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Synthetic addiction extends the productive life time of engineered Escherichia coli populations

Peter Rugbjerg*, Kira Sarup-Lytzen*, Mariann Nagy*, and Morten Otto Alexander Sommer†,‡

*Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2800 Kongens Lyngby, Denmark

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Bio-production of chemicals is an important driver of the societal transition toward sustainability. However, fermentations with heavily engineered production organisms can be challenging to scale to industrial volumes. Such fermentations are subject to evolutionary pressures that select for a wide range of genetic variants that disrupt the biosynthetic capacity of the engineered organism. Synthetic product addiction that couples high-yield production of a desired metabolite to expression of nonconditionally essential genes could offer a solution to this problem by selectively favoring cells with biosynthetic capacity in the population without constraining the medium. We constructed such synthetic product addiction by controlling the expression of two nonconditionally essential genes with a mevalonic acid biosensor. The product-addicted production organism retained high-yield mevalonic acid production through 95 generations of cultivation, corresponding to the number of cell generations required for >200-m² industrial-scale production, at which time the nonaddicted strain completely abolished production. Using deep DNA sequencing, we find that the product-addicted populations do not accumulate genetic variants that compromise biosynthetic capacity, highlighting how synthetic networks can be designed to control genetic population heterogeneity. Such synthetic redesign of evolutionary forces with endogenous processes may be a promising concept for realizing complex cellular designs required for sustainable bio-manufacturing.

Significance

Bioproduction of chemicals offers a sustainable alternative to petrochemical synthesis routes by using genetically engineered microorganisms to convert waste and simple substrates into higher-value products. However, efficient high-yield production commonly introduces a metabolic burden that selects for subpopulations of nonproducing cells in large fermentations. To postpone such detrimental evolution, we have synthetically addicted production cells to production by carefully linking signals of product presence to expression of nonconditionally essential genes. We addict Escherichia coli cells to their engineered biosynthesis of mevalonic acid by fine-tuned control of essential genes using a product-responsive transcription factor. Over the course of a long-term fermentation equivalent to industrial 200-m² bioreactors such addicted cells remained productive, unlike the control, in which evolution fully terminated production.

Author contributions: P.R. and M.O.A.S. designed research; P.R., K.S.-L., and M.N. performed research; P.R., K.S.-L., and M.O.A.S. analyzed data; and P.R., K.S.-L., and M.O.A.S. wrote the paper.

Conflict of interest statement: P.R., K.S.-L., and M.O.A.S. are inventors on a patent application (EP15187150) covering the concept of product addiction. The research leading to these results has received funding from the Novo Nordisk Foundation, Denmark, and from the European Union Seventh Framework Programme (FP7-KBBE-2013-7-Single-stage) under Grant Agreement 613745, Promy.

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Data deposition: Deep-sequencing data from the study have been deposited in ArrayExpress (accession no. E-MTAB-6182).

1To whom correspondence should be addressed. Email: msom@bio.dtu.dk.

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**Results**

To engineer durable and effective product addiction (Fig. 1A), we define three design criteria that address general large-scale bioprocess constraints. Design criterion 1: Cells should be non-conditionally addicted to product, i.e., excluding use of antibiotic resistance and prototrophic selection genes. Large-scale bioreactors operate with strict economic and regulatory requirements prohibitive of inducers or specific nutrient omissions that are normally attractive in laboratory-scale molecular engineering. Accordingly, common inducers such as isopropyl β-D-1-thiogalactopyranoside (1), drop-outs of nutrients, or addition of antibiotics are often not economically viable to implement in large-scale bioprocesses. Design criterion 2: Product addiction should not reduce biosynthetic yield and titer. Pleiotropic effects of product addiction might occur as consequence of perturbing expression of genes involved in central transcription, translation, and glucose metabolism limiting the commercial viability of the addiction system. Design criterion 3: Product addiction should pose no or limited basal fitness cost. A system designed for controlling microbial growth potentially reduces cellular fitness even at the permissive ideal conditions of active production by resource depletion or stresses (21, 22) or suboptimal expression tuning. Such fitness costs might lower biosynthetic performance and could positively select for mutations that inactivate the addiction system, which in turn would permit subsequent production declines.

To render the growth control system nonconditional (design criterion 1), we targeted genes encoding cellular enzymes outside of the primary metabolism and chose the chromosomal gene operon of glmM and folP (Fig. 1B) after testing the direct addiction ability of four different essential operons or single genes (SI Appendix, SI Text 1). Not previously applied for selections, both phosphoglucomutase (GlmM) and dihydropteroate synthase (FoIP) are necessary for cell growth in complex and minimal medium. GlmM supplies essential glucosamine-1-phosphate for biosynthesis of lipopolysaccharide and peptidoglycan of the cell envelope (23). FoIP is involved in tetrahydrofolate biosynthesis (24), and thus is also a possible nonconditional growth regulator.

**Dose-Sensitive Metabolite Addiction with Minimal Burden.** We first tested the dose-sensitivity of the folP-glmM operon for regulating growth when transcriptionally controlled by the AraC transcription factor in response to its well-characterized inducer L-arabinose (25). In our designs, we assumed that careful molecular titration of the actuating essential gene is critical for efficient control of growth: too high basal expression may result in limited or no control of growth, whereas contrasting low basal expression causes growth reductions (fitness cost) even in the presence of intentional intracellular product concentrations. Therefore, to limit the basal burden of the system (design criterion 3) and also balance expression to a level of dose-sensitive growth regulation, we generated a small pool of translational and transcriptional variants in the ribosome-binding site (RBS) strengths for folP and the product-responsive promoter (Fig. 2A). We thus generated clone candidates by swapping the native essential gene operon promoter with two responsive (P_{PhAD}) variants using lambda Red recombineering (26) (Materials and Methods). Within the integration DNA constructs, we simultaneously introduced four different RBSSs (SI Appendix, Table S4) for folP, selected to span an interval in the low-expression region (Fig. 2A) (27) to further increase chances of selecting an ideally tuned clone. Due to our intended, nonconditional product dependency, we directly fed the transformed clone candidates with 0.1–0.5% L-arabinose in LB agar plates and liquid medium (Materials and Methods). Indeed, in minimal M9 medium, we observed different growth rates of the resulting clones, which matched the different RBS strengths (Fig. 2B and SI Appendix, Table S4). This indicated that folP was regulating growth at dynamic levels. Three clones with high fitness, e3.5, e3.5m, and e3.6, displayed addiction to L-arabinose in liquid cultures (Fig. 2B) with various degrees of system fitness cost, compared with the control strain AraC with wild-type folP-glmM promoter. Clones with lower predicted folP RBS strength were still L-arabinose-responsive yet screened out due to much slower growth and, consequently, higher escape propensity. In the absence of L-arabinose, e3.5m displayed >40% reduction in average growth rate (Fig. 2B), indicating significant addiction to L-arabinose. Furthermore, e3.5m appeared to demonstrate our design criterion 3 by very little growth restriction in the presence of the sensed metabolite (Fig. 2B). We further validated the growth response in complex tryptone-based (2xYT) medium to demonstrate that addiction was not dependent on nutrients lacking in our M9 minimal medium (SI Appendix, Fig. S1).

**Synthetic Addition to Mevalonic Acid Biosynthesis.** Utilizing the least fitness-costly design for L-arabinose addiction (e3.5m), we next wanted to develop a mevalonic acid–addicted production strain by shifting the biosensor to the mevalonic acid–responsive AraC_{mev} (28). Mevalonic acid is an attractive chemical building block for products such as fragrances and plastics and with available high-performance heterologous pathways in Escherichia coli (29, 30). AraC_{mev} responds to mevalonic acid starting at 10 mM (1.5 g/L) exogenous mevalonic acid (28). Since intracellular requirements should be lower, we hypothesized that this system could match the productivities of current E. coli mevalonic acid pathways at around 0.3 g/L/h (30). Due to our nonconditional mevalonic acid addiction design, we wanted to ensure early, sensor-saturating levels of mevalonic acid in the production strain. We therefore engineered a constitutively expressed version of a known mevalonic acid pathway based on overexpressed E. coli atoB and Lactobacillus casei mvaS and mvaE (30). Next, we also supplied araC_{mev} and finally recombinered the PhAD-RBS-folP-glmM design of e3.5m into the chromosome. The resulting strain, e3.9, would readily form colonies in the absence
of supplied mevalonic acid, likely owing to efficient biosynthesis of mevalonic acid (21 mM in broth) (Materials and Methods).

**Product Addiction Prevents Production Declines on a Long-Term Scale.** To test the performance of our mevalonic acid addiction system, we experimentally simulated durations of scale-up from the master cell bank to large production bioreactors (>200 m³ volume) by serial passaging (8) and compared the addicted e3.9 strain to the nonaddicted control strain pe1 with wild-type folP-glmM promoter. To avoid stationary-phase cultures not commonly desired in industrial processes, we passaged the four parallel lineages of each strain in exponential phase strictly every 16 h for a total of 14 times, while freeze-storing the four parallel lineages of each strain in exponential phase (70% of the theoretical maximum). However, in accordance with their rising growth rates during the long-term experiment (Fig. 3A), the pe1 lineages gradually lost mevalonic acid production (Fig. 3B), likely due to selective enrichment of nonproducing cells in the populations (8). Overall, these production declines corresponded to a half-life of the population productivity of 50 generations. In all four nonaddicted lineages, mevalonic acid production ultimately fell to below 5% within the accumulated 95 generations studied. In contrast, the four product-addicted lineages of e3.9 retained their initial low growth rates and effectively endured 95 generations without statistically significant improvements of maximum growth rates (Fig. 3C). Coherent with not gaining fitness, the four product-addicted lineages remained >95% productive at the end of the large-scale simulated fermentation at generation 95 (Fig. 3D). Thus, these addicted populations maintained the functional metabolic pathway for a significantly prolonged cultivation period, postponing the beginning of a decline from 50 to at subpopulations of detrimental genetic variants as previously described in long-term cultivations (8).

Next, we recultivated the stocked cell samples to investigate the mevalonic acid production dynamics over the long-term experiment. As designed for criterion 2, initial production in product-addicted and nonaddicted strains was equal at, respectively, 2.9 and 3.1 g/L mevalonic acid (Materials and Methods and Fig. 3 B and D), corresponding to a yield of 0.38 g/g glucose (70% of the theoretical maximum). However, in accordance with their rising growth rates during the long-term experiment (Fig. 3A), the pe1 lineages gradually lost mevalonic acid production (Fig. 3B), likely due to selective enrichment of nonproducing cells in the populations (8). Overall, these production declines corresponded to a half-life of the population productivity of 50 generations. In all four nonaddicted lineages, mevalonic acid production ultimately fell to below 5% within the accumulated 95 generations studied. In contrast, the four product-addicted lineages of e3.9 retained their initial low growth rates and effectively endured 95 generations without statistically significant improvements of maximum growth rates (Fig. 3C). Coherent with not gaining fitness, the four product-addicted lineages remained >95% productive at the end of the large-scale simulated fermentation at generation 95 (Fig. 3D). Thus, these addicted populations maintained the functional metabolic pathway for a significantly prolonged cultivation period, postponing the beginning of a decline from 50 to at
least 85 generations (Fig. 3 B and D). This gain corresponds to a massive increase in terms of functional working volume due to exponential growth: 60 generations of cultivation would match the cell divisions required to saturate a 200-m³ bioreactor at high cell density. This evasion of otherwise evolving production declines strongly indicates that the product addiction design functioned in the manner intended, synthetically confining the cells to the otherwise unfavorable state of high-yield metabolite production.

Product Addiction Limits Accumulation of Genetic Pathway Heterogeneities. Genetic heterogeneities in populations of engineered bioproduction strains can be uncovered by matching mathematical models with deep sequencing and algorithms for population-level assessment of structural genetic variation and SNPs (8). We wanted to explore whether the product addiction system suppressed accumulation of such genetic heterogeneities in the population. Accordingly, we deep-sequenced (>1,000-fold average coverage, SI Appendix, Table S6) the pathway populations at three time points during the long-term experiment using short-read (Illumina) DNA sequencing. We found that mobile elements in the populations of the nonaddicted production lineages enriched over the course of the simulated fermentations. They reached 94–100% presence in the production plasmid populations at the final sampling time (Fig. 4 A), explaining well the simultaneous phenotypic observations of production decline (Fig. 3 A and B). The plasmid disruptions were mediated by several different mobile element subtypes from the host XL1 genome, yet were mainly driven by insertion sequences (ISs) IS1 and IS10, which is in agreement with our previous observations for bacterial mevalonic acid production, by a differently encoded production pathway (8). In contrast, the pathway populations of the product-addicted cells remained genetically intact, and mobile elements were only observed at frequencies below 2% at the experimental end point (Fig. 4 A). These genetic observations support the hypothesis that the product addiction system provides a sufficient synthetic selection pressure to select against accumulation of the otherwise detrimental mobile element insertions that abolish biosynthetic capacity in full agreement with the observed phenotypic dynamics (Fig. 3).

All 14 major position-resolved disruptions (Fig. 4) were mediated by IS1 (0.2–56%) and IS10 (36–93%), while 10 additional mobile element subtypes had transposed from the host chromosome into the plasmid populations at generation 86, however, at frequencies below 0.1% (SI Appendix, Fig. S2).

In the populations of the product-addicted lineages, we detected only four low-frequency disruptions (0.1–1.2%) at the sequenced end point (Fig. 4B). One of these disruptions (IS1 in atoB) was already present in equal frequencies at generation 46, indicating that this allele can reside stably in the populations, perhaps since atoB also exists in a complementary native chromosomal copy and IS1 is known to transcriptionally activate downstream genes (8, 31). These low-frequency disruptions do not appear to affect population-level fitness and mevalonic acid production (Fig. 3 C and D); however, such deep genetic dynamics could indicate the onset of future escapes.

We also analyzed the sensor plasmid populations by DNA sequencing at high depth, as sensor mutations or disruptions might render the essential gene operon constitutive since AraC acts as both a repressor and an activator (32); however, in agreement with the observed production stability, we did not detect genetic heterogeneities in the sensor populations that would explain an escape from engineered addiction.

Discussion

Synthetic product addiction is a concept for linking costly, high-yield metabolite production to cellular growth by a product-responsive biosensor regulating nonconditionally essential genes. We have engineered and shown that such a design can prevent formation of detrimental genetic heterogeneity and be implemented without use of classical selection phenotypes (nutrient prototrophies or antibiotic resistance). Previous biosensor-based designs for product monitoring have harnessed classical conditional selection genes in library-wide selections (18, 19) and for enrichment of phenotypic overproducing subpopulations (5); however, medium amendments such as antibiotics are not feasible for most fermentation products due to cost and regulatory restrictions (33). Instead, using the endogenous selective pressure of carefully tuned essential genes, we have shown that an addiction design can significantly stabilize mevalonic acid production to industrially relevant cultivation scales, despite a considerable production load of a high-yielding pathway.

Although difficult to detect at a population level and on a laboratory scale, evolution is a constraining factor in the performance of bio-production strains (8, 12). Biosensor-based product addiction is agnostic to the mechanisms of production declines: By assessing the product, the concept works at the level of the intended phenotypic outcome and thus redirects evolutionary forces for the benefit of an engineered strain design. Addressing such production declines mechanistically is normally a research-intensive step in bioprocess development that faces two fundamental biological factors, namely the spontaneous escape rate from the engineered pathway (e.g., by mobile element transposition, recombination, and replication errors) and the selective production load (e.g., by metabolic toxicities and burdens
of biosynthesis). A genome-reduced E. coli strain, MDS42, free of mobile elements may, e.g., provide significant life-time extension to dispensable heterologous pathways (34), although not necessarily better starting-point production (8). Classic selection schemes with biosensors (PopQC) can enrich phenotypically better subpopulations (5), although this requires medium amendments while the necessary tuning and potential to ensure favorable evolutionary stability of such systems are unknown. Pathway toxicities may be addressed using adaptive laboratory evolution strategies or dynamic pathway activation (1, 35). Combined, these approaches require significant strain redesign and insight into the specific error modes, which makes systems for continuously maintaining correct fermentation populations appealing. Dynamic, nonconditional addiction to production is therefore an attractive alternative strategy to avoid unpredictable, detrimental error modes from limiting bioprocesses at scale.

Synthetic population maintenance should fundamentally require milder growth penalties than library-wide genetic screens, which prompted our use of endogenous cell processes as growth regulators, which are nonconditional in contrast to classical selection genes (design criterion 1). We successfully used the folP-glMM operon; however, other nonconditionally essential processes such as polymerases, gyrase, or toxin/antitoxin systems might also be useful. In designs for biocontainment of genetically engineered organisms, cells have been addicted to supplemented molecules, thereby preventing unintended environmental release (36–39). Such systems have utilized conditional expression of essential genes in circuits that rendered cells strictly viable only under certain conditions such as enzyme redesign for synthetic dependency on a nonconventional amino acid (37). Using endogenous cell processes, we have demonstrated that synthetic biology can be used to confine a cell to an engineered, costly production genotype. To not unduly compromise bioprocess economics, we evaluated the biosynthetic consequence of our essential process perturbation to ensure that the initial isogenic population did not lose production performance by pleiotropic effects (design criterion 2).

An important consideration for synthetic selection designs are the evolutionary forces that promote mutation of critical control nodes. This risk has previously pointed to a need for redundantly layered or toggled selections to prevent escapes due to a single mutation (18, 19, 36). In product addiction, we aimed for such redundancy by the coupling of two systems (production and its addiction), which must mutate individually before escape, while only the production system confers significant reduction in fitness (design criterion 3). Growth restrictions would also delay biomass formation and hence lower the overall process productivity, which effectively increases the required bioreactor capacity (9). A weakness of our system is the concurrent regulation of two essential genes in an operon, which means that a single mutation in the sensor promoter could cause escape despite two acting genes. However, we did not observe this in the present study, likely due to our tuning of the system to minimize fitness cost.

Product addiction is best suited as a sentinel of product path- ways that are active during phases of cellular growth and therefore costly to maintain. This relation is also important since addiction depends on sufficient sensor saturation. Pathways strictly active in stationary growth phase may inherently experience lower genetic instability and be incompatible with a product addiction scheme. Implementation of product addiction systems requires a product-responsive biosensor with a sensitivity that dynamically matches the operational intracellular concentrations in the ideally biosynthetic cells. Sensitivity becomes especially important as extracellular product theoretically might diffuse to cross-feed nonproducing cheater cells and consequently bypass the addiction system. We therefore also searched for nonproductive subpopulations by time-lapse deep sequencing and phenotypic characterization of the fermentation lineages (Figs. 3 and 4) yet did not observe evolving escapes in the addicted lineages. Industrial practice of passing cultures to seed trains of increasing volumes before final scale likely dilutes the product at early stages (9). However, to bridge the sensitivity gap, sensor tuning may also become important to selectively match the high intracellular concentrations of commercialized cell factory strains, e.g., by directed sensor evolution (40) or signal-processing buffers (41). Furthermore, sensor mutagenesis by rational and random approaches have lately shown success in changing transcription factor specificity for recognition of new biological ligands (42, 43). This will be important to enable more matching pairs of sensors with high-yielding, loaded metabolic pathways and bring molecular biosensors to work in large-scale fermentations. Not only sensing for the pathway end product, recent molecular or whole-cell biosensors may indeed prove their worth in bioprocessing by, e.g., monitoring of lactate, glycolytic flux or pH levels, and accordingly expand the applicability of product addiction strategies (44, 45). In conclusion, we have demonstrated the concept of a synthetic product addiction system, which prevents genetic heterogeneities of nonproducing subpopulations from forming in long-term fermentation with a high-yielding mevalonic acid pathway. We anticipate that utilization of such product addiction systems could significantly aid the robust industrial scale-up of bio-production pathways to maintain a wide range of chemicals and cellular states.

Materials and Methods

**Plasmids.** Plasmids listed in Table 1 were used to generate E. coli strains, as listed in Table 2. pBAD18spec was used for uracil excision cloning (SI Appendix, SI Text 2).

**Strains and Their Construction.** E. coli XL1 (“XL1 Blue,” Agilent) was used as host: recA1 endA1 gyrA96 thi-1 hsdR17 (R trpL60 leuB7,6 galK16,2 recA1 endA1 gyrA96 thi-1 hsdR17 (R trpL60 leuB7,6 galK16,2 recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F lacZAM15 Tn10 (TetR)]. Standard chemical transformation or electroporation was used to introduce plasmids to the strains listed in Table 2, while linear DNA was introduced as described below.

**Media.** Standard M9 medium was M9 medium with 0.8% glucose and 0.4% casamino acids. All media recipes given in SI Appendix, SI Text 3.

**Cloning of Integration DNA for Promoter Replacement.** Linear DNA for replacement of the essential gene promoter including four RBS variants was generated by uracil-excision cloning of PCR fragments (SI Appendix, SI Texts 1 and 2).

**Table 1. Plasmids used to generate strains**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genetic features</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMVA1</td>
<td>$P_{E.coli}$aroB-mvaS-mvaE,t, cam&lt;sup&gt;+&lt;/sup&gt;, p15A</td>
<td>8</td>
</tr>
<tr>
<td>pBAD18</td>
<td>araC, amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>25</td>
</tr>
<tr>
<td>pBAD18-Cm</td>
<td>araC, cam&lt;sup&gt;-&lt;/sup&gt;</td>
<td>25</td>
</tr>
<tr>
<td>pBAD18spec</td>
<td>araCmev, spec&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pKD46</td>
<td>λ-Arabinose-inducible</td>
<td>26</td>
</tr>
</tbody>
</table>

**Table 2. Strains generated and analyzed in this study from an E. coli XL1 parent**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Encoded sensor</th>
<th>folP-glMM promoter and RBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>araC</td>
<td>pBAD18-Cm</td>
<td>AraC</td>
<td>WT, PR&lt;sub&gt;BAD&lt;/sub&gt;RBS1</td>
</tr>
<tr>
<td>e3.5</td>
<td>pBAD18</td>
<td>AraC</td>
<td>PR&lt;sub&gt;BAD&lt;/sub&gt;RBS2</td>
</tr>
<tr>
<td>e3.6</td>
<td>pBAD18</td>
<td>AraC</td>
<td>PR&lt;sub&gt;BAD&lt;/sub&gt;RBS1</td>
</tr>
<tr>
<td>e3.5m</td>
<td>pBAD18</td>
<td>AraC</td>
<td>PR&lt;sub&gt;BAD&lt;/sub&gt;RBS1</td>
</tr>
<tr>
<td>e3.9</td>
<td>pMVA1, pBAD18</td>
<td>AraCmev</td>
<td>PR&lt;sub&gt;BAD&lt;/sub&gt;RBS1</td>
</tr>
<tr>
<td>pe1</td>
<td>pMVA1, pBAD18</td>
<td>AraCmev</td>
<td>WT</td>
</tr>
</tbody>
</table>

RBS sequences are specified in SI Appendix, Table S4. |
Chromosomal Engineering for Biosensor-Dependent Expression of Essential Genes. Cells were engineered for biosensor-based metabolite-dependent growth recombination assisted with pKD46 by transformation with linear PCR-generated integration DNA encompassing the variants of responsive 

Deep DNA Sequencing and Analysis. Production plasmid populations were purified from chosen time points. The plasmid populations were prepared for Miseq sequencing using the Nextera XT v2 set A kit (Illumina) according to the manufacturer's instructions with the addition of two extra "limited-cycle PCR" cycles. Sequencing was performed in a pooled run with a 150-bp paired-end reading (SI Appendix, SI Text 3).

Measurement of Mevalonic Acid Production and Population Growth Rate. Following the simulated fermentation, each population sample from a 25-μL glycerol stock was used to inoculate 15 mL of M9 medium, and the culture was incubated at 30 °C with shaking at 250 rpm in a Labnet 311DS incubator for 100 h. Subsequently, mevalonic acid was quantified on an Ultimate 3000 HPLC running a 5-MM sulfuric acid mobile phase on an Aminex HPX-87H ion exclusion column (SI Appendix, SI Text 3). Maximum population growth rates were measured in a microtiter plate reader inoculated from the production cultures (SI Appendix, SI Text 3).

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