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Design and Control of Extrachromosomal Elements in *Methylorubrum extorquens* AM1

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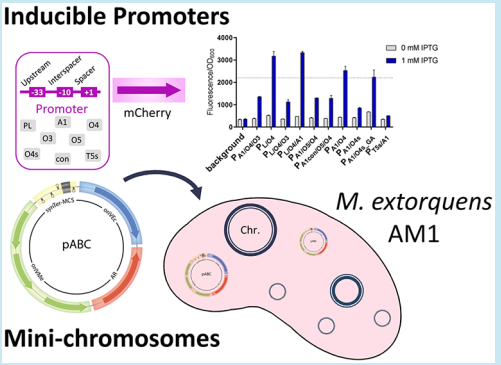
Supporting Information

ABSTRACT: Genetic tools are a prerequisite to engineer cellular factories for synthetic biology and biotechnology. *Methylorubrum extorquens* AM1 is an important platform organism of a future C_1-bioeconomy. However, its application is currently limited by the availability of genetic tools. Here we systematically tested *repABC* regions to maintain extrachromosomal DNA in *M. extorquens*. We used three elements to construct mini-chromosomes that are stably inherited at single copy number and can be shuttled between *Escherichia coli* and *M. extorquens*. These mini-chromosomes are compatible among each other and with high-copy number plasmids of *M. extorquens*. We also developed a set of inducible promoters of wide expression range, reaching levels exceeding those currently available, notably the P_{mxaF} promoter. In summary, we provide a set of tools to control the dynamic expression and copy number of genetic elements in *M. extorquens*, which opens new ways to unleash the metabolic and biotechnological potential of this organism for future applications.



KEYWORDS: *repABC, inducible promoters, synthetic chromosomes, Methylorubrum extorquens, Alphaproteobacteria*

Methylorubrum extorquens AM1 (formerly *Methylobacterium extorquens*) has been used as a model organism to study methylotrophy, *i.e.*, growth on C_1 carbon sources, such as methanol and formate, since its isolation in 1961.[1] The genome of *M. extorquens* AM1 has been fully sequenced, and a genome-scale metabolic network was reconstructed for rational engineering.[2,3] The unique ability of *M. extorquens* to grow on C_1-units has made it an increasingly relevant organism for biotechnology in a methanol- and formate-based bioeconomy.[4] Production of bulk and value-added chemicals such as mevalonate, α-humulene, 3-hydroxypropionate, and 1-butanol has already been realized in *M. extorquens*.[5−8] For the further development of *M. extorquens* as a platform organism in C_1-biotechnology, a broad set of genetic tools are required. Several basic tools are available: one replicative plasmid, *cre-loxP* and *sacB*-based suicide vectors for allelic replacement, and genetic parts including constitutive and, to a lesser extent, inducible promoters.[9−14] Yet, this basic set of genetic tools is far from being complete, and many applications are challenged by the fact that more elaborate genetic tools are missing for this organism.

One particular challenge is the lack of dynamic control of gene expression in *M. extorquens*. The P_{lac} promoter and its derivatives, which are widely used in many bacteria, confer only weak or leaky expression in *M. extorquens*.[10] As an alternative to P_{lac}, cumate-inducible promoters have been created by combining the CuO-CymR system from *Pseudomonas putida* F1 to native P_{mxaF} and $P_{META1p2148}$ promoters.[12,14] While these hybrid promoters are strong upon induction, overexpression of the CymR repressor itself is toxic to the cells causing severe growth defects.

Another, equally important challenge is the availability and control over extrachromosomal genetic elements. Only one broad host range plasmid has been reported that replicates in *M. extorquens* without causing severe growth defects.[9,10] This plasmid (referred to as oriV-traJ′ henceforth) has several disadvantages: a multiple and varying copy number, limited compatibility with other extrachromosomal genetic elements, and the need for constant selective pressure, which restricts its application potential. Genomic integration would circumvent using a plasmid. However, the transformation efficiency is several orders of magnitude lower for genomic integration events, and neutral sites for integration have not been characterized in this organism. It also requires the construction of a suicide vector with the desired DNA cargo flanked by homologous regions (≥0.5 kb) and subsequent verification of integration at the desired locus. All of these steps are work intensive and time-consuming.

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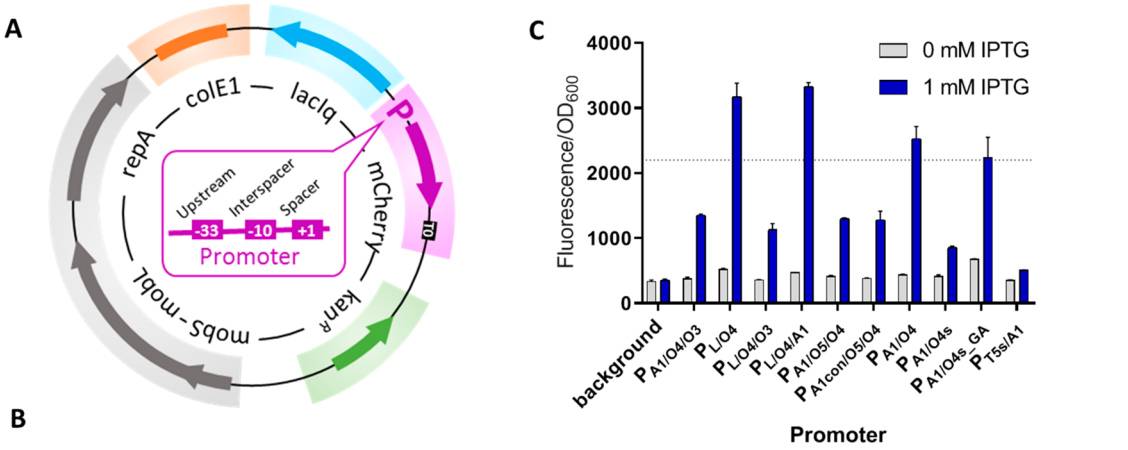

B

Promoter	Upstream region	-33	Interspacer	-10	Spacer	+1
$P_{A1/O4/O3}$	TTTATCAAAAAGAGTG	TTGACT	TGTGAGCGGATAACAAT	GATACT	TAGATTC	AATTGTGAGCGGATAACAATTTCACACA
$P_{L/O4}$	GATAAATTATCTCTGGCGGTG	TTGACA	TGTGAGCGGATAACAAT	GATACT	GAGCAC	ATCAGCAGGACGCACTGAC
$P_{L/O4/O3}$	GATAAATTATCTCTGGCGGTG	TTGACA	TGTGAGCGGATAACAAT	GATACT	TAGATTC	AATTGTGAGCGGATAACAATTTCACACAG
$P_{L/O4/A1}$	GATAAATTATCTCTGGCGGTG	TTGACA	TGTGAGCGGATAACAAT	GATACT	GAGCAC	ATCGAGAGGGACACGGCGA
$P_{A1/O5/O4}$	AATTGTGAGCGGTAACAATTTAG	TTGACT	TGTGAGCGGATAACAAT	GATACT	TAGATTC	ATCGAGAGGGACACGGCGA
$P_{A1con/O5/O4}$	AATTGTGAGCGGTAACAATTTAG	TTGACT	TGTGAGCGGATAACAAT	TATAAT	TAGATTC	ATCGAGAGGGACACGGCGA
$P_{A1/O4}$	TTTATCAAAAAGAGTG	TTGACT	TGTGAGCGGATAACAAT	GATACT	TAGATTC	ATCGAGAGGGACACGGCGA
$P_{A1/O4s}$	TTTATCAAAAAGAGTG	TTGACT	TGTGAGCGCTCACAATT	GATACT	TAGATTC	ATCGAGAGGGACACGGCGA
$P_{A1/O4s\_GA}$	TTTATCAAAAAGAGTG	TTGACT	TGTGAGCACTCACAATT	GATACT	TAGATTC	ATCGAGAGGGACACGGCGA
$P_{T5s/A1}$	TTTATCAAAAAGAGTG	TTGCTT	TGTGAGCGCTCACAATT	TATAAT	TAGATTC	AATTGTGAGCGGATAACAATTTCACACAG

Figure 1. IPTG-inducible promoters for *M. extorquens*. Map of pIND4-derived plasmid for testing new promoters expressing mCherry (A). Sequence of hybrid promoters constructed and tested (B); *lacO* sequences are shown in red. The point mutation within the *lacO4s* region is underlined. Fluorescence/OD_{600} before and after addition of 1 mM IPTG (C). The dashed line marks fluorescence/OD_{600} signal obtained using the strong P_{mxaF} promoter.

Many Alphaproteobacteria have multipartite genomes composed of a main chromosome and one or more megaplasmids. The replication of megaplasmids is integrated into the bacterial cell cycle and occurs once to ensure faithful transmission of genetic information.[15,16] A small region within these megaplasmids, termed the *repABC* cassette, drives vertical transmission of these secondary replicons. *repA* and *repB* encode for partitioning proteins analogous to the well-known ParA and ParB, while RepC is a replication initiator protein. The origin of replication resides within the *repC* coding sequence, whose expression is downregulated by a short, nontranslated counter-transcribed RNA (ctRNA) typically located in the *repB*/*repC* intergenic region.[15−17] On the basis of this distinctive region, a new family of *repABC*-type shuttle vectors was successfully designed for *Sinorhizobium meliloti* mimicking the characteristic features of secondary replicons, *i.e.*, single copy number and stable propagation.[18] Four of these *repABC* regions were shown to be able to replicate in *M. extorquens* but they affected the growth of the organism.[18]

Here, to expand the limited genetic toolbox of *M. extorquens* AM1, we developed a set of inducible, orthogonal promoters of different strengths, which can be dynamically controlled by IPTG. Furthermore, we systematically tested new *repABC* regions to establish a set of extrachromosomal elements ("mini-chromosomes") that are faithfully inherited by daughter cells and compatible with each other. This work provides the tools for the extensive genetic engineering of *M. extorquens* AM1 in the future.

RESULTS AND DISCUSSION

Realization of Tight, IPTG-Inducible Promoters with a Dynamic Range. To overcome the problem of weak and leaky expression from IPTG-inducible promoters in *M. extorquens*, we sought to develop different *lacO*-controlled promoters that are tight and show a wide range of expression levels after addition of the inducer.

The $P_{A1/O4/O3}$ promoter,[19,20] also known as $P_{A1lacO-1}$, was functional in *M. extorquens* with 27-fold induction of the fluorescence reporter gene mCherry (Figure 1C). The $P_{A1/O4}$ and $P_{A1/O4s}$ promoters[21] were also functional and showed 21- and 6-fold induction, respectively. A single point mutation within the O4s region of $P_{A1/O4s}$, resulting in $P_{A1/O4s\_GA}$, showed increased mCherry expression at the expense of leakiness without inducer.

The T5 promoter has been used for many years for protein overexpression in *E. coli*.[22−24] The $P_{T5\text{-}lac}$ promoter from pCA24N was weakly functional in *M. extorquens*, but there was no difference in the fluorescent signal between uninduced and induced states (data not shown). We modified the upstream region and replaced *lacO4* for a *lacO4s* sequence resulting in promoter $P_{T5s/A1}$ that is tightly repressed but only weakly induced (11-fold). While $P_{T5s/A1}$ is not suitable for gene overexpression, it might be interesting for applications where a protein is toxic if expressed at high levels or constitutively within the cell.

We also created three $P_{L\text{-}lacO}$ hybrid promoters, $P_{L/O4}$, $P_{L/O4/O3}$, and $P_{L/O4/A1}$, which are all strongly repressed in the absence of inducer. Upon induction, $P_{L/O4}$ and $P_{L/O4/A1}$ exhibit 15- and 23-fold induction of mCherry, respectively. Notably, these two promoters provide mCherry fluorescence levels that are approximately 1.5-fold higher than those of the P_{mxaF}

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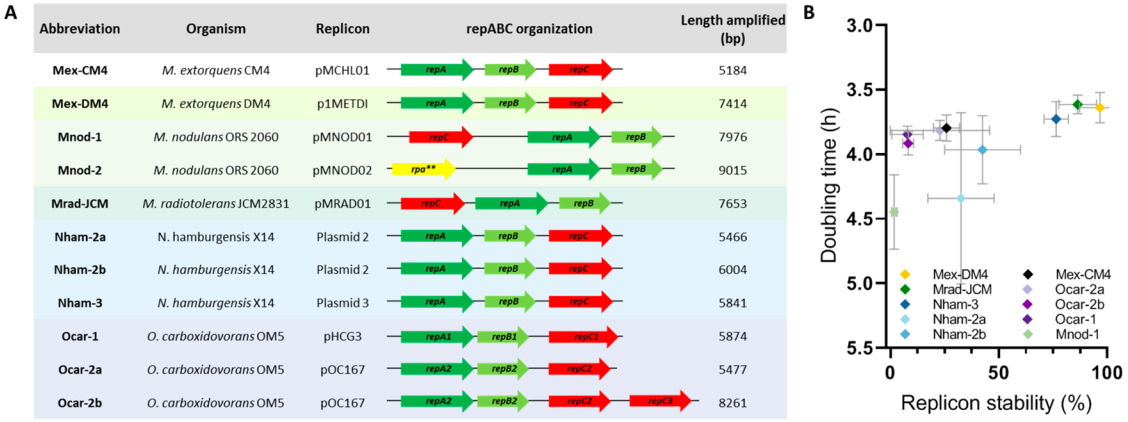

Figure 2. *repABC* regions tested in *M. extorquens*. (A) Size and operon organization. (B) Doubling time *versus* replicon stability (% of kanamycin resistant colonies) of *repABC* regions in the suicide vector pK18mob2. Mean and SD from three biological replicates.

promoter, which drives 9% of soluble protein expression in *M. extorquens*[25] (Figure 1C, dashed line shows P_{mxaF} levels). The dynamic range of the $P_{L/O4/A1}$ promoter is given in Figure S1. See Table S4 and S5 for more details on all inducible promoters tested in this study.

In summary, we created and identified a set of IPTG-inducible promoters for *M. extorquens* that range between 6- and 36-fold induction (Table S4). Additionally, our P_{A1}- and P_L-derived promoters range in maximum strength between 9% and 166% of the strong P_{mxaF} promoter, which opens new possibilities for the controlled overexpression of proteins. Thus far, the highest expression level reported from an inducible promoter in *M. extorquens* was 33% of the P_{mxaF} promoter.[13] To facilitate the use of these new promoters by the scientific community, we created empty vectors featuring the respective promoter and a multiple cloning site with an optional N-terminal Strep-II tag compatible with the Methylobrick system[9] and pET vectors (Novagen).

Identification of Suitable *repABC* Regions for Use in *M. extorquens* AM1. To establish artificial mini-chromosomal elements in *M. extorquens*, we tested 11 *repABC* regions from six different organisms. We introduced these regions into pK18mob2, which is a "suicide" vector unable to replicate in *M. extorquens* (Figure 2A and S2). All *repABC* regions were maintained as independent replicons in *M. extorquens*. The *repABC*-based replicons were recovered for restriction analysis and sequencing of the *repABC* region to confirm their integrity. The only exception was Mnod-2 from *Methylobacterium nodulans*, for which no colonies were obtained after electroporation, likely due to incompatibility of the Mnod-2 *repABC* region with the native genomic system.

Next, we determined the growth behavior of *M. extorquens* carrying each replicon under selective conditions, as well as replicon stability after 96 h under nonselective conditions (Figure 2B). We observed a general trend that replicon stability and growth behavior of *M. extorquens* were correlated. Mnod-1, also from *M. nodulans*, was very unstable, showed the lowest doubling time (Figure 2B) and a long lag phase (data not shown), suggesting that *repABC* cassettes from this organism are generally not well compatible with *M. extorquens*. Similarly, Nham-2a and Nham-2b from *Nitrobacter hamburgensis* are very unstable and affected the growth. Replicons originating from *Oligotropha carboxidovorans* were also unstable in the absence of selective pressure, in particular Ocar-1 and Ocar-2a, discouraging the use of these elements for genetic manipulations of *M. extorquens*.

The *repABC* cassette Mex-CM4 originating from *M. extorquens* CM4 showed an interesting behavior. Mex-CM4 allowed fast doubling of *M. extorquens* AM1 under selective conditions, while cells quickly lost the replicon in the absence of antibiotic pressure (Figure 1B and S4). The *repABC* cassettes of Mex-CM4 and Mex-DM4 (discussed below) are 98% identical to each other, except for the fact that Mex-DM4 has a longer region downstream of *repC* (Figure 1B and Figure S2). It might be the case that Mex-CM4 lacks some *parS* sites required for a faithful transmission of the respective replicon to the daughter cells. This behavior makes Mex-CM4 an interesting system to establish CRISPR-Cas in *M. extorquens* where transient expression followed by fast extinction of the genetic element is a desired feature.

The replicons Mex-DM4 (originating from *M. extorquens* DM4), Mrad-JCM (from *M. radiotolerans* JCM2831), and Nham-3 (from *N. hamburgensis* X-14 plasmid 3) showed the highest stability with 97%, 86%, and 77% of cells still harboring the plasmid after 96 h without antibiotic selection, respectively. The replicons were isolated from kanamycin resistant cells after 96 h without selective pressure and their integrity was verified by restriction analysis (data not shown). These three replicons allowed fast doubling times of *M. extorquens*, reaching almost wildtype-like growth behavior, and were present at a copy number of 1 (Table 1), indicating that they are good candidates for the construction of mini-chromosomes.

Table 1. Copy Number of Stable *repABC* Cassettes in *M. extorquens*[a]

replicon	copy number (mean ± SD)
oriV-traJ′ (pTE101)	8 ± 1.21
Mex-DM4 (pAD1)	1 ± 0.17
Mrad-JCM (pAD3)	1 ± 0.13
Nham-3 (pAD6)	1 ± 0.09

[a]Mean ± SD from three biological replicates.

Assembly and Testing of Mini-Chromosomes in *M. extorquens* AM1. Next, we used the stable *repABC* regions identified in the pK18mob2 system plus Mex-CM4 to construct mini-chromosomes that can be shuttled between *M. extorquens* and *E. coli*. The pABC vector concept and assembly strategy is based on four basic modules each with

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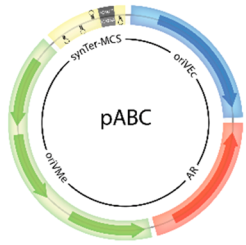
Table 2. Modular Construction of *repABC*-Based Mini-Chromosomes[a]

Name	oriVMe	oriVEc	Antibiotic Resistance	SynTer-MCS	Build up Scheme
pABC-DM4K	Mex-DM4	pMB1	Kanamycin	1	
pABC-JCMK	Mrad-JCM	pMB1	Kanamycin	1	
pABC-X14K	Nham-3	pMB1	Kanamycin	1	
pABC-CM4K	Mex-CM4	pMB1	Kanamycin	1	
pABC-DM4G	Mex-DM4	p15A	Gentamicin	2	
pABC-X14G	Nham-3	p15A	Gentamicin	1	
pABC-DM4T	Mex-DM4	p15A	Tetracycline	2	
pABC-X14T	Nham-3	pSC101*	Tetracycline	3	

[a]Abbreviations: oriVMe, *repABC*-based origin of replication for *M. extorquens* AM1; oriVEc, origin of replication for *E. coli*; SynTer-MCS, multiple cloning site flanked by synthetic terminators.

Table 3. Doubling Times (h) of *M. extorquens* with *repABC*-Based Mini-Chromosomes in Single and Double Compatibility Tests[a]

origin of replication	Mex-CM4	Mex-DM4	Mrad-JCM	Nham-3	oriV-traJ′	pMG160
CM2720 (strain)	4.11 ± 0.02	3.90 ± 0.01	3.85 ± 0.03	3.94 ± 0.02	4.09 ± 0.05	4.31 ± 0.17
Mex-DM4			X	4.41 ± 0.04	4.15 ± 0.03	4.46 ± 0.06
Mrad-JCM				5.44 ± 0.31	4.68 ± 0.21	5.48 ± 0.27
Nham-3					4.43 ± 0.04	4.49 ± 0.03
oriV-traJ′						5.54 ± 0.40

[a]Mean ± SD from three biological replicates. X = not compatible. pTE100/pTE101 = oriV-traJ′, pIND4/pTE1841 = pMG160. Areas were left blank to avoid redundancy.

several standardized parts (see below). Each part is flanked by standardized linker sequences for ligase chain reaction LCR-based assembly. This enables a fast and convenient build-up of custom-made replicons for individual purposes. Additional modules, if necessary, can be implemented with ease. The position and orientation of each module (Table 2) was designed to minimize crosstalk from adjacent regions. The MCS and the promoter of the *repABC* operon are insulated by flanking transcriptional terminators. The antibiotic resistance cassette is located downstream and in the same orientation as the *repABC* transcriptional unit to avoid reverse transcriptional read-through.

We decided to assemble several mini-chromosomes from the four basic modules, *i.e.*, (1) a *repABC*-based origin for *M. extorquens* AM1 (Mex-DM4, Nham-3, Mrad-JCM, Mex-CM4), (2) an *E. coli* origin of replication (pMB1, p15A, or pSC101*), (3) a minimal antibiotic resistance cassette (Kanamycin, Gentamicin, Tetracycline), and (4) a multiple cloning site (MCS1, MCS2, MCS3) flanked by synthetic terminators (Table 2). When we assembled and tested the final mini-chromosomes, our constructs showed similar or higher growth rates of transformed *M. extorquens* compared to replicative plasmids. Replicon stability of the mini-chromosomes was confirmed *via* flow cytometry, showing similar results as before (Figure S3 and S4). We also assembled pABC mini-chromosomes with a mob site for conjugation and the Cre recombinase under control of constitutive (PcoxB) and inducible (PL/O4/A1) promoters as additional fifth modules (see Table S2).

The modular construction of the mini-chromosomes allowed us to test intercompatibility of replicons while avoiding extended regions of high sequence identity, which could lead to homologous recombination events. Nham-3 was compatible with Mex-DM4 and Mrad-JCM, while the two latter were not compatible with each other (Tables 2 and 3). In addition, we also tested the compatibility of Mex-DM4, Mrad-JCM, and Nham-3 with the pIND4 and pTE101 vectors, respectively. All three mini-chromosomes were fully compatible with those plasmids, further expanding the box of available and compatible extrachromosomal genetic elements in *M. extorquens* AM1. Replicon integrity was verified by restriction analysis from single and double compatibility test of pABCs and/or replicative plasmids. There were no instances of recombination, proving all compatible replicons functioned autonomously (data not shown). In all cases tested, double transformations were possible, adding to the convenience of these tools for their use in genetic engineering of *M. extorquens* AM1.

CONCLUSIONS

Here, we provide a set of novel promoters for *M. extorquens* AM1 that are tight and show a dynamic range of different expression levels upon induction, even exceeding the promoter elements available thus far. We also identified a set of *repABC* regions that allowed us to construct different mini-chromosomal elements that can be stably maintained at single copy number and in different combinations with each other. In addition, we also provide *repABC* regions that allow transient expression in the absence of antibiotic selection, which is a prerequisite to establish CRISPR-Cas-based methods in this organism in the future. Altogether, these tools expand genetic tools available for the engineering of *M. extorquens*, an important platform organism for a sustainable C_1-biotechnology. The genetic tools developed in this study are freely available to the community and hopefully leverage the metabolic and biotechnological potential of *M. extorquens* AM1.

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MATERIALS AND METHODS

Strains and Cultivation Conditions. *M. extorquens* AM1 strains (Table S1) were grown at 30 °C in minimal medium with 123 mM methanol.[26] Antibiotics were used accordingly: kanamycin 35 μg/mL for *M. extorquens* or 50 μg/mL for *E. coli*, gentamicin 7.5 μg/mL for *M. extorquens* or 8−15 μg/mL for *E. coli*, tetracycline 4−10 μg/mL for both, chloramphenicol 34 μg/mL for *E. coli*, ampicillin 100 μg/mL for *E. coli*. *E. coli* TOP10 Δ*dapA* strains were supplemented with 0.3 μM diaminopimelic acid (DAP). *E. coli* was grown at 37 °C in LB medium. *E. coli* TOP10 and DH5α (Thermo Scientific) were used for construction and amplification of all plasmids in this study. Solid medium had 1.5% (w/v) select agar.

Strain Construction. Plasmids (Table S2) were transferred into *M. extorquens* by electroporation or triparental mating.[27] The *dapA* gene of *E. coli* TOP10 was disrupted by an FRT-flanked chloramphenicol resistance cassette, amplified from pKD3 with MC261 + MC262 primers, using the Quick and Easy *E. coli* Gene Deletion kit (Gene Bridges K006-GVO-GB) according to manufacturer's instructions. In brief, 20 μL of recipient *M. extorquens* from a well-grown culture were spotted on nonselective medium and incubated at 30 °C for 24 h. Donor and helper *E. coli* TOP10 Δ*dapA* strains were spotted on top of the recipient cells and incubated at 30 °C for 24 h. pRK2013 was used as helper plasmid. Spotted cells were recovered and plated at appropriate dilutions on selective medium. Donor and helper *E. coli* TOP10 Δ*dapA* cannot grow without DAP. Single colonies were screened for chromosomal integration of pTE1899 by colony PCR.

DNA Manipulation and Plasmid Construction. Refer to Table S2 for detailed information on the construction of each plasmid (Supporting Information). Standard molecular techniques were used for amplification, purification, cloning and transformation of DNA.[28] Point mutations were generated by QuickChange Site-Directed mutagenesis (Stratagene, La Jolla, USA). Ligase chain reaction (LCR) and primer hybridization reactions were performed as published before.[18] T4 Polynucleotide Kinase, FastAP, and FastDigest restriction enzymes were obtained from Thermo Scientific, Q5 DNA polymerase was obtained from NEB, and used according to manufacturer's instructions. DNA oligos were obtained from Eurofins Genomics (Table S3). Plasmid isolation and PCR product purification was performed with NucleoSpin Plasmid and NucleoSpin Gel and PCR Clean-up kits (Macherey Nagel), E.Z.N.A. Plasmid Mini Kit (Omega Bio-Tek), or illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences) according to manufacturer's instructions.

Growth in 96-Well Plates. Cultures were inoculated from a single colony or glycerol stocks into Erlenmeyer flasks with 20 mL of the appropriate medium. Cells from late exponential phase were diluted in fresh medium at an $OD_{600} \approx 0.05$ and 180 μL of cell suspension was aliquoted into Nunclon Delta Surface (Thermo Scientific #167008) 96-well plates. The temperature was kept constant at 30 °C and OD_{600} was recorded every 30 min using a Tecan Infinite M200Pro (Tecan, Männedorf, Switzerland). Data was analyzed using the GraphPad Prism 7 software.

qPCR-Based Copy Number Determination. *M. extorquens* strains were grown in selective medium until OD_{600} 1.0, adjusted to 1.38×10^6 cells ($\triangleq$ 10 ng DNA/μL) and boiled at 95 °C for 15 min to serve as templates. qPCR was carried out in a qTOWER Thermal Cycler (Analytik Jena, Germany) using the Takyon No ROX SYBR 2X MasterMix blue dTTP. Reactions were performed according to the manufacturer's instructions in a 5 μL volume. *M. extorquens katA*::pTE1179 was used as a reference strain. Replicon copy number was calculated according to Lee *et al.*, 2006[29] using primer sets JD253 + 254 and MC147 + 148.

Replicon Stability. *M. extorquens* carrying pK18mob2-*repABC* derivatives were grown until OD_{600} = 1.0−1.5 in selective medium. Cell suspensions were diluted every 24 h to an $OD_{600} \approx 0.02$ in nonselective medium. Dilution series were plated on agar with and without kanamycin. Antibiotic resistance was correlated with the presence of the assessed plasmid. The inheritance stability of selected *repABC* replicons expressing mCherry as mini-chromosomes was assessed *via* flow cytometry on a BD LSRFortessaTM SORP flow-cytometer (BD Biosciences, NJ, USA). Fluorescence was detected using a 561 nm laser at 100 mW and a 610/20 bandpass filter.[30,31] Forward and side scatter values were monitored using a 488 nm laser at 100 mW. The acquired data was analyzed using FACSDiva software v8.0 (BD Biosciences). The percentage of fluorescent and nonfluorescent cells was determined from 30 000 gated events each.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.9b00220.

Table S1: Strains used in this study; Table S2: Plasmids used in this study; Table S3: Primers used in this study; Table S4: Fold induction of IPTG-inducible promoters in *M. extorquens* AM1; Figure S1: $P_{L/O4/A1}$ promoter in *M. extorquens* AM1; Figure S2: Overview of the individual *repABC* regions tested in this study; Figure S3: Verification of flow cytometry sensitivity; Figure S4: Replicon stability measured by flow cytometry of mini-chromosomes; Table S5: DNA sequences of inducible promoters characterized in this study (PDF)

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Author Contributions

T.J.E., A.B., M.W., and M.C. designed the research. M.C., M.W., F.P., and A.D. performed the experimental work. M.C. and M.W. analyzed or provided data. M.C. and T.J.E. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SD, standard deviation; h, hours; IPTG, isopropyl β-D-1-thiogalactopyranoside.

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