Next, we added 10 μL of a 2 mM Ru1 stock solution on top of the solidified agar and monitored colony formation over a period of 72 h at 30 °C (Figures 3E–G). Consistent with a limited diffusion of Ru1 and the continuous production of 3nY in its proximity, colonies of addicted E. coli were only observed near the center of the plate after 48–72 h (Figure 3E, Figure S4). Conversely, in the absence of ampicillin, colonies appeared evenly distributed on the plate, while alloc-3nY by itself was not able to rescue the synthetic auxotrophs (Figure 3F,G). Notably, in an effort to mimic the localization observed for Ru1 by adding 3nY onto agar plates (10 μL from a 20-mM stock solution), we observed a distinct halo pattern at the location at which 3nY was provided (Figure 3H and Figure S4). This marked difference with respect to supplying Ru1 is consistent with the previously observed toxicity of this ncAA at high concentrations.

Thus, these results highlight the advantage of producing 3nY over time by a biocompatible catalyst, which does not show any apparent toxicity when applied onto agar plates containing live E. coli.

To further localize growth and allow for the redeployment of a catalyst after its initial use, we prepared heterogeneous palladium nanoparticles entrapped in polystyrene beads (PdNPs). Such PdNPs have previously been used for the uncaging of propargyl-(proc)-protected amines (Figures 4A) in vivo, and accordingly, we synthesized proc-p-chloro-D/L-phenylalanine (proc-p-CIF) and proc-3nY as suitable ncAA precursors. We used the deprotection of proc-p-CIF and the subsequent incorporation of p-CIF into a GFP variant featuring a UAG stop-codon at position Y151 to assess the activity of freshly prepared PdNPs in the presence of live E. coli. As we have shown previously, monitoring GFP fluorescence over time in 96-well plates provides information on both the toxicity of the heterogeneous catalyst and its ability to promote the desired transformation. Adding PdNPs at a concentration > 5 mg mL\(^{-1}\) indeed resulted in robust fluorescence levels when proc-p-CIF was provided as substrate and the comparison to appropriate controls further indicated that the nanoparticles do
not display any appreciable toxicity at these concentrations (Figure S5).

Next, we performed analogous deprotections of proc-3nY to evaluate the ability of PdNPs to rescue pAddict-harboring E. coli cells. Unfortunately, our attempts to further localize growth on solid media proved unfruitful. When placing around 100 polystyrene beads featuring entrapped PdNPs on top of an agar plate on which about 500 cells were spread, we did not observe the formation of colonies after extended incubation times (>5 days, Figure S6), a result we attribute to the problem of overcoming the solid–solid phase separation between catalyst and substrate.

Conversely, when adding PdNPs (5 or 10 mg mL\(^{-1}\)) to 5 mL liquid cultures containing proc-3nY (500 \(\mu M\)), ampicillin (15 \(\mu g\) mL\(^{-1}\)), and addicted E. coli, we observed significant cell growth over a period of 24 h (Figure 4B and Figure S7). While \(O_{D_{600}}\) values for PdNPs were significantly lower than those obtained for cultures that were supplemented directly with 3nY, the proc-protected precursor was not able to support cell growth. To further pinpoint that bacterial growth is the result of the conversion of proc-3nY by the heterogeneous catalyst, we quantified 3nY concentrations after 24 h by reverse-phase HPLC (Figure 4B). Indeed, ncAA concentrations obtained correlated with PdNP concentrations and the observed cell densities.

Last, we envisioned that the use of a heterogeneous catalyst could allow us to reuse PdNPs after their initial deployment (Figure 4C). Specifically, following an initial 23-h period of employment, polystyrene beads were separated from cells and extensively washed before new media containing proc-3nY (500 \(\mu M\)), ampicillin (15 \(\mu g\) mL\(^{-1}\)) and pAddict-harboring E. coli cells was added. After each cycle, we quantified cell growth, determined 3nY concentrations by HPLC, and verified that growing cells remained addicted to 3nY (Figure 4D and Figure S8). Indeed, redeployment of PdNPs was possible, and we observed cell growth and 3nY production for five consecutive deployments. Consistent with the deactivation of PdNPs over time, \(O_{D_{600}}\) values and 3nY yields decreased significantly after the third recycling step. Nevertheless, the ability to reuse PdNPs for multiple deployments provides a proof-of-concept for employing heterogeneous catalysts that promote new-to-nature transformations as recyclable biocontainment devices.

Here, we demonstrate how biocompatible transition metal catalysts with abiological reactivities can add safeguards and new features to synthetic containment strategies. Specifically, we demonstrate that both homogeneous and heterogeneous catalysts are able to rescue synthetic auxotrophs by performing new-to-nature reactions that provide an essential ncAA. Directly deploying a homogeneous, ruthenium-based catalyst on solid media enables spatial control over the growth of E. coli that feature a single plasmid that ensured addiction to an ncAA. When using heterogeneous PdNPs for rescuing these synthetic auxotrophs in liquid media, the catalyst could be successfully deployed up to five consecutive times to ensure bacterial survival.

With respect to their potential application to biocontainment, the combination of recoded organisms and biocompatible small-molecule catalysts has some notable advantages. While relying on an expanded genetic code makes the dispersal of genes to wild-type organisms more difficult, the use of small-molecule catalysts that are not genetically encoded prevents such a spread entirely. Moreover, by relying on reactivities unknown to nature, biocompatible catalysts represent a form of xenobiological sophistication that further increase the barrier between wild-type and designer organisms.\(^{10,15}\) We anticipate this gap to widen once other catalyst/transformation combinations are evaluated for their ability to rescue synthetic auxotrophs dependent on ncAAs and unnatural nucleotides as well as designer organisms that have undergone full-genome recoding.\(^{16,20,23}\) The resulting modularity should allow for flexibility and customization, which are attractive features when considering the variety of environments to which GMOs could potentially be applied.

In addition to biocontainment, generating functional links between man-made catalysts with new-to-nature activities and the survival of E. coli could also find applications in other areas
of synthetic biology, such as creating enzymes with abiological activities. Specifically, as bacterial growth is restricted by the rate a catalyst can provide a ncAA, the recruitment of biocompatible catalysts by promiscuous or designed proteins within E. coli could boost the catalytic performance of these essential, abiological cofactors. Under selective conditions, bacteria harboring such enzyme variants will have a competitive advantage over those featuring inactive variants. As ampicillin (or carbenicillin) concentrations provide a tunable selection pressure, the directed or long-term evolution of such artificial metalloenzymes in vivo is therefore within reach.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00713.

Supporting figures, methods, sequences, HPLC data, and NMR spectra (PDF)

Time course (every 10 min) depicting the growth of addicted E. coli over 30 h across ampicillin and 3nY in the presence of alloc-3nY (MP4)

Time course (every 10 min) depicting the growth of addicted E. coli over 30 h across ampicillin and 3nY in the presence of alloc-3nY (MP4)

Time course (every 10 min) depicting the growth of addicted E. coli over 30 h across ampicillin and Ru1 gradients in the presence of alloc-3nY (MP4)

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R.R. designed and performed experiments. C.M. conceived and supervised the project. R.R. and C.M. wrote the manuscript.

Notes
The authors declare no competing financial interest.

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